PHYTOCHEMICAL, BIOLOGICAL AND TOXICITY STUDIES OF *TERMINALIA SERICEA* BURCH. (COMBRETACEAE)

BY

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A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS OF THE DOCTOR OF PHILOSOPHY DEGREE IN CHEMISTRY

TO THE

DEPARTMENT OF CHEMISTRY

SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES

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March 2018

ABSTRACT

Terminalia sericea Burch. ex. DC (Combretaceae) is one of the 50 most popular medicinal plants in Africa. The fruit, leaves, stems and roots are commonly used for the treatment of cough, skin infections, diabetes, diarrhoea, venereal diseases and tuberculosis. However, the roots are most commonly used in the preparation of traditional medicines. Pharmacological studies have revealed that the crude root extracts display antibacterial activity against several Gram-positive and Gram-negative bacteria. Anolignan b, termilignan b and arjunic acid are reported to be the major antibacterial constituents present in the roots. Other compounds isolated from the roots include resveratrol-3-rutinoside, sericic acid, sericoside and arjunglucoside I.

Authorities worldwide, including the Medicines Control Council of South Africa, have begun to regulate herbal drugs sold in the form of commercial formulations. Quality control of herbal drugs is challenging, since the chemical profiles of the raw materials may vary, depending on the origin of the plant material and the way that it was handled and processed. The chemistry, in turn, impacts on the safety and efficacy of the plant material. To date, there are no available data on parameters that can be used to standardise the quality of *T. sericea* raw materials. The aim of this study was therefore to provide information on the variation of the chemical constituents that contribute to the biological effects of the roots of *T. sericea* and also establish its safety.

Since the compounds previously isolated from the roots were not commercially available, isolation of the major constituents of the roots was undertaken to obtain analytical standards. A crude dichloromethane:methanol (1:1) extract was initially fractionated using silica gel column chromatography, where after, some of the fractions were further purified using silica gel and Sephadex LH-20 column chromatography. Final purification of the enriched fractions was achieved using preparative high performance liquid chromatography coupled with mass spectrometry (prep-HPLC-MS). The structures of these compounds were subsequently elucidated using one- and two- dimensional nuclear magnetic

resonance (NMR) spectroscopy and mass spectrometry and identified as sericic acid (340 g), sericoside (500 g), resveratrol-3-rutinoside (240 mg) and arjunglucoside I (74 mg).

The chemical variation within the crude root extracts of samples (n = 42) from ten populations in the Limpopo Province of South Africa, was determined using ultra performance liquid chromatography-mass spectrometry (UPLC-MS). A method was developed for the simultaneous determination of sericic acid, resveratrol-3-rutinoside, sericoside and arjungluicoside I in the extracts using UPLC with photodiode array detection (PDA). The method was validated according to the guidelines of the International Council for Harmonisation (ICH). A regression coefficient (R^2) of 0.998 was obtained for sericic acid, resveratrol-3- rutinoside and arjunglucoside I, while the R^2 value for sericoside was 0.999, indicating a linear relationship between the concentration and the detector response. Satisfactory limits of detection for sericic acid (25.2 ng/mL), sericoside (11.6 ng/mL), resveratrol-3-rutinoside (23.3 ng/mL) and arjunglucoside I (8.81 ng/mL) were determined. Recoveries of 98 % and 80% were obtained for samples spiked with 12.5 µg/mL and 25 µg/mL of resveratrol-3-rutinoside, respectively, indicating that the method is accurate. The intra- and inter-day variation in resveratrol-3-rutinoside concentration, measured over three days, indicated excellent analytical precision, since all the relative standard deviations were below 0.70 %. The quantitative data revealed that sericic acid (1.59 to 8.45 mg/g), sericoside (2.07 to 20.17 mg/g), resveratrol-3-rutinoside (0.65 to 29.82 mg/g) and arjunglucoside I (0.86 to 8.44 mg/g dry weight) were the major constituents of the root samples, but their concentrations were highly variable. Chemometric analysis of the aligned UPLC-MS data was used to investigate similarities and differences in the chemical profiles of the samples using an untargeted approach. A principal component analysis (PCA) model was constructed and subsequently hierarchical cluster analysis (HCA) indicated the presence of two main groups, which were found to be independent of the populations to which the samples belong. Classes, based on the HCA class identifiers, were subsequently assigned to the samples, and an orthogonal projection to latent structures-discriminant analysis (OPLS-DA) model was then constructed, (R^2 cum = 0.996 and Q^2 cum = 0.967). The corresponding loadings

plot allowed sericic acid, sericoside and resveratrol-3-rutinoside to be identified as biomarkers associated with the first group. Quantitative, rather than qualitative differences were responsible for the observed clustering pattern. Techniques that could be applied in quality control protocols for *T. sericea* root were investigated.

High performance thin layer chromatography (HPTLC) analysis of the root extracts was optimised by testing different developing solvents and visualization reagents. The presence of the sericic acid (R_f = 0.80), sericoside (R_f = 0.49) and resveratrol-3-rutinoside (R_f = 0.36) were clearly visible on the plates. There were visible variations in the concentrations of resveratrol-3-rutinoside in representative samples from the 10 populations, corresponding to the UPLC results. The powdered samples were then analysed by mid-(MIR) infrared spectroscopy. Chemometric analysis of the data revealed no definitive clustering pattern. Partial least squares-discriminant analysis (PLS-DA) calibration models were established from the MIR spectral data combined with the accurate UPLC-values, for the prediction of the sericoside (R^2Y = 0.848, Q^2 = 0.757, RMSEP = 2.70 mg/g) and resveratrol-3-rutinoside (R^2Y = 0.794, Q^2 = 0.695, RMSEP = 4.37 mg/g) concentrations in powdered root samples.

The antibacterial activities of the root extracts, column fractions and isolated compounds were determined using three Gram-positive and five Gram-negative bacteria, all selected due to their ability to cause intestinal and skin disorders. Extracts and fractions containing high concentrations of sericic acid exhibited the highest activities against *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27858), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12223) and *Shigella sonnei* (ATCC 9292). The pure compound (sericic acid) was highly active against *S. sonnei* (MIC 0.078 µg/mL), a Gram- negative bacterium. There were no variations in the activity of the crude extracts against *B. cereus* and *P. aeruginosa*, while the MIC values obtained against *S. typhi* were variable and ranged from 0.25 to 1.0 mg/mL. Sericoside and resveratrol-3-rutinoside did not display any activity.

The anti-oxidant activities were evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) and reducing power assays. The anti-oxidant assays revealed that resveratrol-3- rutinoside exhibited lower activity (DPPH = 186 µg/mL; RP = 184 µg/mL) compared to the crude extract (DPPH = 22.3 µg/mL; RP = 24.4 µg/mL) and ascorbic acid (DPPH = 11.3 µg/mL, RP = 145 µg/mL). Sericic acid and sericoside did not display any anti- oxidant activities. The variation in the anti-oxidant activities (4.58 to 26.0 µg/mL) of the samples from different populations was an indication of chemical variability.

A toxicity study of the raw powdered plant material was conducted using vervet monkeys (*Chlorocebus pygerythrus*). Biochemical analysis (liver function tests, kidney function tests and hematology), physical and physiological examinations were conducted. The subjects were fed a normal diet supplemented with *T. sericea* root powder (2.14 g/kg per day) for 120 days, where after the diet was returned to normal (washout) for another 30 days. The treatment groups presented with elevated serum enzymes at Week 4, followed by the reduction of the elevated serum enzymes levels at Week 12. These results indicate short-term hepatotoxic effects, followed by hepatoprotective activity. Reduction of the serum glucose at Week 4 suggests hypoglycemic potential. However, elevated serum creatinine levels indicated possible nephrotoxicity.

In conclusion, this study has indicated the variability in the chemical constituents of the roots of *T. sericea*, which affects the antibacterial and anti-oxidant activities. Sericic acid, resveratrol-3-rutinoside, and sericoside were, for the first time, identified as biomarkers that can be used for the quality control of raw root material to be used in herbal products. Sericic acid was also found to be the main antibacterial constituent of the roots. The hepatoprotective, nephrotoxic and hematotoxic effects observed in monkeys to which the root powder had been administered is cause for concern.

KEYWORDS

Terminalia sericea

Sericic Acid

Sericoside

Reverastrol-3-rutinoside

Biomarkers

Quality control

Toxicity

DECLARATION

I, Chinedu Prosper Anokwuru (Student no: 11636277) declares that this thesis for the award of Doctoral degree (PhD) in Chemistry of the University of Venda has not been previously submitted to this or any other institution and that all the referenced materials have been duly acknowledged.

Chinedu.Anokwuru
Signature

March 2018
Date

PUBLICATION ARISING FROM THIS STUDY

- Anokwuru, C.P.; Ramaite, I.D.; Bessong, P. Phenolic content distribution and antioxidant activities of *Terminalia sericea* Burch. *Afr. J. Trad. Complement. Altern.Med.* **2015***, 12*, 21-27.

CONFERENCE PROCEEDING ARISING FROM THIS STUDY

- Chinedu P Anokwuru, Ipfani D. I Ramaite. Effect of solvent on the phytoconstituents and antibacterial activity of *Terminalia sericea* root extracts (Oral). SACI North Young Chemist Symposium, University of Limpopo, South Africa. November 23, **2016.**
- Chinedu P Anokwuru, Ipfani D. I Ramaite. Antibacterial activity of *Terminalia sericea* root fractions (Poster). Frank Warren Conference, Rhodes Univeristy, South Africa. December 4 - 8, **2016.**

DEDICATION

I dedicate this study to all parents who are sacrificing everything to make their children great. May you be witnesses to the fulfilment of your dreams.

ACKNOWLEDGEMENTS

- I am thanking God for the grace and opportunity to complete this study. Without life, every plan of man, no matter how great or small, perishes.
- I want to thank Prof IDI Ramaite specially for accepting to supervise this study. Without you, I would not have been able to come this far. Thank you for your emotional and financial support.
- I am thanking Prof PO Bessong for the guidance during my study. You constantly kept me focused especially in discerning what was most important and relevant for the completion of my study.
- For Prof S Combrinck (Tshwane University of Technology, Pretoria South Africa), I say “merci beaucoup.” I lack words to express my gratitude for your impact in my work. You were the compass that directed me, and the shield that protected me from the frustrations that accompany doctoral studies. You are not just meticulous but consistent. You taught me that it is possible to be firm and smile at the same time. You reminded me again and again that good is not enough. I should always strive for the best, even in little things. My mother would not have treated me better than you did. Thank you.
- I am thanking the Research and Publication Committee (RPC) of University of Venda for funding this project.
- I also thank Technology Innovative Agency (TIA), South Africa for monthly stipend and funding of the toxicology studies.
- I am thanking Prof S van Vuuren (Univeristy of Witwatersand, Johannesburg South Africa) for assisting me with the antibacterial activities in her laboratory. Thank you for making me do the microbial testing myself. It was really refreshing. I must say that I was challenged by your punctuality. You are always in the office by 6.30 am every working day. I could hardly keep up to that time during my visit.
- I am thanking Prof A Viljoen (Tshwane University of Technology, Pretoria South Africa) for allowing me use the laboratories and instruments for the analysis of my samples. Sir, the setup of the laboratory is really intimidating, but I was humbled by your simplicity. I have learnt from you that true success comes with humility.

- I want to thank Dr W Chen (Tshwane University of Technology, Pretoria South Africa) for helping me with my UPLC-MS analysis. I really wondered how you were able to attend to everyone at the same time without freaking out. Sometimes, I see the frustration in you when faced with challenges (especially dealing with the instruments). However, what I did not comprehend was the calmness you exuded while resolving whatever the issue was. Thank you for engaging me in the processing of my results. You did not just hand them to me, you challenged my mental capacity to grasp the underlining principles.
- My thanks also goes to Dr S Chaudhary (Tshwane University of Technology, Pretoria South Africa) for assisting me with the prep-HPLC-MS analysis.
- I will like to thank Prof O Taglialatela-Scafati (University of Naples Federico II, Napoli Italy), Dr R Kerssebaum (Bruker Biospin SmbH, Rheinstetter Germany) and Dr S Moleele-Mnyakeni (University of Venda, Thohoyandou, South Africa) for assisting in the structure elucidation of the isolated compounds.
- I am also thanking Mr. Farai Dondofema for helping me GPS location on the Map.
- I want to thank Mr M Fhumulani Mutshaeni for going the extra mile in making my laboratory work less stressful. Even when there were scarce resources, you never turned me back. You never vented on me the frustrations you were absorbing. You treated me like a brother. Thank you.
- I will not forget Mr P Pandelani. Thank you for finding time to run my NMR experiments, especially those heat periods when every student wants to turn in results for submission.
- I am also thanking Mr T Mathoni for his moral, spiritual and material support.
- It was the quality of training and education that my late parents Mr and Mrs John Anokwuru Kamalu afforded me that led me thus far. Their memory still lives with me every day.
- I am thanking Babcock University, Nigeria for granting me the privilege to embark on this study. Thank you for the sponsorship and spiritual support.
- I want to thank my siblings, Uche, Chinwendu and their families. You have been a source of strength to me. With heavy hearts I remember my late little brother Emmanuel, who was taken from us on the 23rd of April 2017. We

were denied the joy of celebrating with him for the graduation of his Master's program on June 4, 2017 and sadly will not be with us to celebrate the completion of my study. Sleep on brother until the resurrection morning.

- I want to thank my children Chibuenyim, Chizurum and Chinemerem for their support and understanding. You have been a source of strength to me during this period. I am sure we will have more time to play now.

- I thank my wife for her support and understanding especially since we both started this study together. You have been my strength all through this period. You were able to balance your studies with the taking care of the family. I pray that God gives you more strength and wisdom to deal with issues as you also climb the academic ladder

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LIST OF ABBREVIATION

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ATTC	American type culture collection
AST	Aspartate aminotransferase
ATR	Attenuated total reflectance
CTR	Center
COSY	Correlation spectroscopy
CK	Creatine kinase
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl–picryl-hydrazyl
ESI	Electron-spray ionization
ECRA	Ethics Committee for Research on Animals
EDTA	Ethylene-diamine-tetra-acetic acid
EU	European Union
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared
GAP	Good Agricultural Practice
GCTP	Good Clinical Trial Practice
GGT	Gamma-glutamyl transferase
GMP	Good Manufacturing Practice
GSP	Good Sourcing Practice
HCT	Haematocrit
Hb	Haemoglobin
HMBC	Heteronuclear multiple bond coherence
HSQC	Heteronuclear single quantum coherence
HCA	Hierarchical cluster analysis
HPTLC	High performance thin layer chromatography
HDL	High density lipoprotein
IC_{50}	Inhibition concentration (50%)
ICH	International Council for Harmonization

LDL	Low density lipoprotein
MS	Mass spectrometry
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MIC	Minimum inhibitory concentration
m/z	Molecular ion
MSC	Multiplicative scatter corrections
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectrometry
OPLS-DA	Orthogonal projection to latent structure-discriminant analysis
PAR	Pareto
PLS-DA	Partial least square-discriminant analysis
PDA	Photodiode array
PCA	Principal component analysis
QToF	Quadruple time of flight
RSA	Radical scavenging activity
RBC	Red blood cell
RDW	Red blood cell distribution
RSD	Relative standard deviation
Rf	Retention factor
RMSEP	Root mean square error of prediction
SANS	South African National Standards
SD	Standard deviation
SE	Standard error
SNV	Standard normal variate
TGA	Therapeutic Goods Administration
UPLC	Ultra-performance liquid chromatography
UV	Univariate
WBC	White blood cell

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Figure 6:8 Average body weight (kg) of vervet monkeys after 12 weeks of exposure to a diet supplemented with *T. sericea* root and additional 4 weeks washout period

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CHAPTER 1
BACKGROUND TO THE STUDY

1.0 INTRODUCTION

Plants have been a source of medicine for man for several centuries (Hubsch et al., 2014). Records of Mesopotamia (3000 BC), ancient China and India (2500 BC) and the Papyrus Ebers (1550 BC) describe the use of herbal medicine for the treatment of a wide range of diseases (Baydoun et al., 2015). There are approximately 350,000-400,000 plants species currently identified globally, of which about 70,000 are used in medicine (Daniel, 2006; Sher et al., 2016). According to the World Health Organization (2003), 80% of people living in developing countries depend on herbal-based alternative systems of medicine. The preference of plant-based medication over synthetic drugs in local communities worldwide can be attributed to their easy accessibility, treatment efficacy, affordability and assumed minimal side effects (Ullah et al., 2013). Natural products from animals, plants and micro-organisms are good sources of new drugs, due to their chemical diversity and biological relevance (Brusotti et al., 2014; Li et al., 2016). The WHO (2003) also reported that about 25 % of modern drugs are derived from plants. Examples of such natural products (lead compounds) from plants include: quinine (antimalarial), morphine (analgesic), vincristine (anticancer), artemisinin (antimalarial), atropine (anticholinergic) and theophylline (bronchodilator) (Sharma et al., 2015). Although herbal medicine has served as a reliable source of medication for centuries, the emergence of synthetic drugs in the 20th century led to the replacement of botanical healing practices due to the greater economic profitability of synthetic drugs (Sushma, 2011). However, side effects, high cost, and treatment failures due to resistance of microorganisms to synthetic drugs have led to an increase in the use of herbal medicines and studies into medicinal plants to seek alternatives to synthetic drugs (Gupta et al., 2013; Sharma et al., 2016; Xin et al., 2017).

A herbal medicinal product is defined as "*any medicinal product, exclusively containing as active ingredient one or more herbal substances or one or more herbal preparations, or one or more such herbal substance in combination with*

one or more such herbal preparation" (Kroes, 2014). Herbal products contain a mixture of complex phytochemicals, which can interact with multiple targets and mostly have low toxicity and few side effects. One of the advantages of multicomponent herbal medicines over single drugs is the possible synergistic effects of the active components, which results in a greater combined effect compared to the sum of the individual effects (Guo et al., 2014; Shi et al., 2016). Herbal products have attracted attention due to increase in their patronage. This is due to the long history of established therapeutic effects, perceived safety due to natural sources of the medicine and the high cost of modern medicine (Maulidiani et al., 2015).

Commonly known herbal medicine systems include Chinese Traditional Medicine (CTM), Ayurveda, Unani, Siddha, Kampo Medicine, Arabic Medicine, European phytotherapy and Traditional African Medicine (Shinde et al., 2009). The use of these traditional medicines involves a holistic health care philosophy, which is concerned with the behavior of the system as a whole (Hu and Xu, 2014; Wah et al., 2012). In contrast, Western medicine is based on the philosophy of reductionism. This is a process where complex entities are understood by reducing them to simpler or fundamental elements. This is the reason why medicinal plants are separated to isolate the active constituents, or a single active drug for a specific ailment is synthesized. Although reductionism has proved to be tremendously successful in the treatment of diseases, it is limited in addressing complex diseases, has undesired side effects and a high cost of research and development (Shi et al., 2016; Xie and Leung, 2009).

1.1 GLOBAL MARKET VALUE OF HERBAL DRUGS

In the last decade, there has been a 10-15 % increase in the global demand for herbal products per annum (Maulidiani et al., 2015). It is estimated that the global market for traditional medicine will reach US$115 billion by 2020 (Quansah and Karikari, 2016). The market value of the Chinese herbal industry was estimated at $83.1 billion in 2012, which was a sharp increase of 20 % from the previous year (Booker et al., 2016; Wah et al., 2012). In Japan, traditional medicine comprises about 2.2 % of the total pharmaceutical products produced, and is worth $1.3 billion (Maegawa et al., 2014). The annual expenditure on traditional medicine in the Republic of Korea was $4.4 billion in 2004 and rose to $7.4 billion in 2009. The out-of-pocket spending for natural products in the United State was estimated at $14.8 billion in 2008, while the estimated market value was $100 billion (Soner et al., 2013; WHO, 2013). In Brazil, the herbal medicine revenue was estimated at $160 million in 2007 (Soner et al., 2013). The estimated worth of the Indian herbal industry is $1 billion (Sahoo and Manchikanti, 2013). The market value of the South African herbal medicine is $75 to 150 million per year (Ndhlala et al. 2011; Street and Prinsloo, 2013).

1.2 CLASSIFICATION AND REGULATION OF HERBAL PRODUCTS

Herbal products are subjected to regulatory processes before they are approved and registered for commercialization. Guidelines are stipulated so as to guarantee quality, efficacy and safety of the herbal products (Kulkarni et al., 2014). The increase in the global use of herbal medicine has led to the movement of medicinal plants and products from one region to another, resulting in adulteration, misidentification, contamination and variation in the constituents of plant materials (Booker et al., 2016; Guzelmeric et al., 2017; Marseglia et al., 2016). This has led to challenges in the quality control, efficacy and safety of herbal products and the desire for internationalization and globalization of herbal medicine (Zeng et al., 2007). For example, *Anemarrhena*

asphodeloides collected from Korea and China, displayed variation in the concentration of three biomarkers, mangiferin, ngasol, and timosaponin III (Kim et al., 2014). It is therefore important to establish internationally recognized guidelines for assessing the quality of herbal medicine.

Another challenge is the difference in the criteria for regulation of herbal products (Sahoo et al., 2010). Criteria for regulation, which include quality control, efficacy and safety, differ in many countries. This results in different regulatory GMP (Good Manufacturing Practice) requirements across regions and countries (He et al., 2015). The classification of herbal products determines the regulatory process required for their approval (Sahoo and Manchikanti, 2013). For example, traditional medicines in China and India are fully integrated into the main stream health care and are therefore treated as regular medicines. In Germany, herbal medicines are integrated into primary health care and a simplified registration process is required before marketing of herbal products. In Australia, herbal medicines are regulated by the Therapeutic Goods Administration (TGA). Herbal products are listed as complementary medicines and are considered to carry only “low risk” of adverse effects and are assessed based on toxicity of the ingredient, dosage form, serious disease claim, side effects/ interactions, and adverse reactions. In the US, herbal products are registered as dietary supplements with no disease prevention claim. These products are not required to comply with the rigorous requirements of the Food and Drug Administration (FDA) for approval. However, herbal products with a specific health claim are subjected to the same process as standard drugs before registration. In Canada, herbal products are registered under the National Health Products Regulation (NHPR). Evidence of safety, efficacy for the proposed health claim and quality of the product must be provided for herbal products (Commisso et al., 2013; Ganorkar and Urade, 2016; Govindaraghavan and Sucher, 2015; Jordan et al., 2010).

The Traditional Herbal Medicinal Product Directive (THMPD) was established by the European Union (EU) in April 2011 to regulate herbal product marketing across member states. Herbal products are only registered in the EU if there is evidence of the use of the product for at least 30 years (including a minimum of

15 years in Europe) for treatment of specific minor ailments. No clinical trials are required to validate the efficacy and safety of the herbal product, since the long history of use is considered sufficient evidence of its pharmacological effects (Commisso et al., 2013).

1.3. STANDARDISATION OF HERBAL PRODUCTS

Herbal products are obtained by extracting single or multiple plant materials with suitable solvents (Bandaranayake, 2006). Due to the complexity of the chemical compositions of the extract, active compounds or characteristic compounds are used to standardize the herbal products before they are approved for commercialization (Huang et al., 2016). According to the American Herbal Product, "standardization" refers to the body of information and control necessary to produce material of a reasonable consistency (Nikam et al., 2012). It is the prescription of a set of standards or inherent characteristics, constant parameters, and definitive qualitative and quantitative values that can assure quality, efficacy, safety and reproducibility (Kumari and Kotecha, 2016).

Standardization involves the development of guidelines or standards through experimentation and observation, so that specific characteristics exhibited by the herbal materials can be used for quality control of the product. Therefore, standardization is a tool used for quality control processes (Sachan et al., 2016). It helps to establish a consistent biological activity, chemical profile or simply a quality assurance programme for the production of herbal drugs (Shivatare et al., 2013). Herbal products made from a single plant material can be standardized by using the active constituents of the plant material. However, in the case of mixed herbal products, where two or more plant materials are used, it becomes more difficult to use all of the active constituents in all the different materials for standardization. Instead, a set of compounds which are representative of the mixture must be used for the standardization. The process is therefore not directed at isolating single bioactive compounds, but towards the identification of constituents of the mixture that provide total information required for consistency in composition of the herbal product (Bele

and Khale). It is very important to standardize the quality of the raw material, in-process materials and final products (Murali et al., 2017).

Standardization of herbal medicine is important, because of the challenges in authentication and quality control of the raw material, complexity and variations in the chemical constituents and poor adherence to processing practices such as Good Manufacturing Practices (Kumari and Kotecha, 2016b). Authentication of the raw material is an important prerequisite to ensure reproducible quality of the herbal medicine (Kushwaha et al., 2010). Processes such as collection period and time, collection and drying methods, packaging, storage and transportation of harvested materials, age and part of plant part collected, must be considered during the standardization of raw material (Hariharan and Subburaju, 2012). The quality of the raw material is also dependent on the presence of foreign matter (part of the medicinal plant material other than those named, or mineral and mixtures like sand, stones, glass, metals etc), organoleptic and microscopic evaluation, volatile matter, ash values, pesticides, heavy metals, microbial and radiation contaminants (Murali et al., 2017; Rasheed et al., 2012). Besides proper identification of the raw material, the process of handling the material until the final product is very important for reproducibility. There are international standardized practices, such as Good Sourcing Practice (GSP), Good Agricultural Practice (GAP), Good Laboratory Practice (GLP), Good Manufacturing Practice (GMP), and Good Clinical Trial Practice (GCTP), which must be adhered to for quality herbal products to be obtained (Chawla et al., 2013; Efferth and Greten, 2012).

In the laboratory, herbal preparations are standardized by using chromatographic techniques and marker compounds. Marker compounds are generally classified as DNA-based molecular markers and chemical markers (Kushwaha et al., 2010). Chemical markers help to identify adulterants and provide confirmation of plant origin and are otherwise known as biomarkers when the compounds contain therapeutic agents, while the DNA markers are used for authentication and breeding of medicinal plants (Hennell, 2012). Chromatographic techniques are used to obtain chemical fingerprints of the

herbal material. The fingerprints are unique patterns that indicate the presence of multiple chemical markers within a sample (Li et al., 2008).

1.4. BIOMARKERS OF SOUTH AFRICAN MEDICINAL PLANTS

There are about 30,000 species of higher plants in southern Africa, of which about 24,000 are indigenous to South Africa. Approximately 3,000 of these species are used as medicine and records indicate that 350 species are commonly used and traded as medicinal plants (Aremu et al., 2010; Van Wyk et al., 1997). There are about 200,000 indigenous traditional healers in South Africa and up to 60 % of South Africans consult healers in addition to using modern medicine (Van Wyk et al., 2013). Although South Africa has a large diversity of herbal products, only a few indigenous plants have been standardized for commercial purposes, due to limited studies on the identification of biomarkers that can be used for quality control (Van Wyk, 2011). The quality of herbal medicine is assessed (quality control) according to the specification of the product (standardization), which details the requirements for identity, purity and content of the characterizing compounds (Choi et al. 2002). Van Wyk (2011), Van Wyk et al. (2013) and Street and Prinsloo (2013) have reviewed extensively 16 South African plants species that have been fully or partially commercialized. *Aloe ferox*, *Harpagophytum procumbens* (Devil's claw), *Hoodia gordonii* and *Sceletium tortuosum* are the only South African medicinal plants that have been fully standardized for export to Europe and America.

Aloin is the biomarker in *A. ferox* and the content in *A.ferox* varies between 8.5 % and 32 %, however the minimum requirement for export to Europe is 18 %. *Harpagophytum procumbens* has been successfully registered as herbal medicine in France and Germany and as a food supplement in the United Kingdom, the Netherlands, the United States and the Far East. The common standard for the content of its main therapeutic compound (harpagoside) is between 0.8 % and 2.3 %, as specified by the European Pharmacopoeia. Data

obtained after Phase II clinical trials (Brien et al., 2006), conducted to determine the efficacy and safety of the extract in the treatment of osteoarthritis of the knee, revealed that Devil's claw was effective in the reduction of pain associated with the condition, but did not provide definitive information to its efficacy and safety. The trial was suspended due to the unexpected withdrawal of funds.

Hypoxis hemerocallidea (African potato) is commonly used in commercial preparations for prostate treatment and as an immune booster. The activity has been attributed to hypoxoside, its aglycone derivative rooperol, and β-sitosterol. *Hypoxis hemerocallidea* has been commercialized and is exported to Europe. *Sutherlandia frutescens* is exported worldwide, but has not been approved by regulatory agencies in Europe or the United States. *Pelargonium sidoides* has been licensed as a herbal medicine for the treatment of respiratory tract infection in Germany, Russia, the Ukraine and Lativa. However, there is insufficient literature on the standardization and quality control of the herbal product. Aspalathin is the major constituent of *A. linearis* (rooibos tea) that is used for quality control. However, there are no reports on the link between the constituents and the health benefits of the tea.

The major constituents and active principles of *Hoodia gordonii* have been reported in literature. However, there are no clinical data to support the appetite-suppressing activity of P57. The standardization and quality control of the herbal product containing P57 (the main active constituent of *H. gordonii*) has been developed using chromatographic and spectroscopic models (Vermaak et al., 2010a; Vermaak et al., 2010b; Vermaak et al., 2011). *Hypoxis hemerocallidea* was first commercialized in 1967 as *Hypoxis* phytosterol and was sold in Germany for the treatment of prostate hypertrophy. However, there is no available literature on the standardization and quality control of the plant or its herbal preparations. Mesembrine is the main active constituent of *Sceletium tortuosum* and its concentration varies between 0.05 and 2.3% of the dry weight. Clinical trials in healthy adults indicated that the herbal extract was well tolerated (Nell et al., 2013). *Sceletium tortuosum* is commercialized and exported under the trade name Zembrin® by HG&H Pharmaceuticals (PTY) Ltd (www.zembrin.com/clinical-studies-page/). *Merwilla natalensis* (Hyacinthaceae),

known as 'inguduza', is at a risk of extinction due to the high demand for the bulb for medicinal purposes. In 2006, 2.1 million bulbs worth R3.87 million were sold. However, there are no reports of specific major constituents or active principles that can be used for standardization and quality control (Street and Prinsloo, 2013).

1.5. SAFETY OF HERBAL DRUGS

Drugs are constantly withdrawn from the market because of reported unacceptable toxicity in humans. Lack of adequate and reliable toxicology screening methods is one of the challenges responsible for poor interpretation of *in vitro* and *in vivo* assays to human toxicity. This has led in the continual search for more improved assays that are good predictors of human toxicity (Astashkina and Grainger, 2014; Green et al., 2010). Medicinal plants exert a broad range of activities on the physiological system due to the variety of complex chemical structures of compounds produced by the plants (Heyman and Meyer, 2012). The pharmacological effects are through complementary or synergistic actions that are usually non-specific and directed towards aiding the body's own healing processes. These activities provide new and important leads against pharmacological targets and are usually assumed safe, based on their traditional use over long periods of time. However, research has indicated that many plants that are used as food ingredients or in traditional medicine are potentially toxic, mutagenic or carcinogenic (Carvalho et al., 2011; Edziri et al., 2011; Fennell et al., 2004). The consumption of medicinal plants without studies of their efficacy and safety can result in several side effects that may affect different organs. The liver and kidneys are prime targets, because they are involved in the degradation and excretion of a myriad of chemical compounds (De Oliveira et al., 2011). Toxicants interfere with the central functions of an organism, for example neurotoxins affect the brain and nervous system, while cytotoxins and metabolic poisons damage the liver, kidneys, heart or the respiratory system (Mulaudzi et al., 2013).

1.6. RATIONALE AND MOTIVATION OF THE STUDY

There are a large number of indigenous herbal products sold in South Africa as crude and unprocessed drugs (Van Wyk, 2011). This is due to potential challenges in the authentication, processing and production of final products due to insufficient scientific data to validate the quality, safety and efficacy of South African medicinal plants. Many of the herbal products do not adhere strictly to good practices such as GAP and GMP and therefore do not meet the high international regulatory standards (Street et al., 2008; Ndhlala et al., 2011). This has led to only a few of medicinal plants from South Africa being commercialized and exported (Street and Prinsloo, 2013).

Terminalia sericea (Combretaceae) is a medicinal plant that is endemic to southern African and it is listed among the 50 most important medicinal plants in Africa (Moshi and Mbwambo, 2005; Van Wyk et al., 1997). This makes *T. sericea* an important plant worthy of further investigation for commercialization purposes. It is used for the treatment of various ailments such as diarrhoea, stomach ailments, venereal diseases, diabetes, cough, asthma, constipation, conjunctivitis, fever, leprosy, constipation, wound infection and cancer (Katerere et al., 2012). Extensive pharmacological studies on the antibacterial activities have been reported on the crude organic and aqueous extracts against both Gram-positive and Gram-negative pathogens. However, only a few compounds (anolignan b, termilignan b and arjunic acid) have been associated with the antibacterial activities of the root and the treatment of specific ailments (Eldeen et al., 2006; Eldeen et al., 2008).

Although Eldeen et al. (2006; 2008) identified anolignan b, termilignan b and arjunic acids as the main antibacterial compounds of *T. sericea* root, the pathogens tested were limited to *B. cereus, E. coli, K. pneumonia and S. aureus.* In addition, the work was done with a single sample and not with many samples from different populations, which may account for different chemical profiles of the plants and their antibacterial activities. It is therefore important to isolate the compounds that are active against pathogens associated with intestinal disorders and wound infections. It is also important to determine the chemical

profiles of *T. sericea* root from different geographical locations. This would provide information on any variability in the chemical profile and the effect on the antibacterial activities.

Studies on the phytochemistry of *T. sericea* root has revealed very few compounds, mainly lignans (anolignan b and termilignan b), stilbenes (resveratrol-3-rutinoside), pentacyclic triterpenoids (sericic acid, arjunic acid and arjunglucoside I), saponins (sericoside and arjunglucoside) and tannins (punicalagin) (Bombardelli, 1975; Bombardelli et al., 1974; Joseph et al., 2007). However, there are no available literatures on the identification of biomarkers or available commercial standards for the quality control of *T. sericea* root. There are also no available phytochemical reports on the quantification of any isolated compound from different geographical locations. Furthermore, isolation methods previously reported in literature (Bombardelli et al., 1974, 1975; Eldeen et al., 2006, 2008; Joseph et al., 2007) were not well described and difficult to follow. Therefore, there is a need to identify biomarkers for the quality control of *T. sericea* root through first isolation and purification of the major constituents, which can then be used as standards.

An *in vitro* study has indicated that *T. sericea* is toxic. (Bessong et al., 2005). However, the *in vitro* study is not sufficient for the evaluation of the toxicity of the roots of *T. sericea*, which is the main plant part used. The toxicology report was limited and cannot be used to determine the safety of the plant. According to the WHO (2007), the use of phytotherapy based on efficacy and safety criteria is not sufficient to guarantee the quality of both the herbal medicine and its use. The report emphasizes the importance of high quality standards so as to guarantee safe use by patients. So far, there is no available literature on the standardization and quality control of *T. sericea*, making it difficult to guarantee efficacy and safety of any herbal product from the plant. There is therefore the need to establish isolation method that is well described and easy to follow towards standardization of the plants. There is also a need to be more specific on the antibacterial work of the isolated compounds towards a specific ailment.

1.7. AIM OF THE STUDY

The aim of this study was to investigate the phytochemisty, biological activities and toxicology of the root of *T. sericea*.

1.8. OBJECTIVES OF THE STUDY

The objectives of this study were to:

i. Isolate and identify the major constituents of *T. sericea* root and investigate the chemical variation of these major compounds within roots of *T. sericea* collected from the same and different populations;
ii. Use metabolomic profiling to obtain a characteristic chemical fingerprint for the species;
iii. Analyse the data from all the samples using chemeometrics analysis to identify marker compounds for quality control of *T. sericea* roots;
iv. Establish a high performance thin layer chromatography fingerprint for quality control of the raw root material;
v. develop a spectroscopic method for the quality control of *T, sericea* root material;
vi. Evaluate the antibacterial activity of various plant extracts and the isolated compounds against bacteria associated with stomach disorders and skin problems;
vii. Evaluate the comprehensive *in vivo* pharmacological activity of *T. sericea* using a non-human primate (vervet monkeys) model.

1.9. STUDY OUTLINE

This study consists of seven chapters. Each chapter consists of an introduction, methodology, results and discussion, and references.

Chapter 1: Several plants in South Africa have been studied and used as medicinal plants, but only a few of these plants have really been standardized and their safety ensured. In this chapter, a general overview of the traditional medicinal plants was looked into. It also provides the aim and objectives of the study.

Chapter 2: This is an extensive literature review of the chemistry and pharmacology of *the plant T. sericea*.

Chapter 3: The isolation and structure elucidation of the major compounds from the root of the plant is described.

Chapter 4: The plants used in the study were collected from different populations, therefore quantification of the isolated major constituents in samples from different population was carried out. In addition, metabolomic profiling of the crude extract, chromatographic and spectroscopic fingerprinting and chemometric analysis of data obtained from the chromatographic and spectroscopic fingerprinting are described.

Chapter 5: *Terminalia sericea* has been used for the treatment of different ailments. In this chapter the methods used to determine the antibacterial and anti-oxidant activities of crude extracts obtained from different populations, and the isolated compounds, are discussed.

Chapter 6: Several works have been done on the *in vitro* toxicology of the plant *T.sericea*. However, little work has been done on the *in vivo* toxicology that will ensure the safety of the plant to humans as a medicinal plant. Adescription of the comprehensive *in vivo* toxicology study to determine the effect of *T. sericea* root on vervet monkeys is presented in this chapter.

Chapter 7: The general conclusions made from the study and the recommendations drawn are presented.

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CHAPTER 2
PHYTOCHEMISTRY AND PHARMACOLOGICAL ACTIVITIES OF *TERMINALIA SERICEA* (LITERATURE REVIEW)

2.1. INTRODUCTION

The family Combretaceae comprises 18 genera. Members of the family are easily recognizable by the wing-shaped appendages of the fruit (Masoko et al., 2005). Combretaceae are used in Africa, Asia and South America for the treatment of numerous conditions, including abdominal disorders, backache, cancer, chest coughs, colds, conjunctivitis, constipation, diarrhoea and dysentery (Katerere et al., 2012). The genus *Combretum* is the largest within the family and consists of 370 species, followed by *Terminalia*, which is represented by 200 species. These two genera are widely used as traditional medicine in western and southern Africa (Ahmed et al., 2014; Masoko et al., 2007). There are at least 40 species of *Terminalia* that are endemic to Africa of which 11 occur in southern Africa (Nair et al., 2012). *Terminalia* species are used as astringents and brain tonics, and to treat coughs, asthma, stomach pain, liver disorders, piles, leprosy and fever. Phytochemical investigations have revealed the presence of saponins, glycosides, flavonoids, tannins, chalcones and pentacyclic triterpenoids (Eloff et al., 2008; Tom et al., 2011). Among the species of *Terminalia* found in southern Africa, only *T. sericea* has been examined extensively for its pharmacological relevance and phytochemistry (Nair et al., 2012).

2.2. BOTANICAL DESCRIPTION

Terminalia sericea Burch ex. DC. is known as "*vaalboom*" in Afrikaans, "silver cluster leaf" in English, "*mangwe*" in isiNdebele, "*moxonono*" in Sesotho, "*mususu*" in chiShona and Tshivenda, "*mongonono*" in Tswana, "*amangwe*" in isiZulu, and as "*namatipo*" or "*mpululu*" in Nyakusa (Tanzania), "*mukenge*" in Katima Mulilo

(Namibia), and as "*nsunsu*", "*nkonola*", "*kondla*" or "*mogonono*" in Canhane (Mozambique).

The tree is widely distributed throughout Africa where it is found scattered throughout most of the savannah woodlands of eastern, central and southern Africa. It also occurs as a dominant or co-dominant species in mixed deciduous forests. The tree is found in abundance in areas characterized by deep sandy soils. It is drought and, to some degree, frost tolerant and moderately adapted to saline soils (Chivandi et al., 2013). *Terminalia sericea* is a small to medium-sized shrub or brush tree of about 5 to 8 meters in height, although some trees reach a height of 23 m. The trunk is erect and has a wide spreading crown, while the bark is grey to pale brown and coarsely fissured. Silvery hairs adorn the leaves that are a blueish-green colour on the axial side and paler below. The flowers are cream-coloured and emit an unpleasant odour. Fruiting occurs between January and May. The fruits are about 30 mm long, with two broad papery wings surrounding the thickened central part. They range in colour from purple-brown to pink-purple when mature (Chivandi et al., 2013; Likoswe et al., 2008).

Planting of the tree from seed has been unsuccessful due to the low germination rate under natural conditions (Amri, 2011). It has a biomophine rooting system that manifests as a superficial root without tap root in drier savannas, or a tap root in mist savannas (Hipondoka and Versfeld, 2006). Wood from the tree is used for fencing posts, fuel, charcoal, carving, building material, tool handles and for medicinal purposes. It is also used to improve sites by draining waterlogged soils, shading out weeds, enriching soil and controlling erosion (Amri, 2011; Likoswe et al., 2008).

Figure 2.1: Photograph of a typical example of *Terminalia sericea.* Photograph taken by C. Anokwuru

2.3. ETHNOMEDICINAL USES

Terminalia sericea is amongst the top 50 most commonly used African medicinal plants (Moshi and Mbwambo, 2005; Nair et al., 2012). Decoctions or infusions of the plant are generally prepared for medicinal use. Infusion is the process of extracting plant constituents into a liquid (such as hot or cold water, edible oil or alcohol) by allowing the material to remain suspended in the liquid for a prolonged period. The preparation of decoctions, on the other hand, involves boiling of the plant material over time (Fotakis et al., 2016). Infusions and decoctions are commonly used to prepare medication that is taken orally (Lall and Kishore, 2014). For topical applications, plant material is dried, ground and applied on the infected area.

Ethnomedicinal information revealed that the fruit, leaves, stem bark and roots of *T. sericea* are commonly used for the treatment of coughs, skin infections, diabetes, diarrhoea, and gonorrhea. A decoction of the ground roots is used in East Africa for the treatment of bilharzia and stomach conditions. In Tanzania, the Hehe tribe of Iringa use the plant to treat diabetes, diarrhoea and gonorrhoea, while Mbeya traditional healers use the leaves, stem bark and roots to treat bacterial infections, diarrhoea, hypertension and fever. The leaves and roots are also used to combat fungal infections. In Malawi, the dried fruit is used in a multicomponent formulation for the treatment of tuberculosis, while the dried leaves are used to treat dysentery. In Mozambique, the Changano ethnic group uses the stem bark to treat burns and wounds. The leaves are used to relieve stomach aches, while the roots are used to alleviate diarrhoea. In Katima Mulilo, Namibia, *T. sericea* is used to cure meningitis.

In Venda, South Africa, a decoction of the dried leaves and roots are used to treat menorrhagia and infertility, while the powdered dried leaves are applied to infected wounds. They also administer a decoction of the dried root, prepared as soft porridge with maize flour, for the treatment of diarrhoea. Root decoctions are used for the treatment of stomach disorders, diarrhoea, pneumonia and eye infections by the Tswana people of South Africa (Amri and Kisangau, 2012; Chinsembu and Hedimbi, 2010; Eldeen et al., 2006; Fyhrquist et al., 2002; Moshi and Mbwambo, 2005; Ribeiro et al., 2010; Steenkamp et al., 2004). Leaves of *T. sericea* are also used in ethno-veterinary practice for treating wounds (Luseba and Van der Merwe, 2006). The roots are administered to treat diarrhoea and placenta retention in cows (Gabalebatse et al., 2013). The use of *T. sericea* in traditional medicine indicates that the roots, in particular, have good activity against bacteria that cause gastrointestinal infections and septic wounds. This indicates that the roots may be a good source of broad spectrum antibacterial compounds.

2.4 PHYTOCHEMISTRY

The concentrations of crude protein (46.2%), lipid (32.6%), ash (6.9%), linoleic acid (68.63%), oleic acid (14.05%), several minerals (phosphorus: 1121.75 mg/100g dry matter) and several amino acid (glutamic acid: 8.07 g/100 g dry matter) have been reported (Chivandi et al., 2013). The family Combretaceae is the source of a wide range of tannins, flavonoids, terpenoids, stilbenes and lignans that are present in the stem bark and roots (Eloff et al., 2008). A brief description of the secondary metabolites produced by *T. sericea* is provided in the following sections.

2.4.1 Terpenoids

Terpenoids are the largest groups of natural products with approximately 25,000 compound structures reported (Gershenzon and Dudareva, 2007). They exist in diverse chemical forms as an array of linear functionalized hydrocarbons or as chiral, carbocyclic skeletons with diverse chemical modifications that include substitution with hydroxyl, carbonyl, ketone, aldehyde and peroxide groups (Ajikumar et al., 2008). Terpenoids can be generally classified as hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetratepenes and polyterpenes (Zwenger, 2008) depending on the number of isoprene units contained in the structure.

Arjunic acid (**1**), an oleanane pentacyclic (Figure 2.2) terpenoid was isolated by Eldeen et al. (2008) from the ethyl acetate root extract of *T. sericea*. The compound was first isolated by Row et al. (1970) from the stem bark of *Terminalia arjuna*. It has also been reported in *Terminalia fagifolia* stem bark (Garcez et al., 2006). Sericic acid (**2**), sericoside (**3**), the glycoside of sericic acid, and arjungenin were isolated from the aqueous methanol root extract of *T. sericea* (Bombardelli et al., 1974; Joseph et al., 2007). Lupeol (**4**) was isolated from the acetone extract of *T. sericea* stem bark (Nkobole, 2011). Other Terpenoids, β-sitosterol, β-sitosterol-3-acetate and stigma-4-ene-3-one were isolated by Nkobole et al. (2011) from the stem bark.

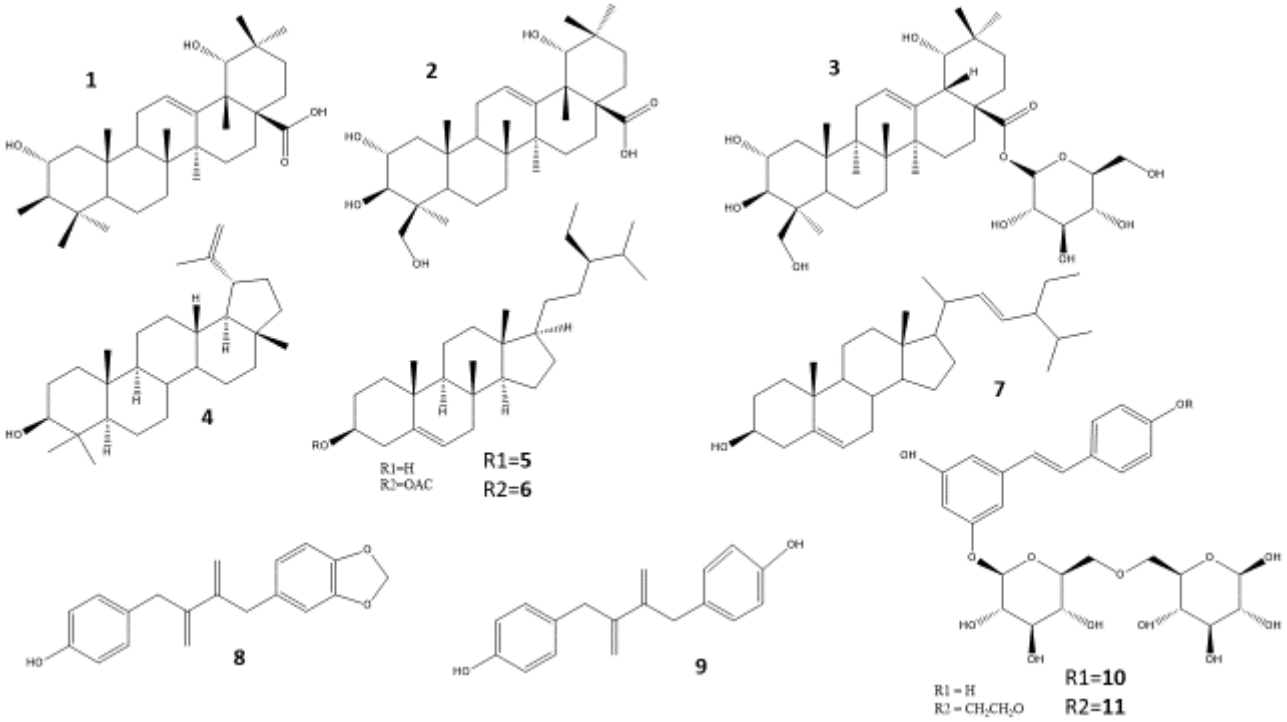

Figure 2.2: Chemical structures of terpenoids i.e. (1) arjunic acid, (2) sericic acid, (3) sericoside, (4) lupeol, (5) β-Sitosterol, (6) β-sitosterol-3-acetate, (7) stigma-4-ene-3-one; lignins i.e (8) termilignan b, (9) anolignan b, and stilbene i.e. (10) resveratrol-3-O-β-rutinoside, (11) 3,5-dihydroxy-4-(2-hydroxy-ethoxy) resveratrol-3-O-β-rutinoside isolated from *Terminalia sericea*

2.4.2 Lignans

Lignans are a group of plant secondary metabolites that are produced by oxidative dimerization of two phenyl propanoid units (Yang et al., 2015). Lignans produced by *T. sericea* are the linear lignan or dibenzylbutane-type (Gnabre et al., 2015). Anolignan B (**5**) was isolated from the ethyl acetate root extract of *T. sericea*. It was first isolated from *Anogeissus acuminata* (Eldeen et al., 2006). Termilignan B (**6**) was first isolated from *T. sericea* root by Eldeen et al. (2008).

2.4.3 Stilbenes

Stilbenes are natural non-flavonoid phenolic compounds that are synthesized through the Phenylpropanoid pathway (Miliovsky et al., 2013). They occur in plants in the form of hydroxylated stilbenes and not as free stilbenes (Simoni et al., 2009). Stilbenes act as phytoalexins that defend the plants against pathogens

and parasites, but also protect against injuries and ultra-violet irradiation (Kasiotis et al., 2013; Paul et al., 2010). Studies have shown that they possess anti-oxidant, antibacterial, antifungal, cardioprotective, neuroprotective, anti-aging and anticancer properties (Frantík et al., 2013; Handler et al., 2007). Joseph et al. (2007) isolated stibenes 3',4,5'-trihydroxyresveratrol-3-*O*-β-rutinoside glycoside (**7**), and 3'5'-dihydroxy-4-(2-hydroxy-ethoxy) resveratrol-3-*O*-β-rutinoside, from an ethanolic root extract of *T. sericea*. 3'5'-Dihydroxy- 4-(2-hydroxy-ethoxy) resveratrol-3-*O*-β-rutinoside was reported as a new compound. Resveratrol-3-*O*-β-rutinoside was previously isolated by Bombardelli, (1975) from the root. None of the stilbenes isolated from the plant has been linked to any of the ethnomedicinal properties of *T. sericea*.

2.4.4 Tannins

The tannin-rich *Terminalia* species owe most of their biological activities such as antimycobacterial activities and antibacterial activity to the tannin content (Ajala et al., 2014; Fyhrquist et al., 2014). In other *Terminalia* species, punicalgin was isolated from *T. oblongata*, while arjunin, casuarinin, and elagic acid were isolated from *T. arjuna* (Eloff et al., 2008; Jain et al., 2009). However, the only tannins to be isolated from *T. sericea* was an inseparable mixture of epicatechin-catechin and gallocatechin-epigallocatechin, isolated from the acetone extract of the stem bark (Nkobole et al., 2011).

2.5 PHARMACOLOGICAL ACTIVITIES

2.5.1. Antibacterial activity

Traditional healers commonly prescribe *T. sericea* for the treatment of coughs, skin infections, diarrhoea, dysentery and general stomach conditions. Many researchers (Cock and van Vuuren, 2015; Cock and van Vuuren, 2014; Eldeen et al., 2006; Eldeen et al., 2005; Eloff, 1999; Fyhrquist et al., 2002; Mabona and Van Vuuren, 2013; Netshiluvhi and Eloff, 2016; Steenkamp et al., 2004; Tshikalange et al., 2005; van Vuuren et al., 2015; York et al., 2012) have investigated the antibacterial activities of the acetone, chloroform,

dichloromethane/methanol, ethyl acetate, ethanol, methanol and aqueous extracts of the leaves, stems and roots against a total of 29 pathogens. Gram-positive bacteria studied: *Bacillus cereus*, *Bacillus subtilis, Bacillus pumilus, Brevibacillus agri, Micrococcus luteus, Enterobacter cloacae, Enterococcus faecalis, Microsporum canis, Propionibacterium acnes, Staphylococcus aureus,* Gentamycin- methicilline resistance *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus, Staphylococcus epidermidis, Sarcina* species and *Trichophyton mentagrophytes*. Gram-negative bacteria include: *Alicaligenes faecalis, Aeromonas hydrophilia, Citrobacter freundi, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Moraxella catarrhalis, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella typhimurium, Serratia marcescens, Shigella flexneri* and *Shigella sonnei.* Both the minimum inhibition assay (Table 2.1) and the disc diffusion method (Table 2.2) were used to evaluate the activities of the crude extracts. Only the bacteria that were tested in the current study are listed in these tables. These bacteria are *B. cereus*, *S. aureus*, *E.coli*, S. *typhimurium*, *S. sonnei*, *K. pneumoniae*, *P. aeruginosa* and *S. epidermidis.*

The antibacterial activity of the roots were most frequently studied using the microdilution assay, (Table 2.1), while the activity of the leaves were the most commonly studied by applying the disc diffusion assay (Table 2.2). No reports on the antibacterial activity of the fruit could be found. The aqueous extract was tested against the most pathogens, probably because it mimics the traditional use (Madikizela et al., 2013), followed by a 1:1 mixture of dichloromethane and methanol. Chloroform extracts were seldom tested and had the weakest activity (Priyavardhini et al., 2009). The aqueous extract exhibited good inhibitory activity (less than 1 mg/mL) against *B. cereus, M. canis, P. acnes, S. epidermidis, S. aureus,* Gentamicin-Methicillin-resistant *Staphylococcus aureus* (GMRSA), Methicillin-resistant *Staphylococus aureus (*MRSA) and *T. mentagrophytes*. The organic extract (dichloromethane/methanol) also displayed good activity against *B. cereus*, *B. agri*. *E. faecalis*, *P. acnes*, *S. epidermida*s, *S. aureus*, GMRSA, MRSA and *T. mentagrophytes*. The stems showed better activity against *S. aureus*, compared to the roots and leaves, in all the studies reported (Fyhrquist et al., 2002).

Several crude extracts of *T. sericea* acts also exhibited good activity against Gram- negative bacteria in the microdilution assay. Aqueous, dichloromethane, and methanol extracts exhibited good activity against *K. pneumonia*, while acetone and dichloromethnane extracts were highly active against *P. aeruginosa*. Aqueous, dichloromethane and methanol extracts displayed good activity against *P. mirabilis*, *P. vulgaris*, *S. typhi and S. flexneri*, when compared to the positive control. In the disc diffusion assay (Table 2.3), methanol and aqueous crude extracts were the most frequently tested against the pathogens. The aqueous extract resulted in an inhibition range of 6-36 mm, while the inhibition range of the methanol crude extract was 7-35 mm. The positive controls used were ampicillin, yielding zones of 11-15 mm, and ciprofloxacin with a range of 6-11 mm. These studies suggest that *T. sericea* is a potential source of broad spectrum antibiotics and indicate that polar compounds are responsible for the antibacterial activity.

Several bacteria including *S. aureus,* MRSA, GMRSA*, S. epidermidas, P. aeruginosa, B. agri, P. acnes, T. mentagrophytes* and *M. caris* are associated with skin-related infections (Mobona et al., 2013). The bacteria *B. cereus*, *E. faecalis*, *E. coli*, *P. vulgaris, S. typhi, S. flexineri* and *S. aureus* cause stomach ailments and diarrhoea (van Vuuren et al., 2015). These studies have validated the use of *T. sericea* for the treatment of stomach conditions and diarrhoea. However, there is limited research linking these activities to specific compounds present in the plant. Although Eldeen et al. (2005) isolated anolignan b, termilignan b and arjunic acids as the antibacterial compounds, their research work was focused mainly on the roots and bark. In addition, they did not have a variety of samples from different geopgraphic areas which will allow the chemical composition and the antibacterial acitivity to be correlated.

Table2.1 Minimum inhibitory concentrations (MIC values) in mg/mL determined for *T. sericea* crude extracts against Gram-positive bacteria as reported in literature

Extract/ Antibiotic Standard (µg/mL)	Plants part used	*Bacillus cereus* ATTC 11778	*Staphylococcus epidermidis* ATTC 2223	*Staphylococcus aureus* ATTC 6538	*Staphylococcus aureus* ATTC 12600	*Staphylococcus aureus* ATTC 25925	References
AC	Leaf Stem Root	- - -	- - -	1.0 0.5 -	- 0.78; 0.75 1.56	- - 1.6	Mongalo et al., 2016
AQ	Leaf Stem Root	0.38 - -	- - 0.25	- - 1.0	- - -	Not Active	Cock and van Vuuren, 2015
CF	Leaf Stem Root	- - -	- - -	- - -	- - -	- - Not Active	Mabona et al., 2013
DM	Leaf Stem Root	- 0.50 -	- - 0.25	1.33 0.50 -	- - -	- - 1.6	Eldeen et al., 2005 Eldeen et al., 2006
EA	Stem Root	- -	1.56 0.39	- -	1.56; 0.31 1.56	- -	Fyhrquist et al., 2002
ET	Leaf Stem Root	- - -	- 0.78 1.56	- - -	- 1.56 1.56	- - -	Eldeen et al., 2006
MT	Stem Root						Cock and Van Vuuren 2015
Ciprofloxacin		0.5	0.47	0.31	0.8	0.69	Cock and Van Vuuren 2015
Ampicillin			1.25			1.25	Mongalo et al., 2016

AC: ACETONE, AQ: AQUEOUS, CF: CHLOROFORM, DM: DICHLOROMETHANE; EA: ETHYL ACETATE, MT: METHANOL, ET: ETHANOL

Table2.2 Minimum inhibitory concentrations (MIC values) in mg/mL determined for *T. sericea* crude extracts against Gram-negative bacteria as reported in literature

Extract	Plant part used	*Escherichia coli* ATTC 8739	*Escherichia coli* ATTC 11775	*Klebsiella pneumoniae* ATTC 13883	*Pseudomonas aeruginosa* ATTC 27858	*Salmonella typhimurium* ATTC 14028	References
AC	Leaf Stem Root	- - -	- - -	- - -	- - 4.0	- - -	Eldeen et al., 2005 Eldeen et al., 2006
AQ	Leaf Stem Root	- 8.0 -	- 0.78 1.56	0.32, 1.0 0.78, 0,83 Not Active	- - -	- 1.00 -	Cock and Van Vuuren, 2015
DM	Stem Root	1.0 -	- -	- -	0.25 -	- -	Mongalo et al., 2016
EA	Leaf Stem Root	- - -	- 1.56 1.56	- 1.56 0.78	- - -	- 0.42 -	Mabona et al., 2013
ET	Stem Root	- -	3.13 1.56	- -	- -	- -	Mabona et al., 2013
MT	Leaf	-	-	0.25	0.31	-	Eldeen et al., 2006
Ciproflaxin	-	0.02	-	0.78, 0.08	-	-	Eldeen et al., 2006
Neomycin	-	-	3.1	-	-	-	

AC: ACETONE, AQ: AQUEOUS, CF: CHLOROFORM, EA: ETHYL ACETATE, MT: METHANOL, ET: ETHANOL

Table 2.3: Zones of inhibition (mm), determined in the disc diffusion assay, of various *T. sericea* crude extracts against Gram-positive bacteria as reported in the literature

Extract/ Antibiotic Standard (µg/mL)	Plants part used	*Bacillus cereus* ATTC 11778	*Staphylococcus epidermidis* ATTC 2223	*Staphylococcus aureus* ATTC 25923	References
AQ	Leaf Stem Root	14.8 - -	11.7 - -	12.3 - -	Cock and van Vuuren, 2015
ME	Leaf Stem Root	- 12.7 -	14 - -	- - -	Cock and Van Vuuren 2015
Ampilicillin		15.3	-	11	Cock and Van Vuuren, 2015
Ciprofloxacin		6.3	-	15	Cock and Van Vuuren 2015

AQ: aqueous, ME: Methanol

Table 2.4 Zones of inhibition (mm), determined in the disc diffusion assay, of various *T. sericea* crude extracts against Gram-negative bacteria as reported in the literature

Extract / Antibiotic Standard (µg/mL)	Plant part used	*Escherichia coli* ATTC 8739	*Klebsiella Pneumoniae* ATTC 13883	*Pseudomonas aeruginosa* ATTC 27858	*Salmonella typhimurium* ATTC 14028	*Shigellla sonnei* ATTC	Reference
AQ	Leaf Stem Root	11.7 - 0	12 - -	- - -	- - -	10	Moshi and Mbwambo, 2005
EA	Leaf Stem Root	- - 0	- - -	0 - -	6.3 - -	- - -	Moshi and Mbwambo, 2005
ME	Leaf Stem Root	0; 12 - -	13 - -	- - -	13.3 - -	11.7 - -	Moshi and Mbwambo, 2005
Ampicillin		51; 14.7	10.3	12.3	11.3	12.0	Moshi and Mbwambo, 2005
Ciprofloxacin		15.3	12.7	13	12	12.3	Moshi and Mbwambo, 2005

AQ: aqueous, EA: ethyl acetate, ME: Methanol

2.5.2 Antidiabetic activity

Root extract of *T. sericea* was found to exhibit higher *in vitro* α-glucosidase and β- glucoronidase activities (IC_{50} = 92 µg/mL) than acarbose (IC_{50} = 131 µg/mL), a known antidiabetic drug (Tshikalange et al., 2008). An in *vitro* study of the antidiabetic activity of the acetone extract of the stem bark (Nkobole *et al*., 2011) indicated that compounds isolated from the extract i.e. β-sitosterol and lupeol, displayed the best inhibitory activity against α-glucosidase (IC_{50} = 54.5 µM and 66.48 µM, respectively) compared to β- sitosterol-3-acetate, stigma-4-ene-3-one and inseparable sets of mixtures of isomers (epicatechin-catechin and gallocatechin-epigallocatechin). Only β-sitosterol and lupeol were tested for their inhibitory activity against α-amylase (IC_{50} = 216.02 µM and 140.72 µM).

2.5.3 Anti-HIV-1 activity

The activity of the crude methanol extract of the leaves against HIV-1 reverse transcriptase was evaluated by measuring the inhibition of the extract against RNA- dependent DNA polymerase and ribonuclease H. The crude extract exhibited strong inhibition of polymerase (IC_{50} = 7.2 µg/mL) and ribonuclease H (IC_{50} = 8.1 µg/mL) (Bessong et al., 2004). Using the HIV-RT colorimetric ELISA kit, the root crude extract exhibited a strong inhibitory activity with lower IC_{50} (43 µg/mL) compared to adriamycin® (100 µg/mL), which was used as the positive control (Tshikalange et al., 2008).

2.5.4 Antimycobacterial activity

The dichloromethane, ethyl acetate and ethanol extracts of the leaves, stem bark and roots were tested against *Mycobacterium aurum* A^+. Of the dichloromethane extracts, only the stem bark was active (3.12 mg/mL) at the tested concentrations. The root extract was the most active of the ethyl acetate extracts (1.56 mg/mL), while the leaf extract was the most active of the ethanol extracts (0.78 mg/mL) (Eldeen and Van Staden, 2007). Termilignan

B and arjunic acid, isolated from the roots, did not show significant activity against *M.aurum* A+ (McGaw et al., 2008). The
acetone extract of the stem back exhibited an activity of 25 µg/mL against MTBH37 Ra, a mycobacterial strain and a clinical isolate (Green et al., 2010). Fyhrquist et al. (2014) reported the activities of leaf and root fractions against *Mycobacterium smegmatis* (ATCC 14468). The activities of the leaf fractions were lower than those of the root fractions. The butanol fraction of the root displayed a higher activity (35.5 mm inhibition zone; 1.562 mg/mL) compared to the crude extract (31.5 mm; 3.125 mg/mL). The antimycobacterial studies reported so far suggest that the root extracts are more active than those prepared from the leaves.

2.5.5 Antifungal activity

The acetone extracts of the root and stem bark inhibited the growth of *Candida albicans* with MIC values of 1.88 mg/mL and 0.94 mg/mL, respectively. The MIC values for the activities of the extracts against Candida *krusei* were root = 0.94 mg/mL; bark = 3.97 mg/mL), *Cryptococcus neoformans* (root > 7.5 mg/mL; bark =1.88 mg/mL), but the activities were not noteworthy (Samie et al., 2010). Acetone, hexane, dichloromethane and methanol leaf extracts displayed noteworthy MIC values (below 1 mg/mL) against *C. albicans*, *C. neoformans, Aspergillus fumigatus, Microsporum canis* and *Sporothrix schenckii* (Masoko et al., 2005). In another study, acetone bark extract was tested against *Fusarium* species. The extract yielded an MIC value of 1.9 mg/mL against *F. verticilloides, F. oxysporum, F. proliferatum and F. graminearum*. Noteworthy activity (MIC 0.95 mg/mL) was obtained only against *F. nygamai* (Samie and Mashau, 2013). Methanol and water extracts of the roots displayed similar inhibitory activity (MIC values 2.65–1.10 mg/mL) against both typed and clinical isolates of *C. albicans* (Steenkamp et al., 2007).

2.5.6 Anti-oxidant activity

Methanolic and ethyl acetate root extracts of *T. sericea* exhibited DPPH radical scavenging activity (IC_{50} = 14.7 µg/mL; > 125 µg/mL, respectively) and

ABTS radical inhibition (IC_{50} = 3 µg/mL; 74.6 µg/mL, respectively) (Adewusi and Steenkamp, 2011). The stem bark also exhibited higher reducing power (IC_{50} = 6.0 µg/mL) than the leaves (IC_{50} = 35.5 µg/mL) and roots (IC_{50} = 35.7 µg/mL). The DPPH free radical scavenging activity of the acetone extract of the stem bark revealed that lupeol (IC_{50} = 3.66 µM) and the inseparable mixtures of epicatechin-catechin (IC_{50} = 2.69 µM) and gallocatechin-epigallocatechin (IC_{50} = 1.64 µM) are responsible for the anti- oxidant activity of the stem bark (Nkobole et al., 2011).

2.5.7 Anti-inflammatory activity

Eldeen et al. (2005) reported on the anti-inflammatory activities of ethyl acetate, ethanol and aqueous stem and root extracts of *T. sericea*, using cyclooxygenase (COX-1 and COX-2) assays. Both plant extracts displayed better COX-1, compared to COX-2 activity. The ethyl acetate extracts of stems and roots were more active than their corresponding ethanol and aqueous extracts. The trend in activity was as follows: ethyl acetate > ethanol > aqueous. The ethyl acetate extract of the stem inhibited COX-1 and COX-2 (90%, 41%, respectively) and the activity was higher than that of the root extract (85%, 37%, respectively). However, the ethanol extract of the root displayed higher COX-1 and COX-2 activity (78%, 32%) compared to stem extract (72%, 20%). It cannot be concluded from the study that the stems exhibit higher anti-inflammatory activity than the roots, since the IC_{50} values of the extracts were not determined.

A further study by the same authors (Eldeen et al., 2006) revealed that anolignan B, isolated from the ethyl acetate root extract exhibited good activity (COX-1: 81%, IC_{50} = 1.5 mM; COX-2: 71%, IC_{50} = 0.186 mM). However, these activities were lower than that of indomethacin, a standard anti-inflammatory drug (COX-1: 78%, IC_{50} = 0.003 mM; COX-2: 70.2%, IC_{50} = 0.186 mM). Termilignan B and arjunic acid, isolated from the ethyl acetate root extract (Eldeen et al., 2008) exhibited a weak anti-inflammatory activity in the COX assays. Termilignan B yielded IC_{50} values of 78 µM (COX-1) and 156 µM (COX-2), while IC_{50} values of 36 µM (COX-1) and 253 µM (COX-2) were obtained for arjunic acid.

Previous anti-inflammatory studies by Eldeen et al. (2006) were based on *in vitro* assays. In contrast, Mochizuki and Hasegawa (2007) studied the anti-inflammatory effect of sericoside, a saponin isolated from the roots of *T. sericea*, using an experimental model of colitis in male rats. The study indicated that the compound was able to attenuate acute inflammatory colitis induced by ethanolic 2,4,6- trinitrobenzene sulfonic acid in male rats.

2.5.8 Anti-obesity activity

Sericoside, exhibited anti-obesity activity by exerting a strong lipolytic effect on differentiated 3T3-L1 cells (Mochizuki and Hasegowa, 2006).

2.5.9 Antiproliferation activity

Terminalia sericea methanolic root extract exhibited anti-proliferation activity against T24 bladder cancer with LD_{50} values of (29.3 µg/mL), HeLa cervical cancer (58 µg/mL) and MCF7 breast cancer cells (53 µg/mL). The leaf methanol extract was less active against all the respective cancer cells (61 µg/mL; 84 µg/mL; and 55.4 µg/mL (Fyhrquist et al., 2006).

2.6. CYTOTOXICITY

Terminalia sericea root extract exhibited significant toxicity against VK (monkey kidney) or Vero cells with an ID_{50} (dose that inhibits 50% cell growth) of 24 µg/mL (Tshikalange et al., 2005). The acetone extract and compounds isolated from the stem bark induced only weak toxicity (β-sitosterol: 197.72 µM; β-sitosterol-3-acetate: 482.25 µM; lupeol: 705.14 µM; epicatechin-catechin: 698 µM and gallocatechin- epigallocatechin: 653.02 µM) against Vero cell lines (Nkobole et al., 2011). The studies reported so far on the toxicity of *T. sericea* do not provide adequate information on the safety of the plant. There is a need for *in vivo* studies where the metabolism and potential biotransformation of the metabolite will occur in the liver. *In vitro* assays assume that parent drugs reach the target site without any transformation and also at concentrations

equivalent to the initial concentration. Limitations posed by transportation, absorption and excretion of drugs are not considered. This information can only be provided through an *in vivo* assay.

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CHAPTER THREE

ISOLATION AND CHARACTERISATION OF MAJOR ROOT CONSTITUENTS OF *TERMINALIA SERICEA*

3.1 ISOLATION OF PLANT METABOLITES

3.1.1 Introduction

Currently, one third of clinically used drugs are of natural origin (Xu et al., 2012). These drugs are either isolated from natural products, synthesized or semi-synthesized by structural modification of natural compounds. For example, aspirin, a known nonsteroidal anti-inflammatory drug was derived from salicyclic acid, a natural product isolated from *Salix alba*, the common willow (Daniel, 2006; Mahdi, 2010). Many widely used drugs, including morphine (pain killer), codeine (antitussive compound), quinine and artemisinin (antimalarial drugs), atropine (parasympathetic inhibitor), physostigmine (anticholinergic), ephedrine (anti-asthmatic), ergometrine (uterine contractor), santonin (anthelminthic), digoxin and deslanoside (cardiac glycosides) were isolated from plants (Xu et al., 2012). Secondary metabolites are purified from plants to determine their structures, physical properties and biological activities (Xu et al., 2012). The process of identifying these compounds involves samples preparation (drying methods), extraction, isolation, purification and structure elucidation of the compounds of interest (Bernard et al., 2014; Pham et al., 2015).

3.1.2 Isolation of compounds from *Terminalia sericea*

Previously, Bombardelli et al. (1974) extracted sericic acid and sericoside from the root of *T. sericea* by refluxing the dried root powder with methanol. The concentrated extract was partitioned between water and chloroform, and then water and butanol, to obtain two fractions. The chloroform fraction was subjected to silica gel column chromatography using ethyl acetate/methanol (95:5

as eluent to obtain sericic acid, while sericoside was isolated from the butanol fraction using chloroform:methanol:water (13:7:2, lower phase). In another study, Eldeen et al., (2006) isolated anolignan b from a root sample by subjecting the ethyl acetate extract to vacuum liquid chromatography by applying gradient elution, comprising various mixtures of hexane, ethyl acetate and finally methanol. The final purification was done by using preparative TLC. However, the method used was not clearly described. Resveratrol-3-rutinoside was isolated from the root by subjecting the ethanol extract to vacuum liquid chromatography, flash chromatography and size exclusion chromatography using Sephadex® LH-20 (Joseph et al., 2007). Arjungenin was isolated by Joseph et al. (2007), but the isolation method was not described.

The processes reported for the isolation of anolignan b, arjunic acid, arjungenin, resveratrol-3-rutinoside, sericic acid, sericoside, and termilignan b, required either the use of large solvent volumes or separation techniques that were not clearly described; making them difficult to follow. There is no available literature on the simultaneous isolation of the major constituents of *T. sericea* root. A relatively simple method for the isolation and purification of the major constituents present in the roots of *T. sericea* is described in the following sections.

3.2 MATERIALS AND METHODS

3.2.1 Sampling and extraction

Samples of *T. sericea* root were obtained from the campus of the University of Venda in June 2015 and were identified by Prof Peter Tshisikhawe of the Department of Botany, University of Venda. A voucher number PC01 was assigned and the voucher was deposited in the herbarium of the Department of Botany. The root samples were debarked and rinsed under running water to remove debris. The root barks were air-dried for two weeks and then ground to a fine powder using a Buchi grinder (Buchi, Switzerland). About 1.2 kg of dried sample was soaked in a mixture of dichloromethane and methanol (1:1) for 48

h. The solvent was removed from the filtrate under reduced pressure using a rotary evaporator (Buchi, Swizerland) at 40 °C to obtain 365 g of crude extract.

3.2.2 Fractionation

A flow diagramme detailing the isolation of the major compounds is presented in Figure 3.1. A portion of the crude extract (140 g) was dissolved in methanol and adsorbed on silica gel (300 g). The dried sample was loaded onto a silica gel (800 g, Kieselgel 60, Merck) column (65 cm x 4.0 cm), slurry packed in ethyl acetate. The compounds were initially eluted with ethyl acetate (2 L), and finally with a mixture of 5 L ethyl acetate and methanol (90:10) to obtain two fractions. It had been previously established in the laboratory that more than 50% of the root extract is soluble in ethyl acetate:methanol (90:10). Fraction 1 (F1), obtained from ethyl acetate (100%), yielded 18.56 g of dry matter, while Fraction 2 (F2), obtained using ethyl acetate:methanol (90:10), yielded 87.4.

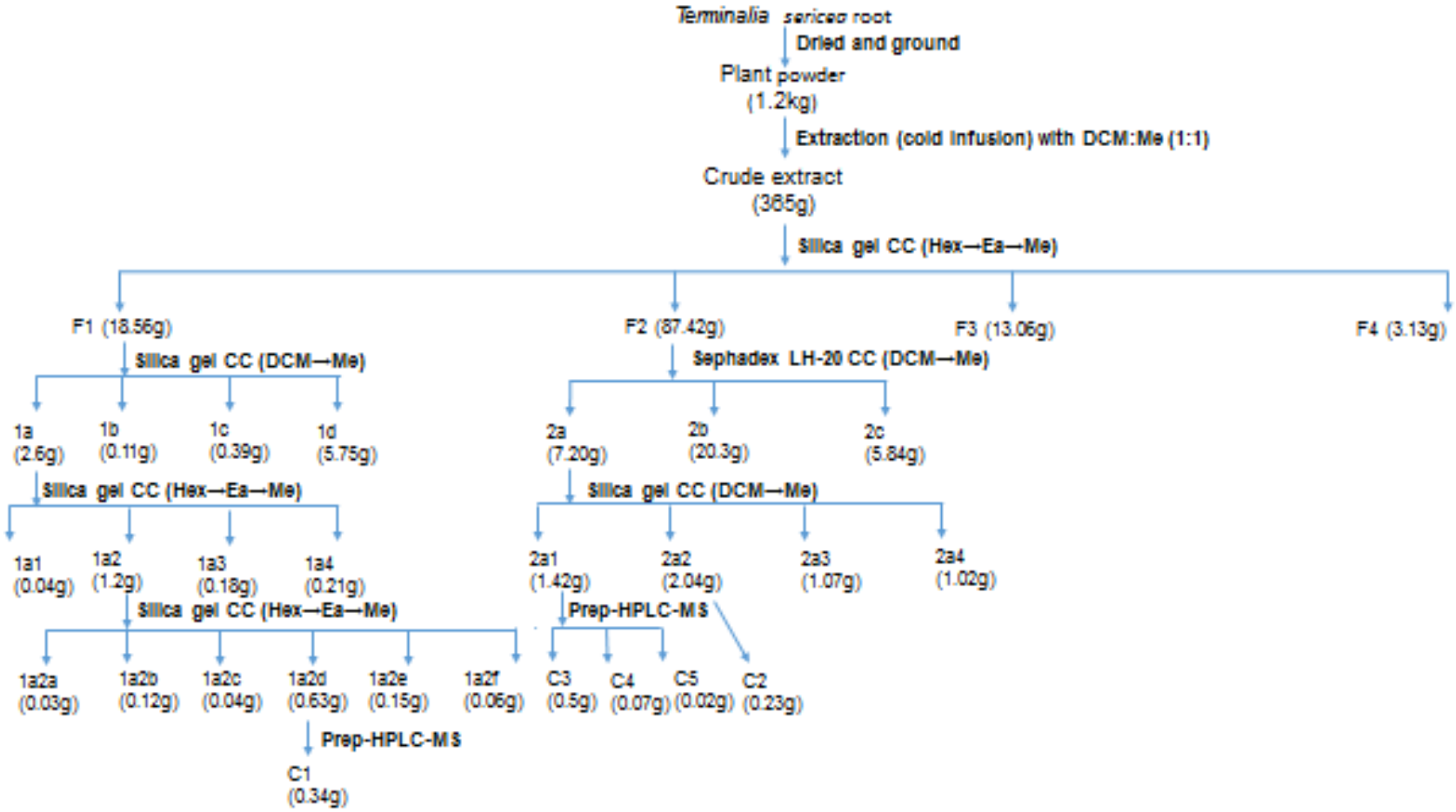

Figure 3.1 Flow chart showing the isolation and purification of pure compounds from the roots of *T. sericea*. DCM: dichloromethane, Ea: ethyl acetate, Hex: hexane, Me: methanol, F: fraction, CC: column chromatography

3.2.3 Purification of fractions

3.2.3.1.Fraction 1

Fraction 1 (12.6 g) was subjected to silica gel column chromatography. The compounds were eluted with mixtures of increasing polarity, consisting of varying concentrations of dichloromethane and methanol, to yield four fractions (F1a-d). The first three fractions i.e. $F1_a$ (2.6 g), $F1_b$ (0.1 g) and $F1_c$ (0.4 g) were obtained following elution with dichloromethane:methanol (9:1), while F1d (5.8 g) was obtained from dichloromethane:methanol (7:3). Fraction TRF1a was further purified on silica gel (Kieselgel 60) by gradient elution with hexane:ethyl acetate and ethyl acetate:methanol to obtain four fractions ($F1_a$1-4). To identify the fraction with the major peaks, each fraction was analysed using UPLC-MS. Fraction $F1a_2$ contained the compound of interest (*m/z* 469) and was further purified by silica gel column chromatography using gradient elution with a mixture of hexane, ethyl acetate and methanol to yield six fractions ($F1_a2_{a-f}$). Of these, $F1_a2_d$ (0.6 g) was further purified using preparative-high performance liquid chromatography-mass spectrometry (prep-HPLC-MS; as described in Section 3.2.5) to obtain pure Compound (**2)** (342 mg).

2,3,19,24-tetrahydroxyolean-12-en-28-oic acid (**2**) *(sericic acid)*. White crystals; HR- ESI-MS *m/z*: 527.336 $[M + Na]^+$ (calcd. for $C_{30}H_{48}O_6Na$, 527.334); IR: ν_{max} 3250, 2936, 1687, 1452, 1380, 1163, 1046, 1026 cm^{-1}. ^1H NMR (400 MHz, methanol-d_4): δ_H 5.34 (1H, t, *J* = 3.2 Hz, H-12), 4.05 (1H, d, *J* = 11.2 Hz, H-23) and 3.42 (1H, d, = 11.2 Hz, H-23), 3.80 (1H, m, H-2), 3.27 (1H, d, *J* = 4.0 Hz, H-19), 3.07 (1H, d, *J* = 9.2 Hz, H-3), 1.29, 1.23, 0.97, 0.96, 0.93, 0.74 (each 3H, s); ^{13}C-NMR (200 MHz, methanol-d_4): δ_C 180.9 (C-28), 143.2 (C-13), 123.1 (C-12), 84.5 (C-3), 80.9 (C-19), 68.1 (C-2), 64.7 (C-23); 27.1, 23.6, 23.5, 22.3, 16.1 and 15.9.

3.2.1.1.Fraction 2

About 60 g of Fraction F2 was subjected to open column chromatography using Sephadex® LH-20 as the stationary phase. Compounds were eluted with a mixture of dichloromethane:methanol by gradient elution, to produce three

fractions obtained with various ratios of dichloromethane:methanol: $F2_a$ (7.3 g) with a ratio of 90:10, Fraction $F2_b$ (20.2761 g) with a ratio of 70:30, and $F2_c$ (5.8414 g) with a ratio of 30:70. Thin layer chromatography and UPLC-MS analysis revealed that $F2_a$ contained the compounds of interest (*m/z* 536, 469_a, 469_b) and further purification was carried out. The latter fraction was further purified using silica gel column chromatography with gradient elution using mixtures of hexane:ethyl acetate and ethyl acetate:methanol. Fractions $F2_a1$ and $F2_a2$ were obtained and these were finally purified using prep-HPLC-MS, as described in Section 3.2.5, to yield Compounds **(10)** (238 mg), **(3)** (500 mg), **(12)** (74 mg) and **(13)** (23 mg).

3',5',4–trihydroxy-resveratrol-3-O-β-rutinoside (**10**). Brown solid; HR-ESI-MS *m/z* 537.1982 $[M + H]^+$ (calcd. for $C_{26}H_{32}O_{12}$, 536.1894). IR: ν_{max} 3285, 1589, 1512, 1445, 1040 cm^{-1}. ^1H NMR (400 MHz, methanol-d_4): δ_H 7.37 (1H, d, *J* = 7.6 Hz, H-2 or H-6); 6.99 (1H, d, *J* = 16.4 Hz, H-α), 6.84 (1H, d, *J* = 16.4 Hz, H-β), 6.77 (1H, d, *J* = 8 Hz, H-3 or H-5), 6.71 (1H, s, H-2'), 6.63 (1H, s, H-6'), 6.44 (1H, s, H-4'), 4.72 (1H,s, glucosyl or rhamnosyl H-1'' or H-1'''), 4.01 (1H, d, *J* = 10.8 Hz, glucosyl H-6''), 3.87 (1H, brs rhamnosyl H-3'''), 1.19 (1H, d, *J* = 5.6 Hz, rhamnosyl H-6'''); ^{13}C NMR (100 MHz, methanol-*d*4): δ_C 158.9 (C-3'), 158.1 (C-5'), 157.1 (C-4), 139.9 (C-1'), 128.8 (C-β), 128.6 (C-2 or 6), 127.5 (C-1), 125.2 (C-α), 115.1 (C-3 or 5), 106.7 (C-2'), 106.3 (C-6'), 102.6 (C-4'), 100.9 (glucosyl C-1''), 100.8 (rhamnosyl C-1'''), 16.5 (rhamnosyl C-6''').

2,3,19,24-tetrahydroxyolean-12-en-28-oic glucopyranoside (**3**) (*sericoside*). White solid; HRESI-MS: $[M + Na]^+$ *m/z* 689.389 (calcd. for $C_{36}H_{58}O_{11}$ 666.398). ^1H-NMR (400 MHz, DMSO-d_6): δ_H 5.24 (1H, d, *J* = 7.6 Hz, H-1'); δ_H 2.92 – 3.73 (glucosemoiety); δ_H 1.22, 1.09, 0.89, 0.87, 0.85, 0.61 (each 3H, S); ^{13}C-NMR (200 MHz, DMSO-d_6): δ_C 176.3 (C-28), 143.7 (C-13), 122.7 (C-12), 84.3 (C-3), 67.5 (C-2), 64.3 (C-24); glucose moiety: 97.6, 78.2, 77.2, 72.9, 69.9, 61.1.

3.2.4 Purity determination of isolated compounds using ultra performance liquid chromatography

The purity of the fractions during column chromatography was also monitored using the UPLC-MS. Targeted masses were identified from the chromatograms and were purified later using prep-HPLC. The purity analysis was evaluated by using a Waters Acquity Ultra Performance Liquid Chromatographic system with a photodiode array (PDA) detector (Waters, Milford, MA, USA) (Figure 3.2).

Figure 3.2 UPLC-QToF-PDA/MS instrument used for analysis of the semi-purified and purified compounds. Photograph taken by C. Anokwuru

An injection volume of 2.0 µL (full-loop injection) was used. Separation was achieved on an Acquity UPLC BEH C18 column (150 mm × 2.1 mm i.d., 1.7 µm particle size; Waters), maintained at 40 °C. The mobile phase consisted of 0.1% formic acid (Solvent A) and HPLC grade (Merck, Germany) acetonitrile (Solvent B) at a flow rate of 0.3 mL/min. Gradient elution was executed as follows: the initial ratio was 90% A:10% B, changed to 50% A:50% B within 4 min, to 50% A:50% B in 6 min, to 5% A:95% B in 2.5 min, maintaining for 0.5 min, before returning to the initial ratio in 0.5 min. The system was equilibrated for 2 min before the next analysis. A Micromass– LCT Premier quadrupole time-of-flight mass spectrometer (QToF-MS) (Waters, Milford, MA, USA) detector was

hyphenated with the UPLC, using the same conditions as before. Both positive and negative electrospray ionisation (ESI) modes were evaluated, but the positive mode resulted in a greater abundance of ions and provided spectra with more information. Therefore, the MS was further operated in the positive mode. Nitrogen (600 L/h) was used as the desolvation gas and the desolvation temperature was maintained at 400 °C. Data were acquired between *m/z* 100 and 1200. The following settings were used for the mass spectrometer: capillary voltage 3500 V; sampling cone voltage 38 V; source temperature 100 °C.

3.2.5 Preparative HPLC-MS

Final clean-up of the isolated compounds was done using prep-HPLC-MS analysis. The chromatographic system comprised a AutoPurification system interfaced with a QDa mass spectrometer (Waters, Milford, MA, USA). Conditions were optimised to achieve chromatograms with better resolution in a short analysis time. An injection volume of 250 µL was applied. The three samples were prepared as 50 mg/mL solutions in methanol (HPLC grade, Merck). Separation was achieved on an XBridge Prep C18 column (250 mm x 19 mm i.d. 1.7 µm particle size, Waters) maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (Solvent A) and methanol (Solvent B) at a flow rate of 20 mL/min. Gradient elution was performed as follows: the initial ratio was 90% A:10% B, held for 1 min, changed to 52% A:48% B within 1 min, changed to 35% A:65% B within 5 min, to 5% A:95% B within 5 min, maintaining for 1.5 min, and back to the initial ratio in 0.5 min. Data were collected by chromatographic software MassLynx 4.1 (Waters, USA). Negative ionisation mode was selected. A probe temperature of 500 °C and a source temperature of 120 °C were selected. The capillary and cone voltages were set to 800 and 10 V, respectively. Data were collected between *m/z* 100 and 750. The eluents were fractionated as 220 drops/tube (about 2 mL) using a fraction collector. The target compounds (*m/z* 535, 469, 711a, 711b, 711c) were collected by the fraction collector. The number of each collected fraction was recorded and was used for the subsequent combination of common fractions, which were concentrated using a Genevac evaporator to give residues, which were subsequently analysed by UPLC- MS.

Figure 3.3 Preparative-HPLC-MS instrument used for the final isolation of compounds from semi-pure fractions of *T, sericea* (http://www.waters.com/waters/en_GB/AutoPurification-System accessed 06/16/2017)

3.2.6 Final purity determination of the isolated compounds

After the prep-HPLC analysis, the final purity of the compounds was determined using the procedure described in Section 3.2.4.

3.2.7 One and two dimensional nuclear magnetic resonance (NMR) spectroscopy

The spectra of the isolated and purified compounds were recorded on Bruker Ultra Shield^{TM} Plus 400 mHz (Biospin) (Bruker, Bellericea, MA, USA). Bruker Topspin 3.2 on AVIII 400 software was used to process the spectral data obtained. One dimensional (1D) ^{1}H NMR spectra were recorded at 400 MHz, while the ^{13}C NMR spectra were obtained at 100 MHz. The ^{1}H and ^{13}C chemical shifts were recorded in parts per million (ppm). Two dimensional (2D) experiments performed were: homonuclear correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC) and nuclear overhauser effect spectrometry (NOESY). The isolated compounds (20 mg) were dissolved in 1 mL of deuterated methanol (Methanol-d_4) or dimethylsulfoxide (DMSO-d_6), depending on the polarity of the

compounds. The dissolved samples were transferred to NMR tubes prior to analysis.

3.2.8 Determination of accurate mass of isolated cmpounds

The accurate masses of the isolated compounds were determined was determined as described in Section 3.2.4

3.2.9 FTIR spectral analysis of the isolated compounds

Fourier transform infrared spectral analysis of the isolated compounds was carried out using an alpha-P Bruker spectrometer mounted with an attenuated total reflectance (ATR) diamond crystal (Bruker OPTIK GmbH, Ettlingen, Germany) (Figure 3.4). Details of the procedure have been described in Section 4.2.6.

Figure 3.4 Fourier transform infrared (FTIR) spectroscopy instrument. Photograph taken by C. Anokwuru

3.3 RESULTS AND DISCUSSION

3.3.1 Isolation and purification of major compounds

A relatively simple method involving column chromatography was used for the isolation of four compounds from the roots. The method involved extraction of the root samples with dichloromethane:methanol to obtain crude extract, which was subjected to silica gel column chromatography to yield two major fractions following elution with ethyl acetate and ethyl acetate:methanol (90:10). Ethyl acetate was able to elute only compound **(2)** without the co-elution of compounds **(10)** and **(3)**. In an earlier isolation process, the inclusion of methanol in ethyl acetate led to the elution of **(10)** and **(3)**, without complete separation from **(2)**. Repeated column chromatography was sufficient to purify all compounds from their fractions, however, prep-HPLC was necessary to obtain pure compounds for analytical purposes, since further columns would have resulted in substantial compound losses.

3.3.2 Characterisation and structure elucidation of the isolated compounds

3.3.2.1 Spectroscopic data for Compound (2)

Compound **(2)** (Figure 3.5) was obtained as white crystals from F1, following elution with ethyl acetate (100%) from the silica gel column and purification with prep-HPLC. Spectral data obtained are similar to those reported in literature (Bombardelli et al., 1974; Hess and Monache, 1999; Rahman et al., 2005; Tchuenmogne et al., 2017). The molecular ion (*m/z* 527.336) obtained from the HR-ESI-MS is consistent with the formula $C_{30}H_{48}O_6Na$ with an exact mass of 527.334. Assignment of the ^{1}H and ^{13}C NMR signals for **(2)** is presented in Table 3.1. Spectra are provided in Appendix 1.

The ^{1}H spectrum (Appendix 1a) of sericic acid **(2)** displays an olefinic proton (1H) on C-12 at δ_H 5.34 ppm (t, J = 3.2 Hz). The triplet observed was due to the

splitting of the methylene proton (CH_2) on C-11. Two doublets at δ_H 4.05 ppm (*J* = 11.2 Hz) and δ_H 3.42 ppm (*J* = 11.2 Hz) were assigned to the hydroxymethylene (-CH_2OH-) protons at C-24. Three hydroxymethine protons (=CHOH-) at δ_H 3.80 ppm (ddd, *J* = 11.2, 9.6, 4.4 Hz), δ_H 3.32 ppm (d, *J* = 15.2 Hz), δ_H 3.04 ppm (d, *J* = 9.2 Hz) were assigned to C-2, C-19, and C- 3, respectively. Six singlet methyl protons observed at δ_H 1.29, 1.23, 0.97, 0.96, 0.93, 0.74 ppm were assigned to C-27, C-23, C-29, C-30, C-25 and C-26, respectively. In the ^{13}C NMR spectrum (Appendix 1b), the carboxylic group (C-28) signal was displayed at δ_H 180.9 ppm. The olefinic carbons, C-13 and C-12, were observed at δ_C 143.2 ppm and δ_C 123.1 ppm, respectively. The three hydroxymethine carbons C-3, C-19 and C-2 were displayed at δ_C 84.6, 81.0 and 68.2 ppm, respectively. The signal for the hydroxymethylene C-24 was observed at δ_C 64.7 ppm. The six methyl carbons C-27, C-23, C-29, C-30, C-25 and C-26 signals were displayed at δ_C 27.1, 23.6, 23.5, 22.3, 16.1 and 15.9 ppm, respectively. The proton and carbon spectral data for **(2)** are indicative of an oleanane-type triterpenoid and are consistent with literature (Gossan et al., 2016). The IR spectrum (Appendix 1g) indicates the presence of a hydroxyl group (3250 cm^{-1}), saturated C-H (2936 cm^{-1}), carboxylic function (1687 cm^{-1}), and olefins (1452 cm^{-1}) (Ali et al., 2006; Jossang et al., 1995; Patnaik et al., 2007).

The cross peak between δ_H 5.34 ppm and δ_H 2.01 ppm in the COSY spectrum (Appendix 1c) indicates a coupling between the methylene proton of C-11 and the methine proton of C-12 (δ_H 5.34 ppm). This is further established by the HMBC spectrum (Appendix 1e), where the cross peak can be observed between δ_H 2.10 ppm (C-11) and δ_C 123.1 ppm (C-12) and δ_C 143.2 ppm (C-13). The proton δ_H 3.04 ppm was assigned to C-3 based on the coupling with δ_H 3.8 (C-2) in the COSY spectrum. However, in the HSQC spectrum (Appendix 1d), δ_H 3.04 ppm displayed a cross peak with both δ_C 43.7 ppm (C-18) and δ_C 84.6 ppm (C-3), indicating that δ_H 3.04 ppm represents both H-18 and H-3. In the HMBC spectrum, the cross peaks observed between δ_H 3.04 ppm and δ_C 68.2 ppm (C-2), 81.0 ppm (C-19), 123.1 ppm (C-12), 143.2 ppm (C-13), and 180.9 ppm (C-28) confirm that δ_H 3.04 ppm are both H-3 (cross peaking with C-2) and H-18 (cross peaking with C-12, 13, 19 and 28).

Table 3.1 ^{1}H and ^{13}C NMR spectral data for sericic acid obtained in this study (exp) and from literature (lit) (Bombardelli et al., 1974; Cumbe, 2015; Hess and Monache, 1999)

Carbon No.	δ_H (ppm) m (*J* in Hz) exp.	δ_H (ppm) m (*J* in Hz) lit.	δ_C (ppm) exp	δ_C (ppm) lit
2	3.80, ddd, (11.2,9.6,4.4)	3.80, ddd, (11.0,9.5,4.4)	68.2	68.4
3	3.07, d, (9.2)	3.06, d, (9.5)	84.6	85.6
12	5.34, t, (3.2)	5.49, s	123.2	123.5
13			143.2	144.6
18	3.07, d, (9.2)	3.07, d, (4.0)	43.8	44.6
19	3.27, d, (4.0)	3.26 (4.0)	81.0	81.2
23	1.31, s	1.25, s	22.3	23.9
24	4.05, d, (11.2) 3.42, d, (11.2)	4.03, d, (11.2) 3.39, d, (11.2)	64.8	65.4
28			180.9	180.5

The location of the hydroxyl group at C-24 was deduced from the chemical shift of C- 23 (bearing a methyl proton δ_H 1.31 ppm) at δ_C 22.3 ppm, which is characteristic of an equatorial position (Ali et al., 2006; Gossan et al., 2016; Hess and Monache, 1999). In the NOESY experiment (Appendix 1f), the correlation between H-3 (δ_H 3.07 ppm) and H-23 (δ_H 1.31 ppm) and the correlation between H-24 (δ_H 4.05, 3.42 ppm) and H-25 (δ_H 0.93 ppm), further confirmed the placement of the hydroxyl function on C-24.

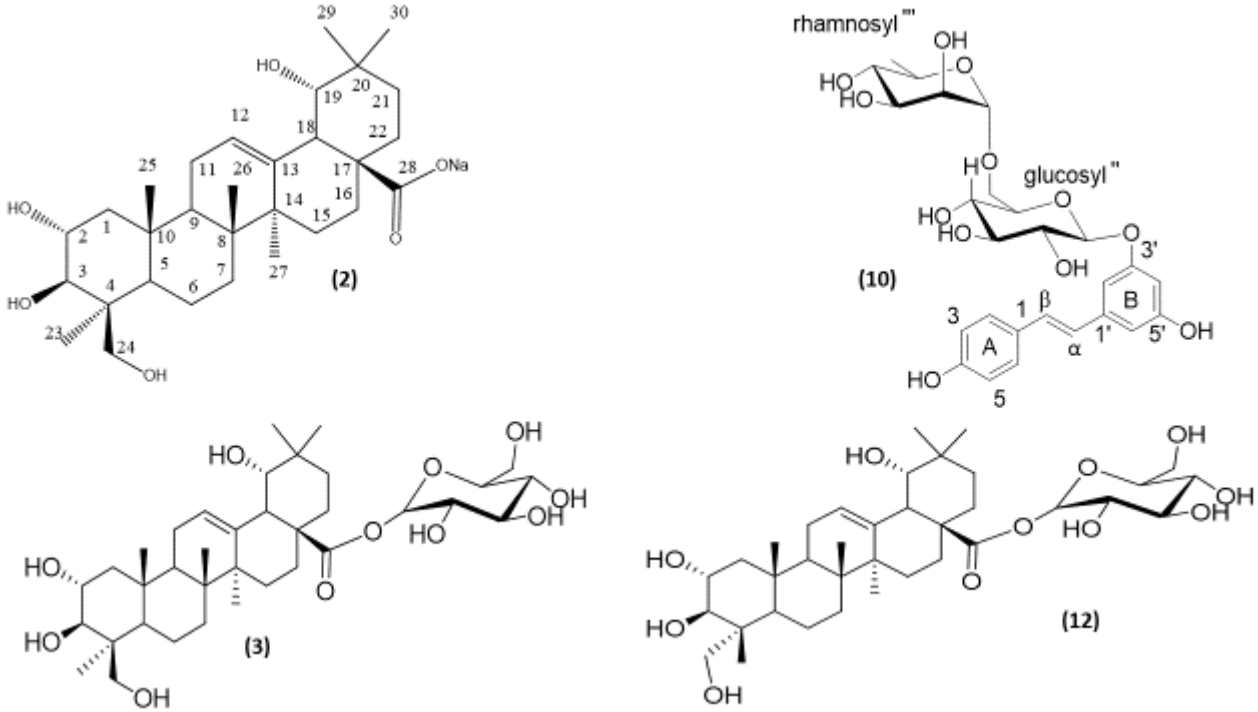

Figure 3.5: Compounds isolated from the roots of *T. sericea* (2) sericic acid, (10) resveratrol-3-rutinoside (3) sericoside (12) arjunglucoside I

3.3.2.2 Spectroscopic data for Compound (10)

Compound **(10)** (Figure 3.5) was obtained as a brown solid from Fraction F2 after elution with ethyl acetate:methanol (90:10) from silica gel. The molecular ion (*m/z* 537.336) obtained from the HR-ESI-MS is consistent with the formula $C_{26}H_{32}O_{12}$ $[M+H]^+$. The accurate mass (536.189) obtained is similar to that reported in literature (Wanjala and Majinda, 2001). The ^1H and ^{13}C spectral data (Appendix 2a and 2b) obtained are similar to those reported in literature (Table 3.2) for the structure of 3',5',4–trihydroxy-resveratrol-3-*O*-β-rutinoside (Bombardelli, 1975; Joseph et al., 2007; Wanjala and Majinda, 2001).

Table 3.2 ^{1}H and ^{13}C NMR spectral data for resveratrol-3-rutinoside obtained from this study (exp) and from literature (Joseph et al., 2007; Wanjala and Majinda, 2001)

Carbon No	δ_H (ppm) m (*J* in Hz) exp.	δ_H (ppm) m (*J* in Hz) lit.	δ_C (ppm) exp	δ_C (ppm) lit
1			127.5	128.8
2	7.37, d, (7.6)	7.49, d, (8.6)	128.6	128.0
3	6.77, d, (8.0)	6.78, d, (8.3)	115.1	115.6
4			157.1	157.4
5	6.77, d, (8.0)	6.78, d, (8.3)	115.1	115.6
6	7.37, d, (7.6)	7.49, d, (8.6)	128.6	128.0
A	6.99, d, (16.4)	6.99, d, (16.3)	125.2	126.0
B	6.84, d, (16.4)	6.85, d, (16.3)	128.8	129.3
1'			139.9	140.4
2'	6.71, s	6.65, s	106.7	107.6
3'			158.9	159.3
4'	6.44, s	6.38, s	102.6	103.7
5'			158.1	158.5
6'	6.63, s	6.65, s	106.3	106.3
Glu				
1''	4.72, s	4.84, s	100.8	101.3
6''	4.01, d, (10.8)	3.86, m	66.2	67.1
Rham				
1'''	4,72, s	4.60, s	100.9	101.5
3'''	3.87, brs	3.68, m	70.7	71.2
6'''	1.19, d, (5.6)	1.12, d, (6.0)	16.5	16.9

In ring A, a hydroxyl group is attached to C-4, while ring B has a hydroxyl group attached to C-5' and a disaccharide unit (glucose + rhamnose) at C-3'. In the A ring, the methine proton (H-2 or H-6) signal is displayed in the ^{1}H NMR spectrum at δ_H 7.37 ppm (d, *J* = 7.6 Hz) while the methine proton H-3 or H-5 signal can be observed at δ_H 6.77 ppm (d, *J* = 8 Hz). The signals for the two *trans*-olefinic protons are displayed at δ_H 6.99 ppm (H-α) and δ_H 6.84 ppm (H-β). In the B ring, singlet signals for H-2' and H-6' protons are evident at δ_H 6.71 and 6.63 ppm, respectively. The singlet signal for H-4' can be seen at δ_H 6.44 ppm. Furthermore, the glucosyl and rhamnosyl anomeric proton signals are both displayed at δ_H 4.01 ppm, while the methyl proton of the rhamnosyl H-6 can be was observed at δ_H 1.19 ppm (1H, d, *J* = 5.6 Hz). The IR spectrum (Appendix 2c) indicates the presence of a hydroxyl group (3285 cm^{-1}) and olefins (1589, 1445 cm^{-1}).

The first three signals of the ^{13}C NMR spectra were assigned to the three substituted carbons (C-3'- δ_C 158.9 ppm; C-5'- δ_C 158.1 ppm; C-4- δ_C 157.1 ppm). The signals for the olefinic carbons were observed at δ_C 128.8 ppm (C-β) and δ_C 125.2 ppm (C- α). The anomeric carbon for the glucose moiety was observed at δ_C 100.9 ppm, while the anomeric carbon for the rhamnose moiety was observed at δ_C 100. 8 ppm. The methyl C-6 signal for rhamnose was displayed at δ_C 16.5 ppm.

3.3.2.3Spectroscopic data for Compound **(3)**

Compound **(3)** (Figure 3.5) was obtained as a white solid from Fraction 2 following column chromatography. Compounds 2 and 3 were very difficult to separate and required repeated fractionation for isolation. Spectral data obtained for Compound 3 are consistent with those reported in literature (Table 3.3). The molecular ion (*m/z* 689.389) is consistent with the molecular formula $C_{36}H_{58}O_{11}Na$. The accurate mass (666.398) obtained is consistent with the literature value (Bombardelli et al., 1974). Furthermore, the presence of proton peaks δ_H = 2.92 – 3.61 ppm (Appendix 3a) and carbon signals δ_C = 94.6, 78.2, 77.2, 72.9, 69.97 and 61.1 ppm (Appendix 3b) in the NMR spectra, are an indication of the presence of a glucose moiety (Asres et al., 2001; Gossan et al., 2016). The glycosidic nature of Compound 3 is validated by the HSQC (Appendix 3c) and HMBC (Appendix 3d) spectral data and confirmed by the IR spectrum (Appendix 3e) indicating the presence of a carboxylic ester (1724 cm^{-1}) (Katerere et al., 2003).

In the HSQC data, there is a correlation between the anomeric proton δ_H 5.24 ppm (1H, d, *J* = 7.6) and the anomeric carbon δ_C 94.6 ppm of the glucose moiety. The HMBC also displays a correlation between the anomeric proton δ_H 5.24 ppm and the carbon (δ_C 176.3 ppm) of the carboxylic ester. Comparison of the carbon and proton spectra of Compound **(2)** and **(3)** indicates that the only difference between the two compounds is the glucose moiety. Therefore, Compound **(3)** is a glycoside of **(2)** and is referred to as sericoside.

Table 3.3 ^{1}H and ^{13}C NMR spectral data for sericoside obtained in this study (exp) and from literature (lit) (Asres et al., 2001; Bombardelli et al., 1974; Gossen et al., 2016)

Carbon No	δ_H m (*J* in Hz) exp.	δ_H m (*J* in Hz) lit.	δ_C exp	δ_C lit
2	3.61, ddd, (12, 10.8, 4)	3.72, dd, (12.5, 9.7, 4.5)	67.5	67.0
3	2.92	3.37, d, (9.7)	84.4	83.9
12	5.16, d, (5.6)	5.36, t, (3.5)	122.7	122.1
13			143.7	143.1
19	3.14, d, (4.8)	3.29, m	80.5	80.0
24	3.75, d,(10.0) 3.30, d, (10.4)	3.38, d, (11.1) 3.29, d, (11.1)	64.3	63.7
28			176.3	175.7
Glu				
1	5.24, d, (7.6)	5.4, d, (8.2)	94.6	94.0
2	2.92-3.61	3.34, dd (8.9,8.2)	72.9	72.3
3	2.92-3.61	3.42, t, (8.9)	77.2	76.7
4	2.92-3.61	3.37, t, (8.9)	69.9	69.5
5	2.92-3.61	3.7, m	78.2	77.6
6	2.92-3.61	3.70, dd, (12.2, 4.2)	61.1	60.6

3.3.2.4 Spectroscopic data for Compound (12)

Compound **(12)** was obtained as a white amorphous solid from Fraction F2 following column chromatography. The proton and carbon NMR spectra suggest that Compound **(12)** is a mixture of a triterpenoid aglycone and its glycoside. The proton spectrum (Appendix 4a) indicates the presence of an olefinic proton δ_H 5.35 ppm, hydroxymethylene protons at δ_H 3.81 and δ_H 3.49 ppm, three hydroxymethine protons at δ_H 3.67, 3.26 and 3.04 ppm and six methyl singlets at δ_H 0.69, 0.74, 0.93, 0.94, 1.02 and 1.29 ppm. These chemical shifts, are consistent with those of sericic acid as presented in Table 3.1. The carbon spectrum (Appendix 4b) indicates the presence of a carboxylic group (C-28) and olefinic carbons (C-13, C-12) with chemical shifts δ_C 177.2, 143.1, and 123.4 ppm, respectively. The carbon spectrum is consistent with that of sericic acid. However, the chemical shift (δ_C 177.2) of C-28 for Compound **(12)** is similar to that of sericoside (δ_C 176.3) as presented in Table 3.3. Other visible peaks on the ^{13}C NMR spectrum were δ_C 94.4, 77.3, 77.0, 76.8, 69.6 and 60.9. These

chemical shifts were consistent with those of sericoside (Table 3.3), indicating the presence of a glucose moiety (Patnaik et al., 2007). The tentative name arjunglucoside I is therefore assigned to Compound **(12).** The 2D spectra of compound **(12)** are not very clear for further confirmation of the structure. The presence of the glucose moiety and the *m/z* value of 527 in Compound **(12),** suggests it is a mixture of arjungenin and its glycoside, arjunglucoside I.

3.3.2.5 Spectroscopic data for Compound **(13)**

The proton NMR signals at δ_H 5.36 (d, *J* = 8.4), 4.02 (d, *J* = 11.6), 3.80 (d, *J* = 11.2), 3.67 (d, *J* = 9.2), 3.44 (d, *J* = 10.8), 3.04 (d, *J* = 9.2) and carbon signals at δ_C 175.5, 137.3, 126.3 and 94.4 ppm (Appendix 5) are characteristic signals of oleanane-type triterpenoid glycoside. These values are similar to the values in Table 3.3. No further analysis was carried out on this compound because it was not identified as a biomarker (Section 4.3.4.2) and it was not obtained as a pure compound (Figure 4.8).

3.3.3 Purity determination and Identification of major compounds using UPLC

The purpose of the isolation was to purify standards that can be used for the quantification of major constituents of *T. sericea* root. The purities of the isolated compounds were determined by analysing solutions prepared from each of the pure compounds (1 mg/mL) using UPLC. The UV response, measured by the PDA was used to estimate the purity of the compounds. The chromatogram of each compound is presented in Figure 3.6. The peaks observed between retention times 1.13 and 1.62 min are due to the solvent (methanol). The purity of the compounds were determined to be: resveratrol- 3-rutinoside (94.1%), sericic acid (100%), sericoside (100%) and arjunglucoside I (94.1%). The chromatograms also indicated the maximum absorption wavelength for each compound i.e. resveratrol-3-rutinoside (λ = 302 nm), sericic acid (λ = 199 nm), sericoside (λ = 201 nm) and arjunglucoside (λ = 201 nm).

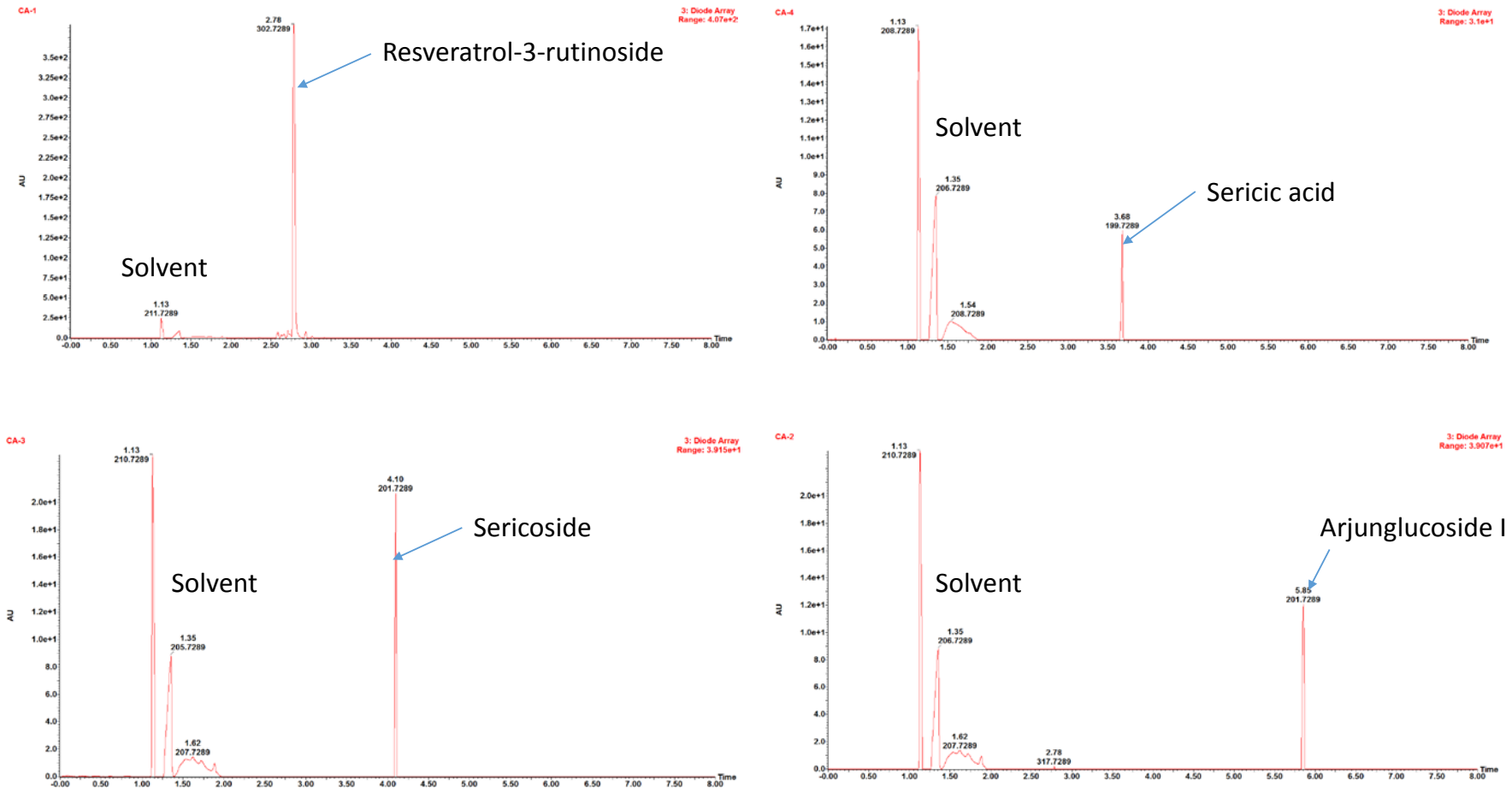

Figure 3.6 Chromatograms indicating the purity of resveratrol-3-rutinoside, sericic acid, sericoside and arjunglucoside

3.3.4 Summary

The following conclusions can be made:

- A simple and reproducible method for the isolation of sericic acid, resveratrol-3-rutinoside, sericoside and arjunglucoside I from the roots of *T. sericea* was developed. Previous methods reported by Bombardelli et al. (1974) and Joseph et al. (2007) required multiple steps and large volume of solvents.
- Resveratro-3-rutinoside, sericic acid and sericoside were obtained as pure standards after rapid final purification with prep-HPLC-MS. This highly sophisticated technique produced highly pure isolates in large quantities. These standards could then be used as analytical standards for the quantification of the compounds in root samples from various populations. The large amounts of each standard obtained also permitted the antibacterial and anti-oxidant activities of the pure compounds to be evaluated. .
- For the first time, resveratrol-3-rutinoside and arjunglucoside I were confirmed as major constituents of *T. sericea* root

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CHAPTER 4

QUALITY CONTROL OF *TERMINALIA SERICEA* ROOT

4.1 INTRODUCTION

The lack of quality control protocols for the majority of herbal medicines is a major challenge to their successful integration into the mainstream healthcare systems of many countries. The correct identification of the plant material and the quantification of the active constituents are the first steps in the quality control of herbal products (Rasheed et al., 2012). Proof of their efficacy and safety are required criteria for licensing the sale of herbal products. These criteria are largely dependent on the quality and chemistry of the raw material. However, it is very difficult to control the factors responsible for the variability of the chemical components (Builders et al., 2015; Eloff et al., 2011; Kaushik et al., 2014).

Quality of the herbal material, in turn, is dependent on the chemical variability of the active constituents, microbial contamination, presence of foreign matter and adulterants (Chun et al., 2010; Govindaraghavan and Sucher, 2015). Unfortunately, the active principles of most herbal medicines are not known and the consistency of different batches may be difficult to control due to differences in their origin or seasonal variations (Rasheed et al., 2012). Intra- and interspecies variations in the chemical constituents of herbal plants result from both genetic and environmental factors. Other factors include the methods of harvesting, drying, storage, transportation and extraction applied to the raw material. The inability to control this variation of the chemical constituents, ultimately makes it difficult to control the quality of herbal products (Eloff et al., 2011).

Chemical markers are chemically defined constituents or groups of constituents intended for the control of quality, regardless of whether they possess any therapeutic activity. These chemical markers are referred to as biomarkers when

they exhibit therapeutic activity. They can also be referred to as analytical markers when they are used mainly for analytical purposes.

Herbal medicines contain a myriad of constituents and in some cases, the therapeutic effects are not limited to the effects of a few isolated compounds, but arise from the combined effect of several compounds. Therefore, the use of a single or a few active markers to characterise the plant material may not be appropriate, since additional compounds may contribute to the additive or synergistic effects of the multiple components in the product (Li et al., 2008; Wah et al., 2012; Yongyu et al., 2011). The efficacy of medicinal drugs is dependent on the relative concentrations of the bioactive constituents. Some of the bioactive compounds may have low concentrations, and could be lost during the process of isolation and purification. It is therefore important to use appropriate analytical tools such as liquid chromatography coupled with mass spectrometry (LC-MS) for chemical profiling and identification of biomarkers of the medicinal plant (Hu and Xu, 2014; Tistaert et al., 2011).

Metabolomics refers to the comprehensive and quantitative analysis of metabolites in a biological system (Arapitsas et al., 2016; Tebani et al., 2016). This technique provides a detailed chemical profile of metabolites in a chemically complex sample, by spreading the information contained over time, thereby revealing underlying information of individual compounds (Bhatia et al., 2013; Tistaert et al., 2011). Metabolomic fingerprinting of herbal extracts can be used to standardise drugs and to establish the scientific basis of their pharmacological actions (Chatterjee et al., 2010). It also provides information regarding the relationship between chemical variation and differences in the corresponding therapeutic effects of samples (Zhong et al., 2016). Due to the complexity and dynamic nature of plant metabolites, a cocktail of platforms are needed to provide, as closely as possible, a full spectrum of metabolites (Xiao et al., 2012). Mass spectroscopy (in combination with gas chromatography or liquid chromatography), nuclear magnetic resonance (NMR) spectroscopy and Fourier transform infrared (FTIR) spectroscopy are techniques that are commonly used for the identification and quantification of metabolites (Vinaixa et al., 2016; Wang et al., 2016).

Gas chromatography-mass spectrometry (GC-MS) is an analytical tool commonly used for isolation and identification of volatile compounds (Tistaert et al., 2011). The technique offers high reproducibility of retention time and a variety of mass spectral databases, including the NIST (National Institute of Standard and Technology) database, are available for the identification of compounds. Although it is suitable for the determination of polar intermediates of primary metabolites, such as organic acids, amino acids, sugars and sugar alcohols, GC-MS cannot be used for the analysis of non-volatile compounds, unless they can be derivatised to form more volatile counterparts (Han et al., 2016; Lee et al., 2013).

Liquid chromatography (LC) is the most common technique for the separation of secondary metabolites, and can be coupled with detectors such as photodiode array, fluorescence, refractive index and mass spectrometers (Cuthbertson et al., 2013). Of these, LC-MS is the most widely used technology in metabolomics, due to its ability to separate and detect a wide range of molecules with high sensitivity. In this technique, the complex chemical mixture is separated into individual compounds that are visible on a chromatogram in the form of peaks, making it possible to discover novel or minor metabolites (Farag et al., 2012). The disadvantages of LC-MS include the poor reproducibility of retention time and mass spectra compared to GC-MS (Cox et al., 2014; Lee et al., 2013).

Nuclear magnetic resonance (NMR) spectroscopy is one of the commonly used analytical tools for plant metabolomics (Lee et al., 2016). This technique is based on the energy absorption and re-emission of the atomic nuclei due to variations in an external magnetic field. The proton (^{1}H) NMR is very useful because of the natural abundance of protons in biological samples. It is a fast and reproducible technique that can be used for simultaneous qualitative and quantitative analysis. In addition, it is non-destructive and requires minimal sample preparation. In NMR fingerprinting, the quantification of compounds can be carried out without any calibration, since the NMR signal of a given compound is proportional to its molar concentration. More so, the 2D spectra can be easily resolved and used to elucidate the structure of chemical constituents,

without the need for further purification (Agin et al., 2016; Khoo et al., 2015; Lee et al., 2016; Shen et al., 2016).

In metabolomic studies, either a targeted or untargeted approach can be followed. The targeted approach refers to the measurement of a set of known or expected metabolites. Analytical standards are used to identify and quantify peaks from the samples (Agin et al., 2016; Gorrochategui et al., 2016). The untargeted approach involves fingerprinting and refers to the measurement of as many metabolites as possible, without any prior knowledge of the nature and identity of the metabolites. In this approach, most of the metabolites are unknown or uncharacterized. The untargeted approach has an advantage over the targeted, because it can detect unexpected changes in the metabolic profile that cannot be detected in the targeted approach, since the focus there is not on the entire metabolite fingerprint (Alan et al., 2015).

Multivariate analysis tools, also known as “pattern-recognition methods”, are statistical methods used to transform and interpret chromatographic or spectroscopic data. These methods establish mathematical criteria that allow similarities between samples or clusters to be expressed quantitatively, giving a visual analysis of patterns or groupings within a dataset (Sandasi et al., 2013; Yi et al., 2016). Principal component analysis (PCA), hierarchical cluster analysis (HCA), and self-organisation mapping (SOM), are used to transform data when using the untargeted or unsupervised approach, while partial least square-discriminant analysis (PSL-DA), linear discriminant analysis (LDA), and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) are supervised methods, which require prior knowledge of the samples (Yi et al., 2016). Phytochemical studies have revealed the presence of anolignan b, termilignan b, arjunic acid, sericic acid, arjunglucoside I and sericoside in *T. sericea* roots. However, as far as could be established, there is no literature available regarding the chemical variability of these compounds. Anolignan b, termilignan b, and arjunic acid have been reported to display antibacterial activities. Chemical variations of these constituents can influence the biological activities of the plant. In this chapter, the chemical variability of the constituents of *T. sericea* root, collected from different populations, was investigated. The effect of the

chemical variability on the biological activities will be reported in the next chapter.

4.2 MATERIALS AND METHODS

4.2.1 Plant material and sample preparation

Root samples from 42 individual *T. sericea* trees were collected from 10 populations (labelled P1 to P10) across Limpopo Province (Table 4.1), between May and June 2015. Voucher specimens (CPA 001-010) were identified by Prof Tshisikhawe, University of Venda prior to their deposition at the Department of Botany, University of Venda. The root samples were debarked and rinsed thoroughly under running water to remove debris. The root samples were air-dried for two weeks and ground using a Buchi mixer (Buchi, Switzerland). A 5.0 g portion of each ground sample was soaked in 200 mL analytical grade (Merck, Germany) dichloromethane/methanol (1:1) for 24 h and filtered using Whatman No 4 filter paper. The filtrates were evaporated to dryness using a Buchi rotary evaporator (Buchi, Switzerland) at 45 ºC. The dried crude extracts were weighed and stored at -20^{o} C prior to analysis.

Table 4.1 Geographical locations and voucher numbers of *T. sericea* root samples

Population	Code	Location	GPS Co-ordinates	Samples	Voucher No
P1	BP	Bela-Bela/Pretoria axis (N1)	S24º47'51.9" E028º27'03.6"	3	CPA001
P2	G	Muyexe, Giyani	S23º11'22.6" E030º55'05.3"	4	CPA002
P3	J	Maila, close to the N1	S23º14'47.0" E029º53'06.8"	4	CPA003
P4	K	Along Punda Maria/ Kruger road	S22º58'22.0" E030º27'27.0"	5	CPA004
P5	MM	Mavambe, Malamulele	S23º00'02.1" E030º39'09.0"	4	CPA005
P6	MP	Mokopong/Pretoria axis along	S24º20'41.6" E028º53'47.4"	5	CPA006
P7	TSA	Tshandama, Thengwe	S22º45'43.0" E030º30'34.3"	5	CPA007
P8	TSH	Tshitavha, Sambandou	S22º44'41.2" E030º38'41.2"	5	CPA008
P9	TZ	Modjadjiskloof, along Tzaneen road	S23º33'14.4" E030º03'55.6"	5	CPA009
P10	V	Vuwani	S23º07'46.8" E030º22'46.9"	2	CPA010

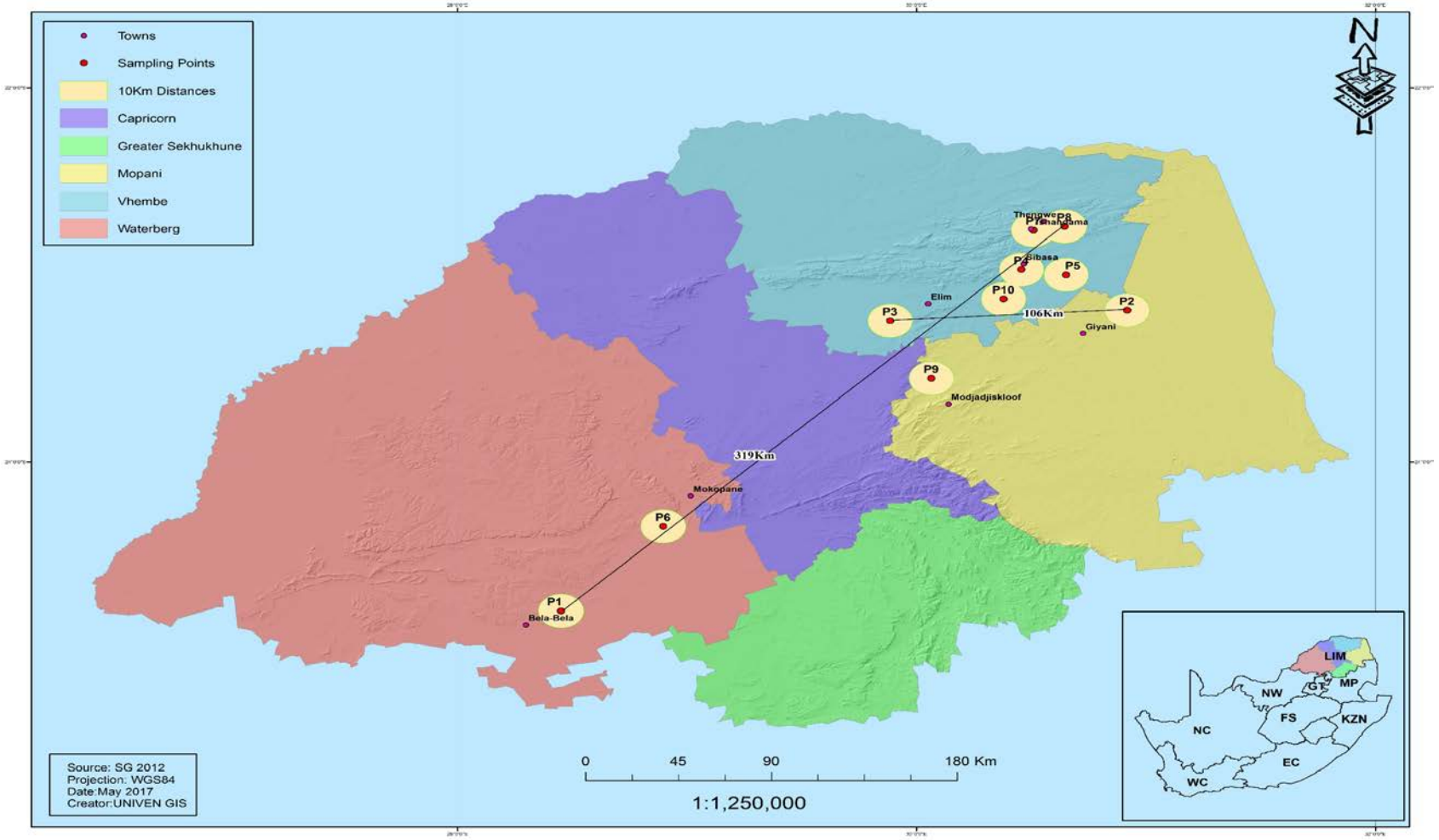

Figure 4.1 Map indicating the 10 sites where *Terminalia sericea* root samples were collected in the Limpopo province. Sampling points close to each other are more than 10 km apart

4.2.2 Method validation and quantification of isolated standards in root samples

A UPLC-QToF-PDA method, described in Section 3.2.6., was developed for the simultaneous quantification of sericic acid, resveratrol-3-rutinoside, sericoside and arjunglucoside I in the crude root extracts of all the samples. The method was validated for linearity, accuracy and precision. Initially, separate stock solutions, each with a concentration of 1.00 mg/mL, were prepared from the individual isolated standards dissolved in methanol. Calibration curves were constructed after analysing a dilution series of a mixture of the four standards over the concentration range 0.500-100 µg/mL. The limit of detection (LOD) and limit of quantification (LOQ) for each standard was calculated, following the construction of a calibration plot and regression analysis of the data, using the following equations (Miller and Miller, 2010):

$LOD = 3.3 \times \delta/S$ (Equation 4.1)

$LOQ = 10 \times \delta/S$ (Equation 4.2)

where δ is the standard deviation of the response and S is the slope of the obtained calibration curve.

The accuracy of the method was determined by evaluating the recovery of resveratrol- 3-rutinoside from an extract solution. An extract, containing a known concentration of resveratrol-3-rutinoside, was spiked with 2.50, 25.0 and 100 µg/mL of the standard solution in triplicate. Following analysis, the percentage recovery of resveratrol-3- rutinoside was calculated using Equation 4.3:

Recovery (%) = (recovered concentration/injected concentration) x 100%

(Equation 4.3)

The instrument precision was determined by intra- and inter-day analysis to establish the reproducibility of the method. A solution of resveratrol-3-rutinoside (10 µg/mL) was analysed three times daily, at different time intervals, over three days. The inter-day precision analysis was done by determining the relative

standard deviation (RSD) of the means obtained on each day. The percentage relative standard deviation (RSD) was calculated using the following equation:

RSD (%) = (SD/χ) x 100 (Equation 4.4)

where SD is the standard deviation and χ is the mean.

The inter- and intra-day repeatability of the method was determined by repeatedly analysing an extract containing a known concentration of resveratrol-3-rutinoside by UPLC-PDA at regular intervals during one day and over three days.

Once the method had been validated, the simultaneous quantification of the four isolated compounds in all 42 samples was carried out. The sample extracts were dissolved in an appropriate volume of HPLC grade methanol to yield 5 mg/mL extracts, which were filtered through 0.25 µm syringe filters (Acrosdisc®, Pall) prior to analysis.

4.2.3 Chemical profiling of crude extracts

The chemical profiles of the crude root extracts from 10 populations were obtained using the UPLC-QToF-MS method as described in Section 3.2.6. The crude extracts (5 mg/mL) were analysed and the PDA and MS chromatograms of individual samples were compared with each other. The peaks were aligned according to the retention times and *m/z* values using MarkerLynx™ software.

4.2.4 Chemometric analysis

The aligned UPLC-MS data and vibrational spectroscopy data were exported to Excel (Sandasi et al., 2011). After assignment of unique sample identifiers and secondary identifiers (population), the samples were imported into Simca 13.0 (Umetrics AB, Malmo, Sweden). For the chromatographic data, a principal component analysis (PCA) model was constructed after applying Pareto scaling. Potential outliers were identified by obtaining a scores plot. Hierarchical cluster analysis of the data was then carried out to determine sample clustering. From the PCA model, a loadings plot was constructed to identify metabolites that

contribute to the clustering observed in each quadrant of the PCA plot. This was followed by the construction of an orthogonal projection to latent structures discriminant analysis (OPLS-DA) model, based on the assignment of class identifiers to the samples. The S-plot and the column loadings plot, constructed from the OPLS-DA model, were used to identify biomarkers.

4.2.5 High performance thin layer chromatography (HPTLC)

The HPTLC system (Figure 4.2) consisted of a TLC 4 sampler, with an automated ADC2 development chamber, a chromatogram immersion device III, a Digistore Reprostar 3, and a TLC scanner 3 (CAMAG, Mutenz, Switzerland). An autosampler, fitted with a 25 µL syringe and connected to nitrogen gas, was used for sample application. The prepared plates were developed in the automatic chamber, while derivatisation was conducted using the chromatogram immersion device. Documentation of the developed plates was made possible by using a Reprostar 3 documentation unit. The entire system was controlled by the WINCATSR Version 1.4.4.6337 (Switzerland) planar chromatography software. The analysis was carried out after applying specific volumes of the crude extracts (10 mg/mL in methanol) and isolated compounds to 20 x 10 cm silica gel pre-coated glass plates (Silica gel 60 F254, Merck, Germany). Several developing solvents were tested, but finally the mixture comprising ethyl acetate:methanol:water (81:11:8) was selected for the analysis of the samples, because it resulted in optimal band separation and compact spots for the visualisation of the crude extracts and isolated compounds. After development, the plates were sprayed with 20 % sulfuric acid in ethanol and heated on a hot plate, until coloured spots became visible on the plate.

Figure 4.2 High performance thin layer chromatography system (CAMAG) consisting of A) automatic sampler, B) automatic developing chamber, C) reproster (scanner), D) plate heater. Photograph taken by C. Anokwuru

4.2.6 Vibrational spectroscopy analysis

Mid-infrared (MIR) spectra were recorded over the range 375-4000 cm^{-1} using an alpha- P Bruker spectrometer mounted with an attenuated total reflectance (ATR) diamond crystal (Bruker OPTIK GmbH, Ettlingen, Germany) as described by Sandasi et al. (2011). Spectra were acquired using OPUS 6.5 software. The ground samples were sieved (500 µm, Endecotts Ltd, United Kingdom) to obtain uniform particle size. Sample powders were placed directly onto the surface of the crystal and spectral data for each sample was captured in the absorbance mode. A total of 32 scans were accumulated for each sample with a spectral resolution of 4 cm^{-1}. The procedure was carried out in triplicate and the average spectra obtained in MS Excel®.

Near-infrared spectra were recorded over the range 4000-10000 cm^{-1}, in reflectance mode. A total of 32 scans were accumulated and averaged for each sample to obtain the average spectrum. All measurements were done in triplicate and the average spectra calculated in MS Excel®, before exporting to SIMCA P+

13.0 for chemometric analysis (Baranska et al., 2005). Each powdered and sieved sample (500 µm, Endecotts Ltd, United Kingdom) was transferred to a clear vial (Thermo Scientific Chromacol) and loaded into a NIRFlex N500 solid cell spectrometer for recording of NIR spectra. The vibrational spectroscopy data was handled in a similar fashion, but here various sample pre-processing methods (Multiplicative Scatter Correction, Standard Normal Variate and First, Second and Third derivatives) were applied, and the resulting models were evaluated by comparing the model statistics. The number of principal components (PCs), cumulative variation within the X (R^2X-cum) and the predictive ability of the model (Q^2-cum) were used to evaluate the models (Nsuala et al., 2017). Finally, PLS-DA models were constructed by using the spectral data as the X-variables and the concentration values obtained though UPLC- PDA for the respective isolated compounds, as the Y-variables (Sandasi et al., 2013).

4.3 RESULTS AND DISCUSSI

4.3.1 Sampling and extraction yield

The sampling sites of *T. sericea* root in Limpopo Province is illustrated on the map in Figure 4.1. Two populations each were collected from the Waterberg and Mopani districts, respectively. The most samples (six populations) were collected from the Vhembe district. The shortest distance between the closest populations is 10 km. The furthest distance was observed between P1 and P8 (319 km). Sampling was done based on the abundance of the trees in a locality.

Root samples from the 10 populations were extracted with dichloromethane:methanol to obtain crude extracts and the percentage yields are presented in Table 4.2. Initially, acetone, dichloroemethane:methanol (1:1), ethyl acetate, methanol and water were used for the extraction of metabolites from the root samples obtained from Population 4.

Table 4.2 Extraction yield (% dry weight) of dichloromethane:methanol (1:1) crude extract from *T. sericea* roots harvested from 10 populations (n = 42)

Population	Yield (%)
P1	25.9 ± 4.08
P2	24.1 ± 3.10
P3	19.9 ± 3.99
P4	33.7 ± 3.04
P5	25.8 ± 3.29
P6	17.9 ± 1.87
P7	20.7 ± 2.58
P8	25.1 ± 2.85
P9	15.1 ± 3.56
P10	20.7 ± 3.22
Overall mean	22.9 ± 5.22

The highest yield was found for the dichloromethane:methanol and methanol extracts. However, preliminary antibacterial activities (Section 5.3.2) indicated that the dichloromethane:methanol extract displayed better antibacterial activity compared to the methanol extract. Therefore, dichloromethane:methanol was used for the extraction of samples from the ten populations for both quantitative analysis and determination of antibacterial activities. This was to allow the direct comparison of the antibacterial activities with the chemical composition of the root samples. Furthermore, the mixture is also a good choice for the extraction of a combination of both non-polar and polar compounds from plants (Van Vuuren et al., 2015).

The highest extract yield was found for Population P4 (33.7%), while the lowest yield was obtained for samples representing Population P9 (15.1%). The highest variation within a population was observed for Population P1 (SD 4.08%). This could be as a result of genetic differences between the trees (Sampaio et al., 2016). The variations observed among the population could be an indication of the effects of geographical origin on the quantities of metabolites produced (Cirak et al., 2015), resulting in variations in the matrices of the samples collected (Muraina et al., 2008).

4.3.2. Method validation and quantification of isolated compounds in *T. sericea* samples

4.3.2.1 Linear regression analysis

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of the analyte in the sample (ICH, 2005). The regression coefficient (R^2) obtained from the calibration curve constructed for each compound is presented in Figure 4.3.

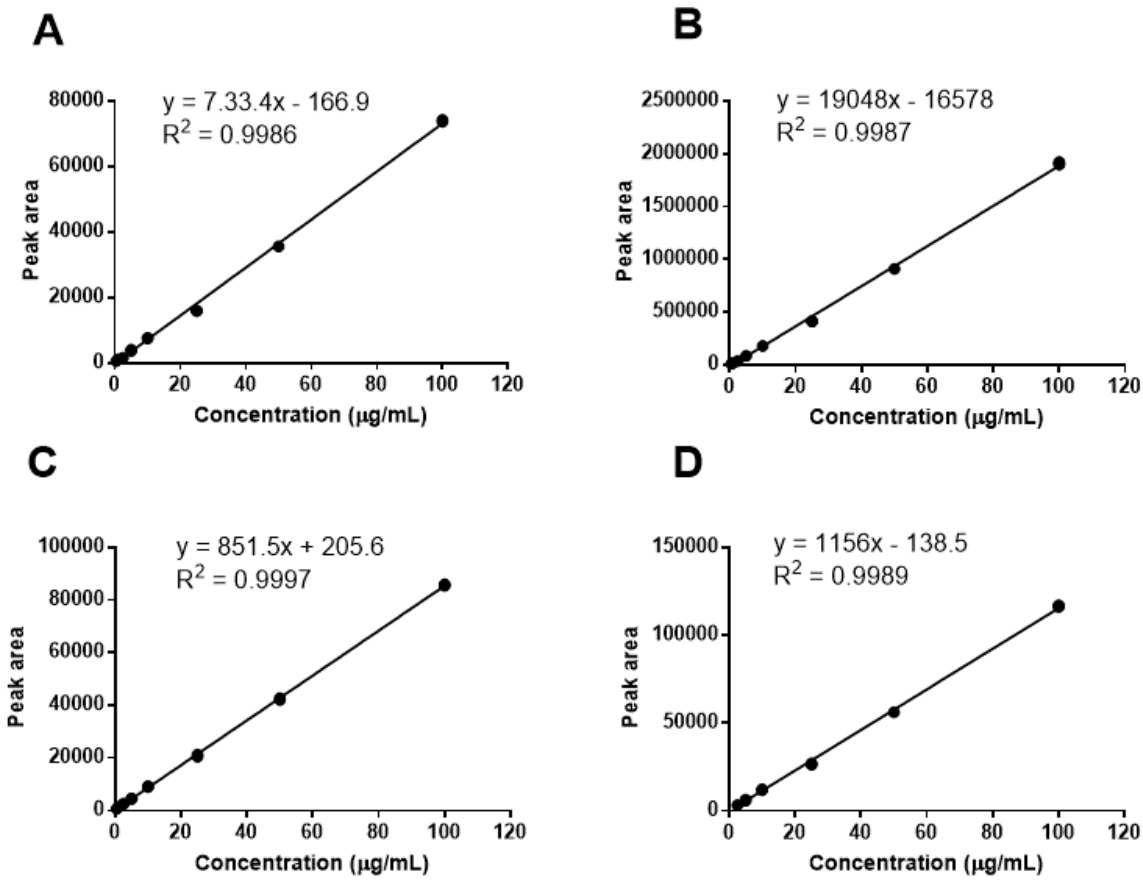

Figure 4.3 Linear calibration curve for each standard compound obtained from regression analysis of the UPLC-PDA data of the four metabolites over the concentration range 0.500 to 100 µg/mL for A) sericic acid, B) resveratrol-3-rutinoside, C) sericoside, and over the range 2.50 to 100 µg/mL for D) arjunglucoside I

The calibration curves for the four analytes were all characterised by a regression coefficient of at least 0.998, indicating a good relationship

between the tested concentration range and the detector response (peak areas) at a 95% confidence level. The summary of the regression results, as well as the LODs and LOQs determined for each analyte, are presented in Table 4.3. The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the test. In contrast, the limit of quantification is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of the test (Shrivastava and Gupta, 2011). The determined LODs for the UPLC-PDA method ranged from 11.6 to 25.2 ng/mL, while the corresponding LOQs ranged from 35.3 to 76.2 ng/mL.

Table 4.3 Line formulae, LOD and LOQ values for sericic acid, resveratrol-3- rutinoside, sericoside and arjunglucoside I isolated from *T. sericea* root

	Sericic acid	Resveratrol-3-rutinoside	Sericoside	ArjunglucosideI
Rt (min)	3.67	2.78	4.09	5.82
Calibration range (µg/mL)	0.500-100	0.500-100	0.500-100	2.50-100
Regression equation	y = 733.4x-166.9	y =19048x-16578	y =851.5x+205.6	y=1156x-138.5
R^2	0.998	0.998	0.999	0.998
δ	5.61	134	3.00	8.81
LOD (ng/mL)	25.2	23.3	11.6	25.2
LOQ (ng/mL)	76.5	70.8	35.2	76.2

Rt: retention time; R^2: regression correlation coefficient; δ: standard deviation of intercept y; LOD: limit of detection; LOQ: limit of quantification at 95% confidence limit

The results for the intra- and inter-day repeatability analysis of resveratrol-3-rutinoside (10 µg/mL) using the UPLC-PDA are presented in Table 4.4. The intra-day analysis was done for three consecutive days and the RSD-values were calculated as 0.58% for the first two days and 0.59% for the third day. The inter-day analysis revealed a RSD of 0.70%. Overall, the RSDs were all well below 1%, indicating that the precision of the instrument was good and that the analyte is stable (Santos et al., 2017).

Table 4.4 Results for the intra-day and inter-day precision analysis of resveratrol- 3-rutinoside (10 µg/mL) using UPLC-QToF-PDA

		Intra-day		Inter-day
	Day 1	Day 2	Day 3	
χ (µg/mL)	9.93	9.97	9.83	9.91
SD (µg/mL)	0.06	0.06	0.06	0.07
RSD (%)	0.58	0.58	0.59	0.70

χ: mean; SD: standard deviation, RSD: relative standard deviation

The recoveries of resveratrol-3-rutinoside from spiked samples are presented in Table 4.5. There was 98% recovery when the crude extract was spiked with a low concentration of resveratrol-3-rutinoside (2.5 µg/mL). Unexpectedly, there was a poor recovery (67%) when a high concentration of resveratrol-3-rutinoside was used to supplement the extract. Recoveries between 80 and 120% are acceptable for the evaluation of accuracy in the method validation of plant materials (Kanama et al., 2015). The values obtained in this study are therefore acceptable since they fall within the acceptable range.

Table 4.5 Results for the recovery of resveratrol-3-rutinoside in spiked sample C10 using UPLC-QToF-PDA

Sample	SampleContent (µg/mL)	Spike (µg/mL)	Sample + Spike	Recovery	% Recovery
C10	38 ± 0.85	2.50	39.8 ± 0.0	1.80	98
		25.0	50.3 ± 0.2	12.8	80
		100	91.9 ±0.3	53.9	67

Following the validation of the method, the concentrations of the four isolated standards were determined in the 42 root samples. The results indicated that resveratrol-3- rutinoside was the most abundant compound in the root samples of eight of the ten populations, while sericoside was the main component in the two remaining populations (P3 and P9). (Table 4.6). Overall, resveratrol-3-rutinoside was the most abundant (overall mean = 15.2 mg/g). No sericic acid was detected in any of the samples from P7 and three other populations (P2, P5 and P8) each contained at least one sample wherein sericic acid was not detected.

The highest concentrations of sericic acid were produced by a sample (P4.2; Appendix 6) from Population P4 (12.6 mg/g) while the highest concentration of resveratrol-3-rutinoside was found in a sample from Population P5 (29.8 mg/g). A sample from Population P4 (P4.2) also contained the highest sericoside concentration (20.2 mg/g), while a sample from P3 (P3.3, Appendix 6) was found to have the highest concentration of arjunglucoside (8.44 mg/g).

Populations P3, P4 and P5 were collected from Vhembe district (Figure 4.1), indicating that root samples from that district have the highest concentrations of the four isolated standards. Comparing the extraction yields and the concentrations of the isolated compounds, resveratrol-3-rutinoside and sericoside are most likely the compounds responsible for the high extraction yield observed for P4 and P5. Arjunglucoside I has been previously quantified (Singh et al., 2002) in an ethanol bark extract of *T. arjuna* using HPTLC (0.102%). This previously reported value is at least eight times lower than the concentration of 8.44 mg/g (0.84%) for arjunglucoside I reported in the current study for P3.

There is no available literature on the quantification of sericic acid, resveratrol-3-rutinoside, sericoside and arjunglucoside I in *T. sericea*. The previously reported concentration of arjunglucoside I from *T. arjuna* was from a single sample and not from different populations. The current study reports for the first time, the quantification of sericic acid, resveratrol-3-rutinoside, sericoside and arjunglucoside I in root samples. The study has also demonstrated that the major constituents of *T. sericea* root can be quantified for quality control purposes using the developed models.

Table 4.6 Concentrations (mg/g) of sericic acid (C2), resveratrol-3-rutinoside (C10), sericoside (C3) and arjunglucoside (C12) in *T. sericea* roots from 10 populations (n = 42)

Population	C1		C2		C3		C4	
	Mean	(Min-max)	Mean	(Min-max)	Mean	(Min-max)	Mean	(Min-max)
P1	5.63	1.82-8.64	21.80	17.2-24.7	5.15	2.58-6.75	2.42	1.04-3.56
P2	1.50	ND-1.78	16.50	11.1-18.7	10.9	9.70-12.2	3.86	3.18-5.49
P3	1.62	0.68-3.28	2.170	0.650-5.05	8.61	6.66-9.57	6.51	4.41-8.44
P4	8.45	6.14-12.6	23.20	18.9-25.6	17.40	12.3-20.2	3.46	3.12-3.79
P5	5.74	0.00-8.39	19.70	12.4-29.8	7.94	6.51-10.3	4.09	3.09-5.28
P6	5.48	3.29-7.51	16.80	10.2-21.02	4.09	3.55-4.87	1.60	0.86-2.41
P7	ND	ND	15.70	12.03-21.6	11.20	9.01-12.3	5.59	3.99-6.70
P8	1.59	ND-1.68	14.60	10.4-17.3	10.80	9.30-12.9	4.54	3.41-7.11
P9	3.62	1.77-6.49	6.67	2.22-14-3	6.89	2.07-10.3	3.67	3.28-4.09
P10	5.29	2.80-7.78	18.90	13.3-24.5	5.76	3.77-7.75	2.22	2.30-4.44
Overall mean	**4.69**		**15.20**		**9.26**		**3.90**	

ND: Not detected; P: Population; Min: Minimum; Max: Maximum

4.3.3 Chemical profiling of the root extracts using UPLC-MS

Large peaks with corresponding molecular ions and retention times were evident in the chromatogram of the crude extract (Figure 4.4A), confirming that the compounds isolated are major compounds of the roots. A mixture of the four isolated compounds were analysed together with the crude extract (Figure 4.4B) using UPLC-QToF-MS. Resveratrol-3-rutinoside was identified with the molecular ion *m/z* 537 at retention time 2.83 min. Sericic acid, sericoside and arjunglucoside I shared the molecular ion *m/z* 469, but they eluted at different retention times (3.73, 4.14 and 5.88 min), respectively. Other peaks in the chromatogram of the crude extract (Figure 4.4A) with fragmentation ions *m/z* 917 (Rt 1.67 min), *m/z* 452 (Rt 1.79 min), *m/z* 471 (Rt 4.58 min) and *m/z* 209 (Rt 5.29 min) are yet to be identified.

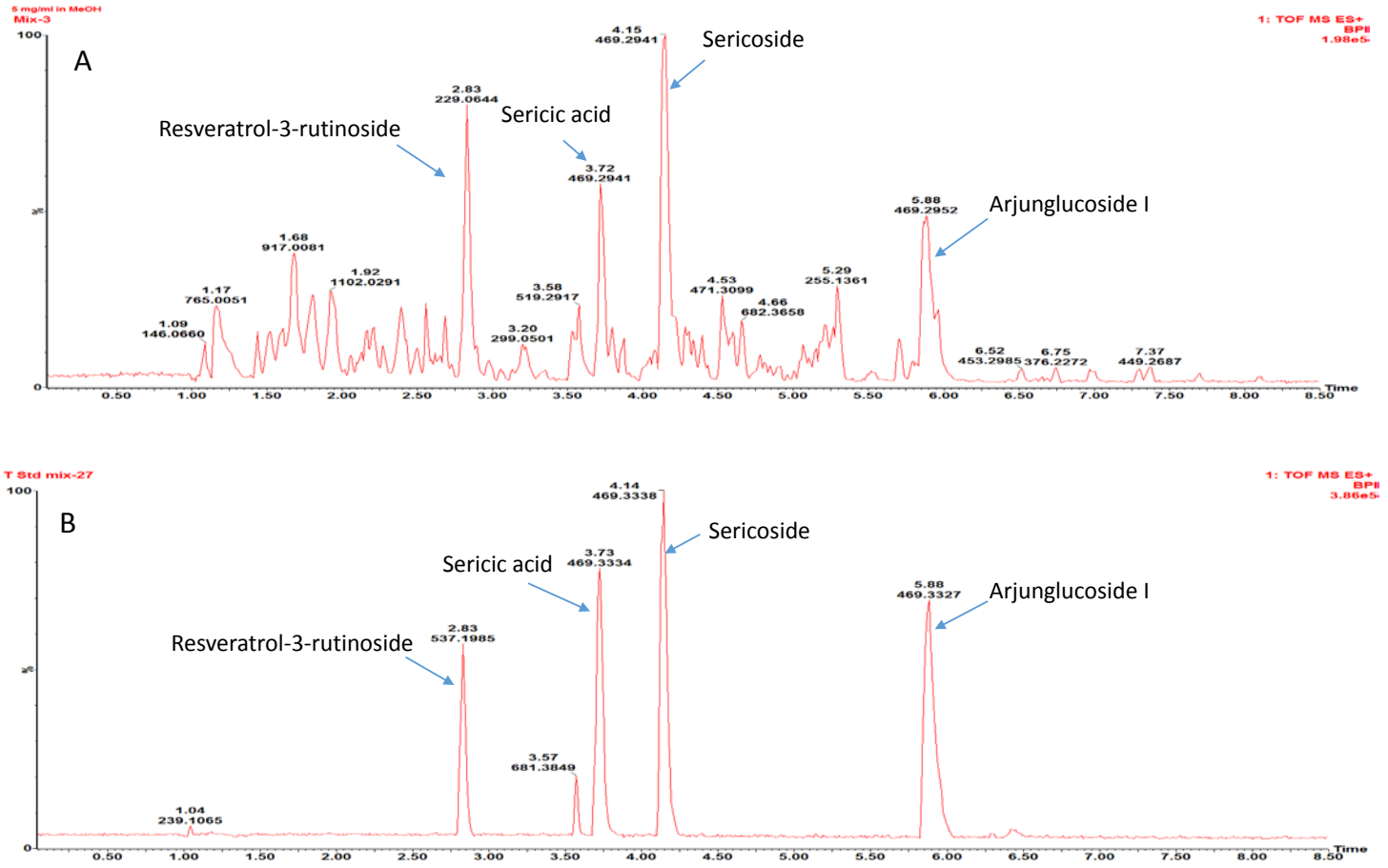

Figure 4.4 Chromatograms of A) the dichloromethane:methanol crude extract of *T. sericea* roots and B) a mixture of the compounds isolated from the roots

4.1.1 Quality control method using UPLC-MS

4.3.4.1 Untargeted UPLC-MS analysis

Raw data obtained from the UPLC-MS analysis, converted and transferred to an Excel spread sheet and imported into Simca 13.0, was first pretreated using Pareto scaling. A six-component PCA model was constructed from the data. The scores plot was used to identify potential outliers. Samples lying outside the Hotelling's T^2 ellipse were investigated as possible outliers (Wold et al., 1987). After studying the raw data, Sample BP2, a strong outlier, was removed from the dataset and a new PCA model was constructed. The model statistics were carefully studied to determine whether the model was over-or under-fitted, by adding and removing principal components and observing changes in the model statistics. A new six component PCA model was constructed and the cumulative variation in the X-matrix was found to be 65.6% (R^2Xcum = 0.656), while the cumulative predictive ability of the model was 37.8% (Q^2cum = 0.378). A good model should have both the R^2(cum) and the Q^2(cum) values > 0.5 (Sandasi et al., 2010). The predictive ability of this model was less than 0.5, therefore making it less accurate.

Hierarchical cluster analysis (HCA) was performed to confirm the groupings observed on the scores plot. A dendrogram constructed from the UPLC-MS data of 39 samples from the 10 populations is presented in Figure 4.5A. The level at which the branches split, relative to the root of the tree, is related to the dissimilarity of the samples. The dendrogram displays two major classes within the dataset, thereby defining clearly the two clusters observed on the scores plot. The two classes can be seen to have different chemical compositions, although these may be quantitative and not necessarily qualitative. Samples in P3 (J1-J5) were all clustered in Group 1 (depicted in green) and were found in the same subclass, indicating greater similarity. Samples in Population P9 (Samples TZ1-5) were also chemically homogenous, since they grouped in Class 2 (depicted in blue). However, Samples TZ1, TZ2 and TZ5 were clustered in a different subclass to Samples TZ3 and TZ4. The greatest variability was reflected by Population P5 (MM1-MM5). Samples MM1 and MM3 clustered in Group 1,

while MM4 and MM5 clustered in Group 2. This result clearly indicates that the clustering of the samples is not based on geographical location (Figure 4.5C).

The PCA scatter plot was coloured according to the groupings observed on the HCA dendrogram (Figure 4.5B). The first component (PC1) explained about 26% (R^2X = 0.263) of the variation responsible for the clustering pattern. In addition, 13.8% (R^2X = 0.138) of the data accounted for the variation observed along PC2. A loadings plot (Figure 4.5D) was constructed to identify variables (retention times and corresponding *m/z* values) that are responsible for the clustering observed in the HCA and the PCA plots. Most of the variables were centered on the X and Y intercept, indicating that the concentration of these variables are similar in all samples. Variables that are far from the centre have higher concentrations in some samples or occur only in some samples (Tankeu et al., 2013).

In the first quadrant of the loadings plot (Figure 4.5D) along the positive PC1, retention times 4.1395 and 2.8362 min represents sericoside and resveratrol-3- rutinoside, respectively. This indicates that the samples present in the upper positive quadrant of the scores plot contain higher concentrations of sericoside and resveratrol-3-rutinoside (Figure 4.5B, C). In the lower positive quadrant, retention time 3.7216 min represents sericic acid. This implies that Samples TZ1, TZ2, TZ5, MP3, MP4 and MP5 have higher concentrations of sericic acid or lower concentrations of either sericoside or resveratrol-3-rutinoside, compared to the other samples. This variation was further confirmed by the chromatograms (Figure 4.6) of some typical samples selected from each quadrant of the positive PCA scores plot.

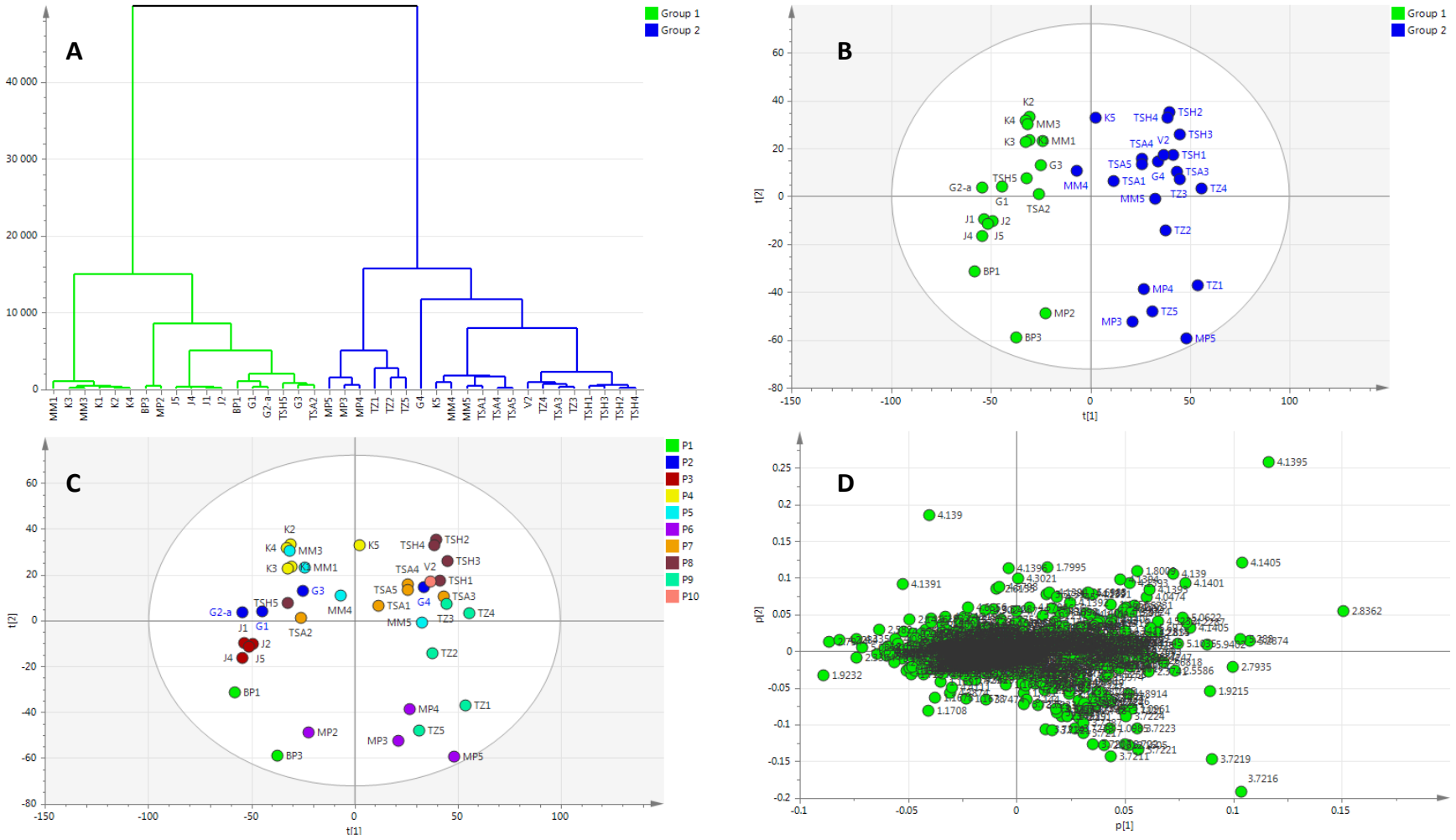

Figure 4.5 Various plots constructed following chemometric analysis of the UPLC-MS data obtained from analysis of root samples from ten populations. A) Dendrogram constructed following hierarchical cluster analysis, B) the PCA scores plot coloured according to HCA dendrogram, C) the same PCA scores plot coloured according to population, D) the loadings scatter plot obtained from the PCA mode

Sample TSH4 (P8) (Figure 4.6A), located in the upper positive quadrant of the scatter plot, is characterized by high a concentration of sericoside (12.9 mg/g) and a low concentration of sericic acid (1.68 mg/g). Sample MP5 (P5), located in the lower positive quadrant (Figure 4.6B), is characterized by a high concentration of sericic acid (7.51 mg/g) and a low concentration of sericoside (4.87 mg/g). Similar trends were observed for other samples occurring in each quadrant of the positive plot.

Although Samples TZ2 and TZ3 (Figure 4.6 C and D) are both from Population (P9), Sample TZ3 clusters in the upper positive quadrant of the PCA scores plot (Figure 4.5B), while Sample TZ2 is found in the lower positive quadrant. The quantitative data (Appendix 6) revealed that TZ3 contained a higher concentration of resveratrol- 3-rutinoside (11.56 mg/g) compared to TZ2 (2.22 mg/g). However, TZ2 contained a higher concentration of sericic acid (5.23 mg/g) compared to TZ3 (1.98 mg/g). The retention times (4.139 and 4.1391 min) observed in the upper negative quadrant correspond to the molecular ions *m/z* 1009.69 and 632.379, respectively. In the lower negative quadrant, the retention times (1.9232 and 1.1708 min) correspond to molecular ions *m/z* 1102.05 and 765.06, respectively. These *m/z* values do not correspond to the compounds previously isolated from *T. sericea* (Section 2.4). The compounds corresponding to these molecular ions are therefore yet to be identified.

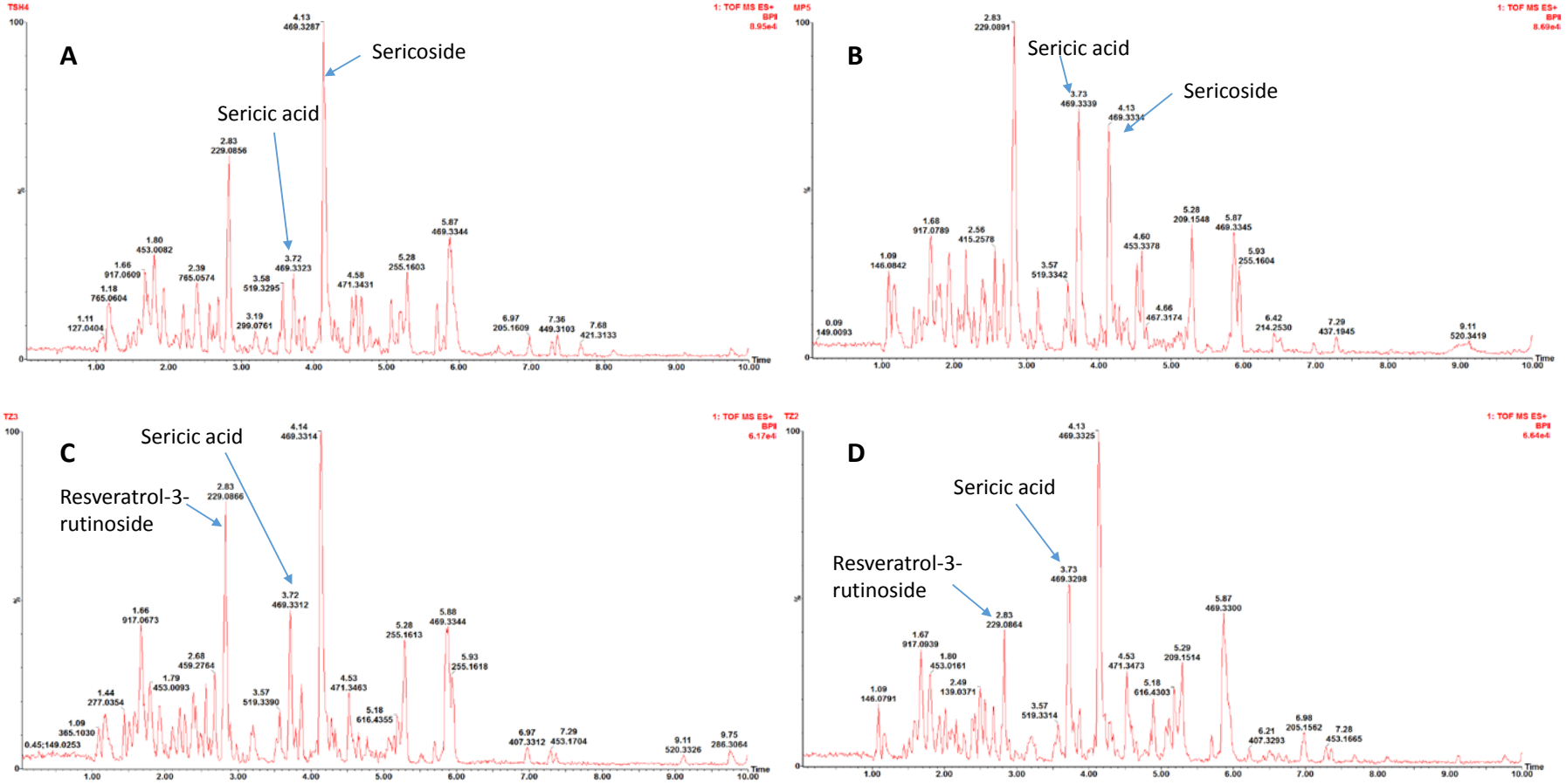

Figure 4.6 Chromatograms derived from UPLC-MS analysis of A) Sample TSH4, B) Sample MP5, C) Sample TZ3, and D) Sample TZ2, indicating variation in the sericic acid, resveratrol-3- rutinoside and sericoside concentration

4.3.4.2 Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) of UPLC-MS data

Unsupervised PCA of the dataset indicated two major clusters. However, for maximum class separation, an orthogonal projection to latent structures-discriminant analysis (OPLS-DA) was constructed, based on the unsupervised PCA model. Classes were assigned to each model based on the classes distinguished by the HCA dendrogram, rather than based on the population or area of origin. The five- component (one predictive component and four orthogonal components) OPLS-DA model obtained indicated that 54.6% (R^2X = 0.546) and 99.6% (R^2Y= 0.996) of the variation in the X-matrix and Y-matrix, respectively was accounted for, and a 96.7% predictive ability (Q^2 = 0.967). The Q^2 value was higher than 0.5 indicating that it is a good model (Mavimbela et al., 2014).

There was a distinct separation of the two classes (Figure 4.7) along the first predictive component tp [1], which accounted for 23.5% of the variation in the data and orthogonal variation of 31.1% (to[1] = 0.311) explained. There was a clear separation between the upper and lower negative quadrant in the OPLS-DA plot (Figure 4.7A). In the PCA scores plot (Figure 4.5B and C), Samples G1, G2-a and G3 were clustered in one quadrant. However, in the OPLS-DA scatter plot, there was a clear separation between G1, G2-a (upper negative quadrant) and G3 (lower negative quadrant). The concentration of sericic acid could be responsible for the distinct separation of the two classes in the negative quadrants. All the samples in the upper negative quadrant have low concentrations of sericic acid. The OPLS-DA scores plot indicates that there are three groupings of *T. sericea* root from Limpopo Province and that these groupings are not related to the population to which they belong (Figure 4.7B).

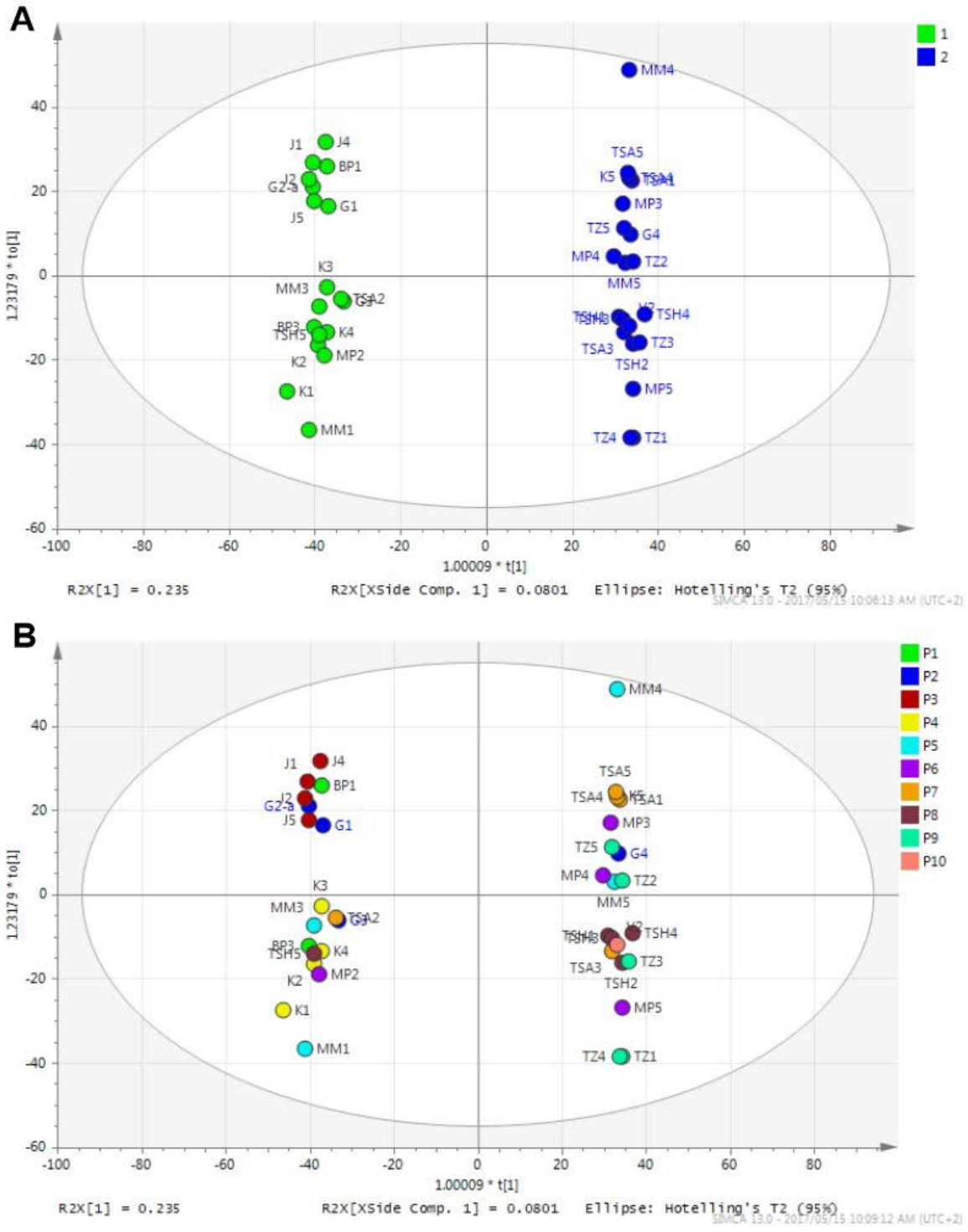

Figure 4.7 OPLS-DA scores plots indicating clusters coloured, according to A) assigned classes, B) population

The S-plot was constructed to identify the biomarkers responsible for the separation of the samples into two major clusters (Sandasi et al., 2012). Variables clustered along the center of the S-plot (Figure 4.8A) have similar concentrations in all the samples, while those at the extreme ends contribute significantly (either qualitatively or quantitatively) to the discrimination between the two classes (Tankeu et al., 2013). Variables along the positive PC1 with retention times (Rt) 2.8363 and 4.1395 min, representing resveratrol-3-rutinoside and sericoside, were the furthest from the centre. This indicates that these compounds are biomarkers that are significantly defining Class 2. The variables along the negative PC1 with Rt 4.2284, 4.335 and 2.7944 min, representing molecular ions *m/z* 544.295, 503.773 and 632.351, define Class 1.

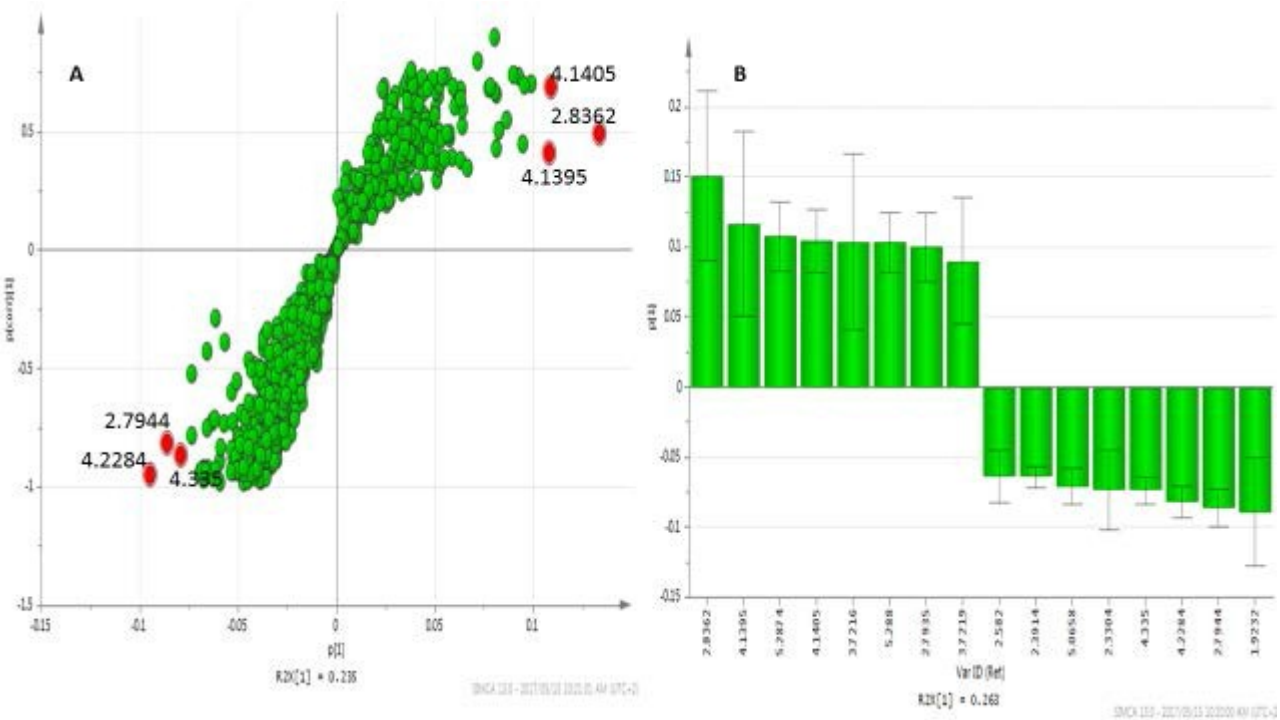

Figure 4.8 Various plots derived from the OPLS-DA model constructed from the UPLC-MS data A) S-plot, B) loadings plot indicating variables (retention times) contributing to the discrimination of the two major classes distinguishing the root extracts

Construction of the loadings column plot (Figure 4.8B) further defined the variables contributing significantly to the two classes. The column loadings plot revealed that resveratrol-3-rutinoside (Rt 2.8362 min) and sericoside (Rt 4.1395 min) were the most significant biomarkers for Class 2. Sericic acid (Rt 3.7216 min) contributed as the fifth most significant contributor responsible for the distinction of Class 2. Class 1 was characterised by the molecular ion *m/z* 1102.05 (Rt 1.9232 min). This is the first report detailing the identification of any biomarker for *T. sericea* roots. A reference to the sampling map (Figure 4.1) indicates that samples collected from Vhembe district are characterised by high concentrations of resveratrol-3-rutinoside and sericoside. Most of these samples are found in the upper positive quadrant along PC1 (Figure 4.5B and C).

4.3.5 Quality control methods using high performance thin layer chromatography (HPTLC) and mid-infrared (MIR) spectroscopy

4.3.5.1 Benefits of HPTLC and vibrational spectroscopy techniques

In the previous section (Section 4.3.3) the UPLC-MS methods used for the identification of biomarkers of *T. sericea* root samples were described. However, UPLC-MS methods are very expensive, due to the high cost of the instrument, the time consuming steps involved for sample pre-treatment and analysis, and the requirement for a skilled person to operate the instrument (Li et al., 2013; Maree and Viljoen, 2011; Mokgalaka-Matlala et al., 2013). On the other hand, HPTLC is a rapid and economical chromatographic technique that is simple to operate, involves low costs, can be used to analyse many samples simultaneously and the results are simple to interpret (Riffault et al., 2014; Vermaak et al., 2010). Vibrational spectroscopy also has some advantages over conventional methods because it is robust, efficient, non-destructive of the sample, and is cost effective. No sample pre-treatment steps are required and samples in any state (solid, liquid and gas) can be analysed (Mncwangi et al., 2014; Xu et al., 2013)

4.3.5.2 High performance thin layer chromatography analysis

The results of the HPTLC analysis of representative samples from each population and the isolated compounds are illustrated in Figure 4.9. After testing several solvent mixtures for the development of the chromatogram, an ethyl acetate:methanol:water (81:11:8) solvent mixture was used to develop the plates. Several visualization reagents were tested, but dilute sulfuric acid (20%) in ethanol, followed by heating on a hot plate until the compounds became visible as different coloured bands, was found to yield the best results. The choice of heating after the application of dilute sulfuric acid was due to the weak chromophores present in sericic acid, sericoside and the unidentified terpenoids (C5). The isolated compounds were used as standards and their purities were also evaluated. Sericic acid (Rf 0.80), sericoside (Rf 0.49) and arjungenin/arjunglucoside 1 (Rf 0.78/0.47) were not visible on the TLC plate when viewed under UV light (254 nm). They became visible after spraying with dilute sulfuric acid and heating.

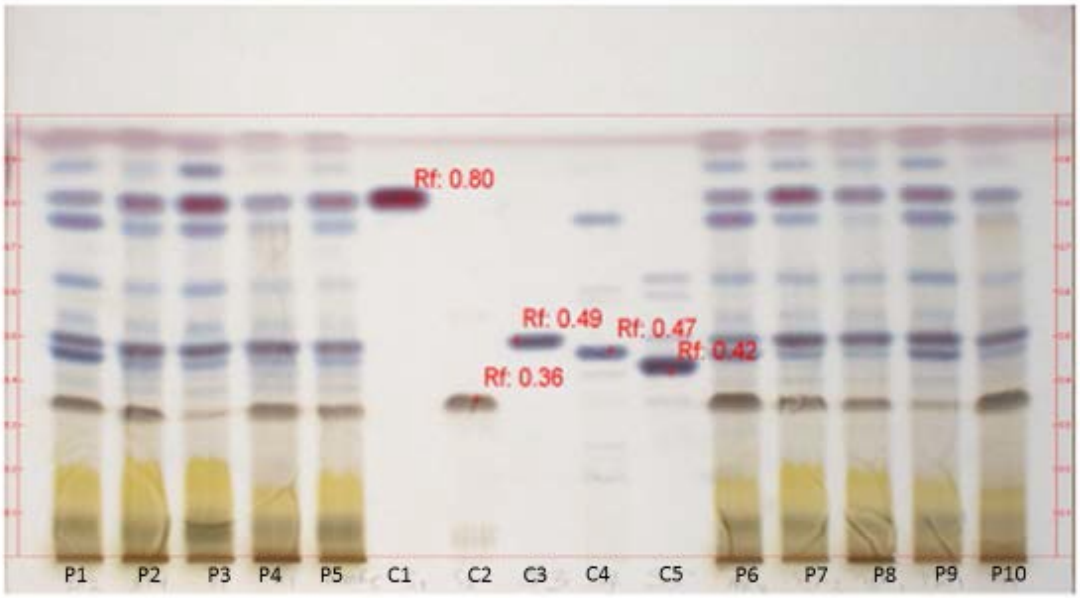

Figure 4.9 HPTLC plate of *T. sericea* crude root extracts showing that sericic acid, resveratrol- 3-rutinoside, sericoside and arjungenin are major compounds of the root and their concentrations are variable. A representative sample was selected from each of 10 populations. C1: sericic acid; C2: resveratrol-3-rutinoside; C3: sericoside; C4: arjunglucoside I; C5: triterpenoid glycoside

The results of the HPTLC (Figure 4.9) demonstrated that sericic acid, resveratrol-3- rutinoside, sericoside and arjunglucoside I are major constituents of *T. sericea* root and their concentrations are variable in the populations. However, the unknown triterpenoid glycoside (C5) isolated from Fraction 2 (Section 3.3.2) could not be seen in any of the 10 samples. It is therefore not suitable as a marker for the identification of *T. sericea* root. The concentrations and variability of the compounds are indicated by the intensity of their bands. They can be easily identified on the TLC plate in all the representative samples. This result corresponds with the quantitative results (Section 4.3.2).

Quantitative analysis of the compounds (Table 4.6) revealed that the concentration of resveratrol-3-rutinoside was the lowest in Populations P3 and P9. In the HPTLC, the intensity of the resveratrol-3-rutinoside band (Rf 0.36) was low in P3 and P9. This agreement in results is an indication that the HPTLC method is a reliable method for quality control (qualitative and quantitative) of *T. sericea*. The chemometric analysis revealed that sericic acid, resveratrol-3-rutinoside and sericoside are biomarkers for the roots. The HPTLC result confirms that these compounds are major constituents with variable concentrations in the 10 populations and can be used for quality control. There is no available literature on the chemical analysis of *T. sericea* roots using HPTLC, except for one report (Fyhrquist et al., 2014). However, their study reported on the possible presence of catechins in the aqueous, aqueous-insoluble and butanol fractions of the roots. The present study therefore is the first to indicate the use of HPTLC for the fingerprinting of *T. sericea.*

4.3.5.3 Multivariate analysis of the MIR data

The partial least square (PLS) method was used to construct the regression model (Lau et al., 2009). Three scaling methods (Centered, Pareto and Univariate) were tested comparatively to choose the most suitable for the model development. Partial least square (PLS) is the most important linear calibration method used for the simultaneous prediction of response variables from spectral profiles (Balabin and Smirnov, 2011; Ni et al., 2011).

4.3.5.3.1 Spectral preprocessing

Vibrational spectroscopy techniques have been used for quantitative and qualitative purposes in the quality control of herbal products (Sandasi et al., 2011). Advantages of infrared spectroscopy over traditional techniques (such as GC-MS and LC-MS) is the high analytical speed, ease of operation, non-destructive nature, robustness, efficiency and cost effectiveness (Li et al., 2013; Mncwangi et al., 2014). Common interferences such as high-frequency noise, baseline drift, stray light and high sample background are associated with spectral signals. These interferences must be eliminated or reduced by a pretreatment process before a reliable and stable model can be developed for quality control purposes (Yan et al., 2011). Spectral filters such as Multiplicative Scatter Correction (MSC), Standard Normal Variate (SNV) and derivatives (First, Second and Third) are used for preprocessing or pretreatment of MIR spectra data before building suitable models for the prediction of variables (Chen et al., 2008; Maree and Viljoen, 2011). Chemometric models were constructed from the spectral data of the powdered root samples with the aim of building a suitable model for the quantification of the biomarkers. The effect of applying different types of scaling and spectral filters to the spectral data on the partial least square regression analysis of the four isolated compounds is presented in Table 4.7.

Three scaling methods (UV, Par and Ctr) and five pretreatment methods (MSC, SNV, First, Second and Third derivatives) were applied and the most suitable combination was used to build the model using partial least square (PLS) method. A model is considered good when the value of the cumulative variation within X (R^2X- cum) and the predictive ability (Q^2-cum) is greater than 0.5 (Sandasi et al., 2011;Tankeu et al., 2013). For sericic acid (C1), the pretreatment with Center scaling and First derivative as the spectral filter gave the best correlation coefficient (R^2 = 0.883) and predictive ability (Q^2 = 0.397).

However the calibration curve for predicting sericic acid in external samples was not constructed since the predictive ability was less than 0.5. The root mean square error of prediction (RMSEP) and the correlation coefficient of the validation set determine the predictive capacity of a PLS regression model.

A reliable model is determined by the number of PLS factors. Increasing the PLS factor improves the predictive ability (Q^2) of the model, but may lead to poor prediction results for samples not in the calibration set (external samples). This is known as 'over-fitting' of the model (Li and Qu, 2010).

Table 4.7 Model statistics obtained after applying various combinations of scaling (Center, Pareto, Univariate) and pretreatment methods (Multivariate Scatter Correction, Standard Normal Variate and derivatives) to the MIR spectral data of powdered root samples

Pretreatment method	Number of principal components	R^2X(cum)	R^2Y(cum)	Q^2(cum)
C2				
CTR+dydx	4	0.883	0.708	0.397
Par+MSC	1	0.708	0.573	0.171
UV+SNV	3	0.660	0.703	0.154
C10				
CTR+MSC	4	0.761	0.825	0.456
CTR+SNV	4	0.760	0.825	0.457
Par+MSC	6	0.923	0.861	0.643
Par+SNV	6	0.922	0.861	0.645
UV+dydx2	1	0.086	0.733	0.403
C3				
CTR+dydx2	3	0.423	0.867	0.700
Par+dydx	3	0.591	0.865	0.654
UV+dydx	3	0.428	0.907	0.722
C12				
CTR+MSC	6	0.755	0.476	0.336
Par+MSC	6	0.713	0.502	0.359
UV+MSC	6	0.684	0.519	0.343
UV+SNV	6	0.681	0.521	0.343

C2: sericic acid; C10: resveratrol-3-rutinoside; C3: sericoside; C12: arjunglucoside I; CTR: Center, PAR: Pareto, UV: Univariate; MSC: Multivariate Scatter Correction, SNV: Standard Normal Variate, dydx: first derivative, dydx2: second derivative, dydx3: third derivative

The best calibration model is the one with the lowest RMSEP values and the highest correlation coefficient R^2 (Luo et al., 2008).

For the quantification of resveratrol-3-rutinoside (C2), Par+MSC and Par+SNV (Table 4.7) were suitable for the construction of the calibration curve, since the values of their predictive ability was greater than 0.5. The calibration curve for the quantification of resveratrol-3-rutinoside was constructed using Par+SNV (Figure 4.10A). This model was the most suitable due to the good predictability (Q^2 = 0.695), correlation coefficient (R^2 Y = 0.794) and a lower RMSEP (4.37 mg/g) compared to Par/MSC, which displayed a higher RMSEP value of 5.00 mg/g (Figure 4.10B).

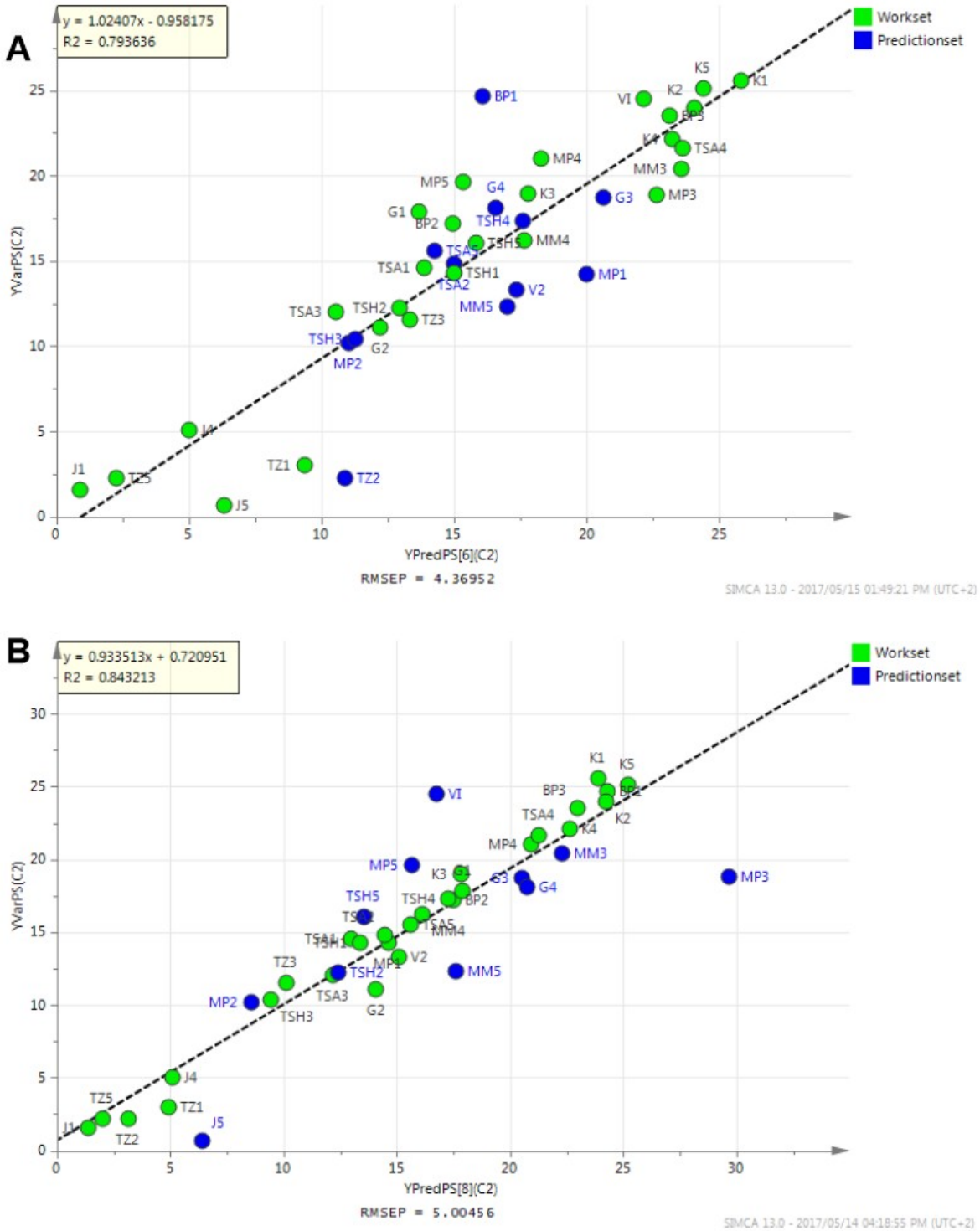

Figure 4.10 Calibration curve for the prediction of resveratrol-3-rutinoside using A) Par+SNV and B) Par+MSC models

After the construction of two calibration curves for the prediction of resveratrol-3- rutinoside, three external samples were quantified using the calibration curve

with the lower RMSEP. The results of the predicted samples are presented in Table 4.8.

Table 4.8 Prediction of resveratrol-3-rutinoside (C2) concentration in three external samples

Sample	PModxPS+[6]	DModXPS+[6]	YpredPS±RMSEP	UPLC/PDA (mg/g)
J2	0.998523	0.692466	0.683 ± 4.37	1.43
MM1	0.804880	0.899673	28.6 ± 4.37	29.8
TZ4	0.878407	0.865583	9.35 ± 4.37	14.3

The predicted values for the samples were within the error margin, except for sample J2. The result has indicated that the model chosen was suitable for the prediction of resveratrol-3-rutinoside in *T. sericea* root samples. Although proof of concept has been demonstrated, for quality control purposes, more samples should be included to construct the model. This would result in a calibration model with a better prediction ability.

For the prediction of sericoside, second derivate was found to be the best spectral filter for Center scaling, while First derivative was the most suitable spectral filter for Pareto and Univariate scaling methods. The three models were used for the construction of the calibration curve since they all had predictive abilities (Q^2) higher than 0.5 (Table 4.7). However, the Par+dydx and UV+dydx calibration curves (Figure 4.11) were considered for the prediction of the sericoside, due to their lower RMSEP values. The calibration curve constructed from the UV+dydx model was finally selected for the prediction of sericoside due to the lower RMSEP value (2.70 mg/g) and higher correlation coefficient (R^2 = 0.847921), compared to the Par+dydx model

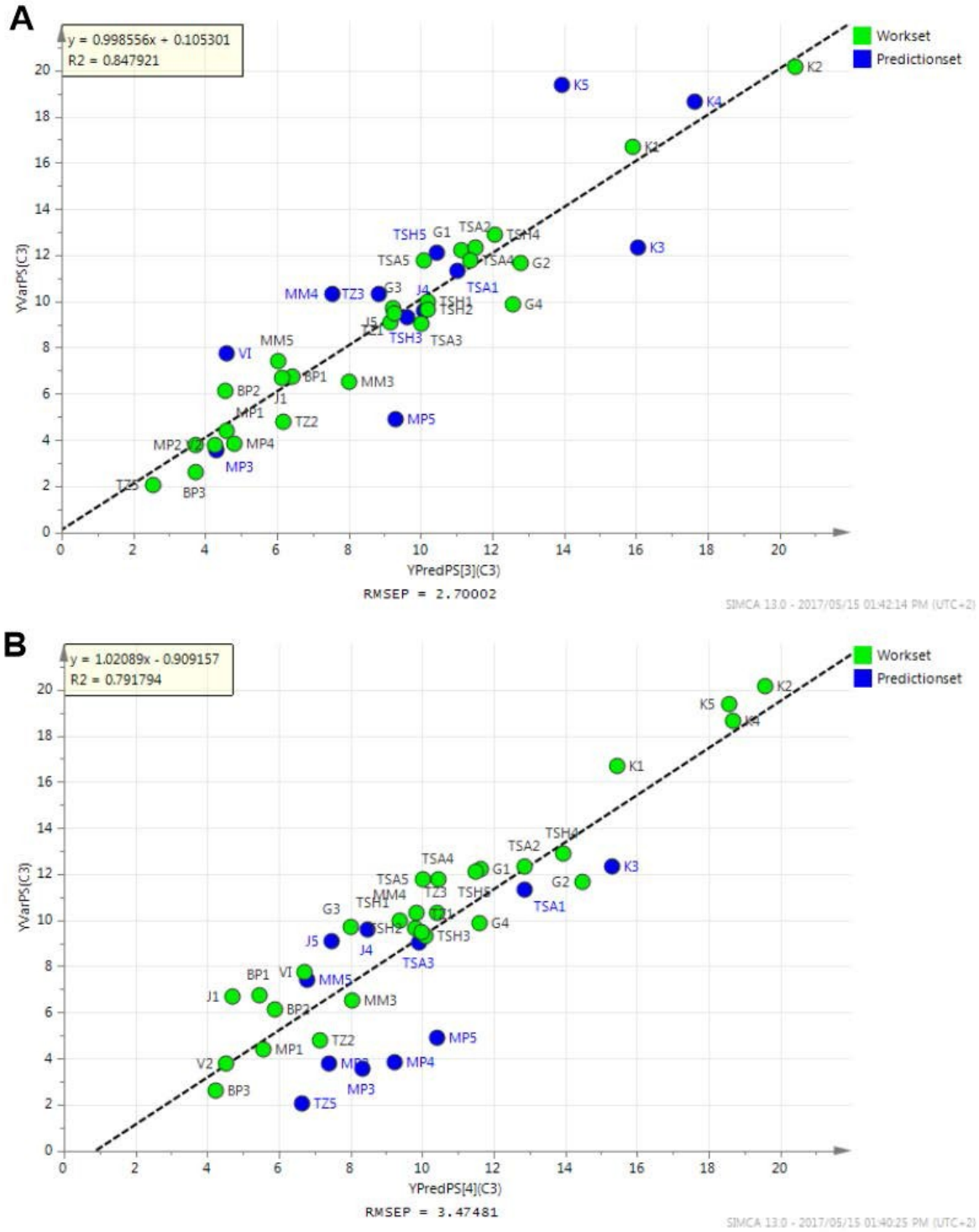

Figure 4.11 Calibration curves obtained from the PLDS-DA models constructed for the prediction of sericoside in three external samples using A) UV+dydx B) Par+dydx pretreatment

The results for the prediction of the concentration of sericoside in three samples (J1, MM1 and TZ4) using the UV+dydx model are presented in Table 4.9. The predicted concentrations were within the error margin (2.70 mg/g) when

compared with the concentrations previously obtained from the UPLC-PDA analysis

Table 4.9 Prediction of sericoside (C3) concentration in three external samples

Samples	PModXPS+[4]	DModXPS+[4]	YPredPS±RMSEP	UPLC-PDA (mg/g)
J2	0.331567	1.05986	6.54 ± 2.70	9.12
MM1	0.888443	0.863176	7.61 ± 2.70	7.54
TZ4	0.903901	0.854197	6.51 ± 2.70	7.78

The arjunglucoside I (C12) concentration was not predicted in the three external samples due to poor prediction ability (Q^2) of all the models. The models obtained were characterized by a predictive ability (Q^2) range of 0.33 to 0.35. These values were lower than 0.5 indicating that the models are not suitable for prediction.

Mid-infrared (MIR) spectroscopy was found to be a reliable technique for the prediction of resveratrol-3-rutinoside and sericoside concentrations in the roots of *T. sericea*. Vibrational spectroscopy has been extensively used for the prediction of biomarkers from plant samples, making it a reliable technique for control of herbal product. Models constructed from the NIR data were characterized by poor model statistics and could not be used for the construction of calibration models. The same strategy was applied to the data i.e. the use of different scaling and pre-processing methods, with no significant improvement in model statistics. None of this data has therefore been included in this report.

4.3.6 Summary

The following conclusions can be made:

- A method for the quantification of arjunglucoside I, resveratrol-3-rutinoside, sericic acid and sericoside was developed and validated.
- The metabolites obtained from UPLC-QToF-MS analysis of root samples from 10 populations were classified into two groups, reflecting different chemical compositions, using hierarchical cluster analysis.
- Resveratrol-3-rutinoside, sericic acid and sericoside were identified as biomarkers responsible for the clustering of Group 2

- HPTLC and MIR methods, suitable for quality control of *T. sericea* root, without the need for any sample pre-processing other than grinding and sieving, were developed.

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CHAPTER 5
ANTIBACTERIAL AND ANTI-OXIDANT ACTIVITIES OF *TERMINALIA SERICEA* EXTRACTS AND COMPOUNDS

5.1 INTRODUCTION

5.1.1 Bacterial infections

Bacterial infections have remained the most prevalent cause of morbidity and mortality worldwide, despite progress in scientific knowledge and medical technology (Tekwu et al., 2012; Dzotam et al., 2016). According to the World Health Organization (WHO), diarrhoea ranks second amongst the leading causes of death globally, in children under five years of age (Rahman et al., 2015). Although preventable and treatable, it is particularly widespread in children in developing countries (Chola et al., 2015; Umer et al., 2013). In South Africa, diarrhoea was established to be the third most common cause of child mortality, after HIV/AIDS and low birth weight, in 2003. However, in 2007, diarrhoea took the first place in the country, accounting for 21% of deaths in children, while respiratory infections and ill- defined natural causes accounted for 16% and 13% of deaths, respectively (Nannan et al., 2012).

Diarrhoea is a common gastrointestinal condition that has fatal implications and therefore poses a serious public health challenge. Common causes include infection, food poisoning, intestinal disorders and a side-effect of medication (Zhang et al., 2013). Enterogenic *Escherichia coli* is the most important pathogen responsible for diarrhoea in infants, children and adults. It is also the most common cause of traveler's diarrhoea that affects millions of tourists visiting developing countries (Chen et al., 2009). Although the bacterium is considered part of the normal intestinal flora of humans and animals, some enteropathogenic strains cause acute diarrhoea. Other pathogens associated with the condition include *Salmonella* spp, *Bacillus cereus*, *Staphylococcus* spp. and *Klebsiella pneumonia*. *Staphylococcus aureus* produces a heat stable

enterotoxin that commonly causes food poisoning or enterocolitis, characterized by projectile vomiting, diarrhoea, fever, abdominal pain, electrolyte imbalance and fluid loss (Cock and Van Vuuren, 2015; Tekwu et al., 2012). *Shigella* species are Gram-negative bacteria that are associated with serious intestinal diseases, such as bacillary dysentery. *Shigella dysenteriae* may lead to ulceration, a condition presenting with bloody stools characterised by high concentrations of neutrophils (Tekwu et al., 2012).

Staphylococcus epidermidas is a Gram-positive bacteria that forms part of the skin flora and mucosal membranes of animals. However, it is an opportunistic pathogen associated with acquired infection, and when it penetrates epithelial barriers, it causes nosocomial infections (Agarwal et al., 2016; Chovanová et al., 2013). It is the leading cause of infections related to medical devices in hospitals, as well as of infections in immune-compromised individuals (Abidi et al., 2015; Gomes et al., 2012). The major entry point for infection is the skin, especially during surgical procedures involving the implantation of medical devices (Agarwal et al., 2016). The organism has the ability to produce biofilms, which increases its resistance against antibiotics and assists with immune system degradation of the host. It has displayed some degree of resistance to methicillin, aminoglycosides, macrolides, tetracycline, chloramphenicol, clindamycin and vancomycin (Agarwal et al., 2016; Betanzos- Cabrera et al., 2015).

Pseudomonas aeruginosa is a Gram-negative bacterium that forms part of the flora of nasal and pharyngeal mucosal surfaces. However, it can become an opportunistic pathogen following penetration into the submucosal tissues of the skin, particularly after sustaining burn injuries. This process gives rise to serious wound infections (Jahani et al., 2016; Taghizadeh et al., 2011). This organism is the most common Gram-negative pathogen associated with hospital-acquired wound infections globally (Kamel et al., 2011; Yang et al., 2015). During skin burn injury, the protective skin barrier is broken, making the skin susceptible to infection, and often resulting in wound sepsis. The challenge in treatment is exacerbated when sepsis is caused by drug-resistant strains (Singh et al., 2014; Yang et al., 2015).

The emergence of multidrug resistance of pathogenic microbial strains to antibiotics has contributed significantly to the impact of infectious diseases on public health (Prakash et al., 2016; Ulloa-Urizar et al., 2015). Although many synthetic and semi- synthetic antibacterial drugs are available, the resistance of bacteria to drugs are rapidly increasing throughout the world (Stanković et al., 2016), leading to an increase in morbidity and mortality as a result of ineffective treatment regimens. This results in longer periods of hospitalization and an increase in the cost of treatment. For example, intravenous drugs may now be required to treat cystitis (a common bacterial infection in women), which in the past was easily treated by oral administration of antibiotics (Brambilla et al., 2017; Quave et al., 2008; World Health Organization, 2014). As a result of multiple drug resistance, stronger antibiotics or combined therapies are employed for effective treatment, which could cause severe side effects. For example, gentamycin is commonly used to treat severe infections of *S. aureus, E. coli, P. aeruginosa,* and *Enterobacter*, *Klebsiella* and *Seratia* spp., but has been reported to be nephrotoxic and ototoxic when administered at a high dosage, particularly when associated with *S. aureus* bacteremia (Vzquez et al., 2016).

The overuse and misuse of antibacterial drugs have resulted in the emergence of resistant strains, not only to the specific drug, but also to other drugs in the same class (Huh et al., 2016; Li et al., 2016; Suwito et al., 2016; World Health Organization, 2014). It has been estimated that approximately 70% of bacterial pathogens, resulting in the patient being hospitalised, are resistant to one or more of the commonly used drugs for treatment (Ramasubburayan et al., 2015). Some of the known resistant bacteria include methicillin-resistant *Staphylococci aureus* (MRSA), vancomycin-resistant enterococci (VRE), extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumonia*, and carbapenem-resistant enterobacteriaceae (CRE), metallo-β-lactamase (MβL)-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Chen et al., 2016; Lai et al., 2014; Valle et al., 2015). Although *Staphylococcus* is a normal human skin and mucosal microorganism, it can cause infection when carried asymptomatically to other body sites. *Staphylococcus aureus* is one of the most serious human pathogens, causing infections such as bacteraemia, severe

pneumonia and various skin conditions (Chomnawang et al., 2009; Hassan et al., 2017). Many of the strains have developed resistance to β- lactam-containing drugs, including methicillin, oxacillin, nafcilin, cloxacilin and dicloxacilin. The bacterium is also resistant to aminoglycosides, fluorquinolones and macrolides. However, it still remains sensitive to the glycopeptide antibiotics (vancomycin and teicoplanin), which are considered the last few effective agents (Chomnawang et al., 2009; Milyani and Ashy, 2012; Zuo et al., 2008).

When new drugs are approved and released into the market, they are initially very expensive and not readily accessible, particularly in developing countries. This, however, does not stop the increase in resistance to already available drugs. This gap created leads to the emergence of further resistance. More so, the indiscriminate prescription of antibiotics has contributed significantly to drug resistance (Aumeeruddy-Elalfi et al., 2016). A further challenge is the time it takes for the approval of new antibiotics when there is failure in the available treatment drugs. Linezolid and daptomycin are the only two antibiotics that have been developed over the last 40 years (Ajiboye et al., 2016; Mundy et al., 2016). However, these improved drugs are also prone to the development of bacterial resistance, and so the cycle continues. According to the World Health Organization (2014) report, antimicrobial resistance is now considered a 'global threat' causing people to die after merely contracting common infections and sustaining minor injuries.

The current crisis surrounding bacterial resistance has directed research towards combination therapy, in an attempt to enhance the efficacy of existing drugs, and to search for alternatives to synthetic antibiotics using natural sources, such as plants (Hubsch et al., 2014). It is evident that there is an urgent need to identify new antimicrobial compounds that can be tested against normal and multidrug resistant bacterial strains (Abdallah, 2011; World Health Organization, 2014). Plants protect themselves against microbial invasion by producing secondary metabolites (Gershenzon and Dudarewa, 2007). They are able to withstand microbial invasion due to their complex defense systems (James and Dubery, 2009). Through the induced defence system, plants fight against microbial infection by increasing the concentration of phytoalexins

through *de novo* enzyme synthesis (Mazid et al., 2011). The antimicrobial activities of plant extracts have been extensively investigated as potential sources of new antibiotics to combat drug resistance. These complex chemical entities often exert their therapeutic effects through additive effects or synergism, and in many cases they are effective against a range of pathogens (Ayoub et al., 2014; Guo et al., 2014). However, not all plant secondary metabolites are involved in the defence system (Pavarini et al., 2012; Wink, 2013), making it difficult to identify compounds with activity.

In spite of the widespread use of a myriad of medicinal plants to combat bacterial infections in many parts of the world, there are no reports available of any compound of plant origin that is clinically significant for treatment of microbial infections. Challenges encountered in drug development from natural sources are many, and include the loss of activity of isolated compounds compared to that the crude extract during purification. The current approach of identifying potential drugs or lead compounds, is focussed mainly on the activity of a single bioactive compound, rather than on the additive or synergistic collaboration of the secondary metabolites present in medicinal plants (Ulrich-Merzenich, 2014).

Several studies have indicated that isolated compounds from plant sources generally have lower antimicrobial activities compared to standard antibiotics (Biva et al., 2016; Fyhrquist et al., 2017; Mushtaq et al., 2016). In some cases, crude extracts exhibited higher antibacterial activities compared to standard antibiotics or isolated compounds (Salih et al., 2017; van Vuuren et al., 2015). Since plants make use of compound combinations to fight infections, the use of whole extracts rather than isolated compounds may provide useful insight into the prevention or cure of infections in humans. Extracts from different specimens of the same plant may differ in their efficacy against a particular organism, depending on the chemical profile of the specimen. This is due to qualitative and quantitative variations in the secondary metabolites that respond to the specific pathogen causing the infection (Wink, 2003). Marker compounds associated with the observed therapeutic activities of a medicinal plant can be identified, isolated and used for quality control and to test the efficacy of herbal products. For example, gensinoside, a saponin, was identified as a suitable marker for the quality control and efficacy determination of *Panax*

ginseng obtained from China, South Korea and Canada (Qiu et al., 2016; Xie et al., 2006). Five isolated bioactive isoquinoline alkaloids were identified as appropriate compounds to establish the quality of *Rhizoma coptidischinensis* (Kong et al., 2009). Quality control of herbal drugs, based on the presence of specific compounds, must be done to ensure that extracts with efficacy are sold. It is therefore necessary to test isolated compounds against appropriate bacteria to determine which compounds contribute to the efficacy of the crude root extract of *T. sericea.*

5.1.2 Anti-oxidants

In the past few decades, researchers have become interested in the role of natural anti-oxidants as part of the human diet and for their potential health benefits (Riahi et al., 2013). Oxidative stress, caused by the accumulation of free radicals and reactive oxygen species (ROS), or the expense of endogenous anti-oxidants, has been linked to the pathogenesis of many diseases, including diabetes, cancer, inflammation, Alzheimer's and atherosclerosis (Jayathilake et al., 2016; Jimnez-Zamora et al., 2016; Nicolai et al., 2016; Tauchen et al., 2016). Anti-oxidants are known to protect biological molecules (DNA) from oxidation, scavenge free radicals or terminate chain reactions initiated by free radicals; thereby reducing the risk of developing chronic degenerative diseases (Limmongkon et al., 2017; Mndez-Lagunas et al., 2017). Studies have revealed that plants (herbs, spices, vegetables, fruits) are good sources of anti-oxidants, because they are rich in phenolic compounds (Kalaycıoğlu and Erim, 2017). These compounds augment the activity of the depleting endogenous anti-oxidant system (Muddathir et al., 2017; Perera et al., 2016). The total concentration of phenolic compounds in an extract is usually a good indication of the anti-oxidant potential of the extract (Baba and Malik, 2015; Hossain and Shah, 2015).

Macrophages are activated within the host organism as a first line of defence against invading organisms, (Kumawat et al., 2016). This results in the ingestion and destruction of the pathogens, a process associated with oxidative stress (Almshawit and Macreadie, 2017; Chakraborty et al., 2016). A variety of

ROS, such as the superoxide anion, hydrogen peroxide and hydroxyl radicals are generated by macrophages to oxidise the bacterial membrane and crucial biomolecules of the pathogen, including DNA, proteins and lipids (Biazus et al., 2017; Zhang and Lu, 2015). Excessive amounts of oxidants produced during phagocytosis are neutralized by the host's anti-oxidant system, of which the main role-players are catalase, superoxide dismutase (SOD), and glutathione peroxide (GPx). However, prolonged production of ROS results in the depletion of the anti-oxidant system of the host, resulting in an imbalance between the production of free radicals and the levels of the anti-oxidants. This leads to oxidative stress that can cause damage to cellular components, including DNA, proteins and lipids, within the host cells (Baronetti et al., 2013; Castaneda et al., 2016; Chakraborty et al., 2016; Fu et al., 2015; Shu et al., 2013). Plants are a good source of exogenous anti-oxidants that can augment the activity of the depleting endogenous anti-oxidant system (Muddathir et al., 2017; Perera et al., 2016).

5.1.3 Study rationale

Ethnobotanical studies have revealed that *T. sericea* is commonly used for the treatment of diarrhoea and stomach-related ailments (Moshi and Mbwambo, 2005), indicating that the plant produces compounds with antimicrobial activity. Ground powders of the dry leaves, stems and roots are applied topically for treatment of burns and wound infections (Lall and Kishore, 2014; Ribeiro et al., 2010). In initiation schools, the leaf is applied for preventing infections after circumcision (Mutsa, 2015). A study on different plant parts of *T. sericea* indicated that the plant could be a good source of broad spectrum antibiotics (van Vuuren et al., 2015). A review by Mongalo et al. (2016) indicates that both aqueous and organic extracts of the root exhibit antibacterial activities with minimum inhibitory concentration (MIC) values of below 1 mg/mL against Gram-positive *Staphylococcus aureus,* gentamicin-methicillin resistant *S. aureus* (GMRSA), *Staphylococcus epidermidis* and the Gram-negative *Escherichia coli* and *Klebsiella pneumoniae.* However, there are no available reports on the antibacterial activities of the major constituents of the roots with emphasis on diarrhoea and skin infection pathogens. There is also no available

literature on the effect of chemical variability on the antibacterial and anti-oxidant activities of *T. sericea* root. The aim of this part of the study was to evaluate the antibacterial and anti-oxidant activities of extracts and isolated compounds from the roots of *T. sericea*

5.2 MATERIALS AND METHODS

5.2.1 Evaluation of the antibacterial activity of *Terminalia sericea*

5.2.1.1 Bacterial isolates

The following pathogens were used in the study and were provided by Prof Sandy Van Vuuren, Department of Pharmacy and Pharmacology, University of the Witwatersrand (Johannesburg, South Africa): *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27858), *Shigella sonnei* (ATCC 9292), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), and *Staphylococcus epidermidis* (ATCC 12223). The bacterial cultures were maintained at 4 ^{o}C to maintain their viability.

5.2.1.2 Preparation and evaluation of plant material

Methanol extracts were prepared from the leaves, stem bark and roots of two randomly selected samples from two populations of *T. sericea* to determine the plant part with the best antibacterial activity against the selected pathogens. The initial choice of methanol as an extracting solvent was based on the traditional use of decoctions prepared from *T. sericea* (Moshi and Mbwambo, 2005). After the selection of the root as the plant part with the best antibacterial activity, acetone, aqueous (cold infusion), dichloromethane, ethyl acetate, dichloromethane:methanol (1:1), and methanol were used for extraction of the root samples from one of the populations. These extracts were tested against the selected pathogens to identify the solvent yielding the best antibacterial activity.

To determine the effect of geographical variation on the antibacterial activity of *T. sericea* root, dichloromethane:methanol (1:1) was selected for the extraction of the samples from all the populations. These extracts were tested against *B. cereus, S. typhimurium, S. sonnei* and *P. aeruginosa*, which were selected because they were the most susceptible towards the dichloromethane:methanol (1:1) extract. Finally, the antibacterial activities of column fractions and pure compounds (resveratrol-3- rutinoside, sericic acid and sericoside), isolated from the root as described in Section 3.2.3, were also evaluated against all of the pathogens. Stock solutions of the crude extracts were prepared at a concentration of 32 mg/mL, while the pure compounds were prepared at 5.0 mg/mL for the antibacterial assay. The samples were dissolved in either 20% DMSO or acetone (both AR grade; Sigma Aldrich), depending on the solubility of the samples or compounds in the solvent.

5.2.1.2 Minimum inhibitory concentration assay

The minimum inhibitory concentration (MIC) assay was used to determine the antibacterial activity of each dichloromethane:methanol (1:1) extract as described by Eloff (1998), with modifications as described by van Vuuren et al. (2015).
Cultures were inoculated in Tryptone Soy broth (TSB) and incubated at 37 ^{o}C for 24 h to prepare overnight cultures for the assay. Microplates were aseptically prepared by placing 100 µL sterile broth in each well with a micropipette. Thereafter, 100 µL of the individual test samples (32 or 5.0 mg/mL) were added to the first row of each plate. Serial dilution was performed by removing 100 µL of the mixed extract and broth from each well and transferring to the well in the next row, resulting in a final volume of 100 µL. Ciprofloxacin (0.01 mg/mL) was used as the positive control, while acetone or 20% DMSO (depending on which solvent the samples were dissolved in) were used as negative controls.

The overnight culture of each pathogen was diluted with fresh sterile TSB at a 1:100 ratio to provide an approximate inoculum size of 1 x 10^6 colony-forming units (CFU)/mL. The inoculum size was estimated by comparing the turbidity of the inoculum visually with McFarland standard solution. A 100 µL volume of the culture was added to each well and the plates were covered with sterile adhesive

film. They were then incubated at 37 ^{o}C for 24 h (EcoTherm, Hartkirchen, Austria). After incubation, 40 µL of 0.2 mg/mL *p*-iodonitrotetrazolium (INT) violet (Sigma-Aldrich, St Louis, MI, USA) was added to the incubated wells. The plates were maintained at ambient temperature for 2 h before being examined for antibacterial activity. The experiments were carried out in duplicate and the average MIC values were recorded.

5.2.2 Evaluation of the free radical scavenging and reducing power antioxidant activities

Free radical scavenging activity using DPPH and the reducing power assays were used to evaluate the anti-oxidant activities of various extracts. Aqueous (cold infusion), acetone, ethyl acetate, acetone:methanol, dichloromethane:methanol and methanol leaf, stem and root extracts, prepared from one sample in each of the populations, were tested to determine the most suitable solvent for extracting compounds with anti-oxidant activity from the roots. Initially, the anti-oxidant activities of the organic (methanol:acetone 1:1) and aqueous (infusion) extracts of the leaves, stems and roots were evaluated to identify the plant part with the highest anti-oxidant activities. After identifying the most suitable solvent for the anti-oxidant activity, dichloromethane:methanol extracts from the 10 populations were evaluated for the anti-oxidant activity.

Free radical scavenging assay:

The free radical scavenging activity of the dichloromethane:methanol root extracts was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay as reported by Motamed and Naghibi (2010), with adjustment for a 96-well plate. Ascorbic acid (Merck (Pty) Ltd, Modderfontein, South Africa) was used as the reference standard. A 100 µL volume of each crude extract (0.50 mg/mL), pure compound or ascorbic acid (0.10 mg/mL), were transferred using an automatic pipette, to the wells of the first row of a 96-well plate, each containing 100 µL of deionised water. The extracts were serially diluted by transferring 100 µL of the samples to the second row and then diluting to subsequent wells. Thereafter, DPPH (Sigma-Aldrich) solution (0.3 mM; 200 µL) was added to all the wells. The contents were mixed and allowed to stand at room temperature in the dark

for 30 min. The blank contained deionised water instead of extract. The absorbance of each plate was measured at 517 nm using a microplate reader (Versa Max, China). The radical scavenging activities (RSA) of the crude extract or pure compounds were determined using the following equation:

$\%RSA = [(A_b - A_S)/A_b] \times 100$ (Equation 5.1)

where A_b is the absorbance of the blank, which contained DPPH solution and distilled water only, and A_S is the absorbance of the sample (crude extracts, pure compounds, or ascorbic acid) and DPPH. The ability of the extracts to inhibit 50% of the free radical (IC_{50}) was extrapolated from a graph of percentage RSA as a function of concentration.

Reducing power assay:

This assay was applied as described by Pereira et al. (2013), with adjustments for a 96-well plate. The crude extracts, pure compounds or ascorbic acid (0.5 mg/mL or 0.1 mg/mL; 100 µL) were transferred into the wells of the first row of a 96-well plate, each containing 100 µL of deionised water. The extracts were serially diluted by transferring 100 µL of the samples to the second row and then diluting the mixed content into subsequent wells. A 50 µL aliquot of sodium phosphate (Merck) buffer (0.2 M, pH 6.6) and 50 µL 1% aqueous potassium hexacyanoferrate [$K_3Fe(CN)_6$] (Merck) were added to each well. Following incubation for 20 min at 45 °C, 50 µL of 10% trichloroacetic acid (TCA; Merck) was added and the contents of the wells were mixed. An 80 µL portion of the mixture was transferred to a fresh 96-well plate, with each well containing 80 µL deionised water and 16 µL $FeCl_3$ (0.1% w/v; Merck). The blank contained distilled water instead of sample. Absorbance was measured at 690 nm using a microplate reader (Versa Max, China). Gallic acid (Sigma) was used as the reference standard. The effective concentration ($EC_{0.5}$) value was obtained from linear regression analysis of the absorbance values as a function of concentration. The $EC_{0.5}$ value is the effective concentration of the extract to yield an absorbance of 0.5. All extracts were tested in triplicate.

5.2.3 Determination of total flavonoid content

The flavonoid content of the root samples was determined for correlation analysis with the anti-oxidant activity. Firstly, the flavonoid contents of the organic (acetone:methanol 1:1) and aqueous (infusion) extracts of the leaves, stems and roots were evaluated to determine the plant part with the best anti-oxidant activity. After that, dichloromethane:methanol extracts of root samples from 10 populations were used for evaluating the effect of geographical location on the total flavonoid content. The total flavonoid content was determined according to the method reported by Boulanonuar et al. (2013), with minor modification. A 100 µL volume of 2% $AlCl_3$ (Merck) in ethanol (Merck) was added to 100 µL of each filtered crude dichloromethane:methanol root extract (1.0 mg/mL) in a 96-well microplate. The mixture was allowed to stand at room temperature for 60 min before reading the absorbance using a microplate reader (Versa Max, China) at 420 nm. The blank was prepared by adding 100 µL of $AlCl_3$ to 100 µL of deionized water. Quercetin (Sigma) was used as the reference standard. The total flavonoid content was expressed as milligram quercetin equivalents per gram of the extract (mg QE/g).

5.2.4 Statistical analysis

Mean and standard deviation (SD) values for the anti-oxidant activities and flavonoid contents were calculated for data (n = 3) using Excel software. To determine whether differences were significant ($p < 0.05$) or not, one-way ANOVA (Graph pad prism 6) was applied.

5.3 RESULTS AND DISCUSSION

5.3.1 Antibacterial activities of *T. sericea* plant parts

Terminalia sericea is commonly used to combat stomach and skin conditions associated with infection and has been reported to have antibacterial activity against Gram-positive and Gram-negative pathogens (de Wet et al., 2010; Lall and Kishore, 2014; Mabona and Van Vuuren, 2013). However, compounds

associated with the activity have not been isolated or identified. *Bacillus cereus, S. aureus, E.coli, K. pneumoniae, S. typhi* and *S. sonnei* were selected as test pathogens based on their involvement in diarrhoea (Van Vuuren et al., 2015). *Pseudomonas aeruginosa* and *S. epidermidis* are associated with skin infections and were selected for this reason (Mabona and Van Vuuren, 2013). Minimum inhibitory concentration (MIC) values lower than 1.0 mg/mL and 0.10 mg/mL are considered to be noteworthy for crude extracts and pure compounds, respectively (Van Vuuren et al., 2007; Van Vuuren et al., 2015).

The root of *T. sericea* is the main organ used traditionally for the treatment of diseases (Van Wyk et al., 2013). The first aim was to confirm if the root exhibited better antibacterial activities than the stems and leaves. Plant parts used were collected from two different locations to account for variations that may arise from different populations. Methanol was selected as the extracting solvent for the preliminary screening, since the polarity is similar to that of water, which is used traditionally for preparation. The antibacterial activities of the methanol extracts of the leaves, stem bark and roots from two samples representing different populations are presented in Table 5.1. The root extracts exhibited better activity against all of the pathogens when compared to the other extracts, as reflected by the lower MIC values obtained against all the pathogens tested. This finding corresponds to a report by Fyhrquist et al. (2002), who found that *T. sericea* root extracts (aqueous, acetone, ethyl acetate, methanol) displayed better antimicrobial activity (hot-plate agar diffusion method), compared to the methanolic leaf extract against *S. aureus* (FOMK), *E. coli* (ATCC 8739), *Enterobacter aerogenes*, *S. epidermidis* (ATCC 12228), *Bacillus subtilis* (FOMK), *Micrococcus luteus* (YMBO), *Sarcina sp.* (FOMK) and *Candida albicans* (ATCC 10231).

Table 5.1 Antibacterial activities of methanol extracts of *T. sericea* leaves, stem bark and roots from two populations (1 and 2) as reflected by the average MIC values (mg/mL). The boldface indicates MIC values that represent noteworthy activity

Sample	*B. c*	*E. c*	*P. a*	*S. a*	*S. e*	*S. s*	*S. t*	Average
LS1	8.0	4.0	2.0	4.0	2.0	8.0	4.0	
LS2	8.0	4.0	2.0	1.0	4.0	4.0	8.0	
Average	8.0	4.0	2.0	2.5	3.0	6.0	6.0	4.5
RS1	4.0	2.0	1.0	1.0	1.0	2.0	2.0	
RS2	3.0	2.0	**0.50**	2.0	2.0	2.0	2.0	
Average	3.5	2.0	**0.75**	1.5	1.5	2.0	2.0	1.9
SS1	6.0	4.0	2.0	4.0	2.0	2.0	4.0	
SS2	2.0	2.0	**0.50**	1.0	2.0	4.0	2.0	
Average	4.0	3.0	1.3	2.5	2.0	3.0	3.0	2.7
Cipro*	1.3	1.3	0.63	0.63	2.5	0.16	0.040	

***B. c*: *Bacillus cereus*, *E. c*: *Escherichia coli*, *P. a*: *Pseudomonas aeruginosa, S. a*: *Staphylococcus aureus*, *S. e*: *Staphylococcus epidermidis, S. s*: *Shigella sonnei, S. t*: *Salmonella typhimurium,* Cipro: *Ciprofloxacin (µg/mL). LS: Leaf sample, RS: Root sample, SS: Stem sample**

The best inhibition overall was observed for the root extracts against *P. aeruginosa* with an average MIC value of 0.75 mg/mL. This finding is of significance, since in general, plants are more active against Gram-positive bacteria than against their Gram-negative counterparts, due to the protection afforded by the thick outer murein layer of the Gram-negative organisms, which prevents the entry of the antibacterial agent (Ndhlala et al., 2013) The result also displayed variation in the antibacterial activities of the leaf, root and stem extracts between the two populations. The leaf extracts displayed variation in antibacterial activities against *S. aureus, S. epidermidis, S. sonnei* and *S. typhi*, while the root displayed variations against *B. cereus, S. aureus, S. epidermidis* and *P. aeruginosa*. The stem extracts displayed variable activity towards all the pathogens, except for *S. epidermidis*. The observed variations in the antibacterial activities are due to differences in the chemistry of the extracts prepared from samples of the two populations (Saei-Dehkordi et al., 2010). Preliminary TLC (data not shown) analysis of the plant parts (leaf, stem and root) indicated that the stem and the root shared similar chemical profiles, which were different from that of the leaves, especially when stained with Natural Product reagent. Flavonoids present in the leaf extracts were not visible in the stem or the root extracts.

5.3.2 Effect of extracting solvent on the antibacterial activity of *T. sericea* root

After confirming that the root was the plant part with the highest antibacterial activity, it was decided to evaluate the ability of various solvents to extract antibacterial constituents from this plant parts. Previous reports on *T. sericea* indicated the use of methanol, acetone, water, chloroform, ethyl acetate, ethanol, dichloromethane, dichloromethane:methanol (1:1) as solvents used to extract constituents for various biological activities (Eldeen et al., 2005; Samie and Mashau, 2013; Tshikalange et al., 2005; van Vuuren et al., 2015). In our study (Table 5.2), ethyl acetate and acetone were found to be the best solvents for the extraction of antibacterial agents from the roots. The ethyl acetate extract (EAE) displayed noteworthy activity against four pathogens of which three are Gram-negative bacteria. The best activity was against *K. pneumoniae* with an MIC value of 0.25 mg/mL. The acetone extract (AE), displayed noteworthy activity (MIC 0.5 mg/mL) against two Gram-negative bacteria (*K. pneumoniae* and *P. aeruginosa*) and one Gram-positive bacterium (*S. aureus*). Despite being the traditionally used extractant, the aqueous extract gave the poorest results with the corresponding MIC values ranging from 0.50 mg/mL against *P. aeruginosa* to 8.0 mg/mL against *B. cereus, E. coli, S. aureus, S. sonnei and S. typhi.* Considering the susceptibility of the pathogens, *P. aeruginosa,* a Gram-negative bacterium, was the most susceptible pathogen to all of the extracts. Anolignan b, termilignan b and arjunic acid, previously isolated from *T. sericea* root ethyl acetate extract, have been reported to be active against *Bacillus subtilis, E. coli, K. pneumoniae* and *S. aureus* (Eldeen et al., 2005; 2008). In the study by Eloff, (1998b), acetone was reported to be the best solvent for the extraction of antibacterial compounds from *Anthocleista grandiflora* and *Combretum erythrophyllum* as evaluated using the bioautography method. This results obtained in the current study confirm that organic solvents are more effective extractants of antibacterial constituents from *T. sericea* root compared to water. This result corresponds to those of several other studies that found that the organic extracts of *T. sericea* have better antibacterial activities than traditionally used water extracts (Mabona and Van Vuuren, 2013; Van Vuuren et al., 2015). According to the Snyder (1974) classification of solvents, ethyl

acetate and acetone are in group VIa due to their similarities in polarity strength and selectivity (Barwick, 1997). This means that acetone and ethyl acetate are able to interact with similar solutes (de Juan et al., 1997). The difference in the characteristics of water and ethyl acetate suggests that the major antibacterial constituents of *T. sericea* are of intermediate polarity.

Table 5.2 Antibacterial activities of crude extracts prepared from the roots of *T. sericea,* using a variety of solvents with different polarities, against selected pathogens. The activities are reflected by the average MIC values (mg/mL) obtained. Values in boldface indicate noteworthy activity

Pathogens	AE	AQE	DME	EAE	ME	Cipro*
B. c	2.00	8.00	2.00	1.00	2.00	0.31
E. c	1.00	8.00	2.00	1.00	2.00	0.63
K. p	**0.50**	1.00	1.00	**0.25**	1.00	0.04
P. a	**0.50**	**0.50**	1.00	**0.50**	1.00	0.63
S. a	**0.50**	8.00	2.00	1.00	2.00	0.63
S. e	1.00	2.00	**0.50**	**0.50**	1.00	0.63
S. s	1.00	8.00	1.00	**0.50**	1.00	0.63
S. t	2.00	8.00	2.00	1.00	2.00	0.31
Average	1.06	5.43	1.43	**0.71**	1.5	

***B. c*: *Bacillus cereus*, *E. c*: *Escherichia coli*, *K. p*: *Klebsiella pneumoniae, P. a*: *Pseudomonas aeruginosa, S. a*: *Staphylococcus aureus*, *S. e*: *Staphylococcus epidermidis, S. s*: *Shigella sonnei, S.t*: *Salmonella typhimurium.* AE: acetone extract, AQE: aqueous extract, DME: dichloromethane/methanol extract, EAE: ethyl acetate extract, ME: methanol extract. Cipro: *Ciprofloxacin (µg/ml)**

5.3.3 Antibacterial activities of column fractions and isolated compounds

The column fractions obtained during the purification steps, as well as the isolated compounds (Chapter 3) were evaluated for their antibacterial activity to identify compounds with good activity. The results (Table 5.3) indicate that *Salmonella typhimurium* was the most susceptible pathogen towards Fractions F1-4 (all with an MIC value = 0.50 mg/mL), eluted successively from the silica gel column (Section 3.2.2). The crude extract applied to the column also displayed noteworthy activity against the organism, as well as towards *P. aeruginosa*, *S. sonnei* and *S. typhi* with the same MIC value (0.50 mg/mL). Comparing the activities of Fractions F1-4, which were obtained directly from the crude extract, F1 and F4 were the most active, since these fractions displayed noteworthy activities of less than 1.0 mg/mL against six and four pathogens, respectively. However, F2 and F3 displayed MIC values of below 1.0 mg/mL

against only one pathogen (*S. typhi*). Furthermore, F1 exhibited better activity (lower MIC values) compared to the crude extract against all the pathogens, while F4 displayed better activities compared to the crude extract against *K. pneumoniae* and *P. aeruginosa* only. Notably, F2 and F3 displayed lower activity against *B. cereus*, *E. coli, P. aeruginosa, S. aureus* and *S. sonnei* compared to the crude extract. Comparing the activities of the crude extract and fractions suggests that the major antibacterial agents were present largely in F1. The marked increase in the activity of column fraction $F1a_2$ (Figure 3.1), derived from further purification of F1, against several of the pathogens compared to that of F1, suggested that its main component had good activity against many of the pathogens tested. The two fractions ($F2a_1$ and $F2a_2$), obtained during further purification of Fraction F2, were found to be considerably less active than the crude extract. This loss in activity of $F2a_1$ and $F2a_2$ could be due to loss of the active compound or separation of the components responsible for the activity into successive fractions during the purification process. Once isolated from Fraction 1a, sericic acid **(1)** exhibited a substantially higher antibacterial activity (Table 5.3) than Fraction 1a against all of the selected pathogens, with the exception of *S. typhi*. Surprisingly, $F1a_2$ yielded an MIC value of 0.31 mg/mL against *S. typhi* compared to that of sericic acid (>1.3 mg/mL).

This suggests that the compound responsible for the activity against *S. typhi* is not sericic acid and that the active constituent(s) was lost during the purification process. To the best of our knowledge, this is the first report on the activity of *T. sericea* root and sericic acid against *S. typhi*. The only report available in literature on *T. sericea* crude extract was the activity of the organic and crude extracts of stem bark against *S. typhi* by Van Vuuren et al. (2015).

Table 5.3 MIC (mg/mL) values indicating the antibacterial activity of crude extracts, fractions and pure compounds against a range of bacterial pathogens. Boldface indicates samples with noteworthy activities

Samples	*B. c*	*E. c*	*K. p*	*P. a*	*S. a*	*S. e*	*S. s*	*S. t*
CE	2.0	2.0	1.0	**0.75**	1.0	1.0	**0.50**	**0.50**
F1	1.0	1.0	**0.50**	**0.75**	**0.50**	**0.50**	**0.50**	**0.50**
F2	4.0	>8.0	1.0	1.0	2.0	1.0	1.0	**0.50**
F3	3.0	>8.0	1.0	1.0	2.0	1.0	1.0	**0.50**
F4	4.0	>8.0	**0.50**	**0.50**	>8.0	1.0	**0.50**	**0.50**
$F1a_2$	1.3	0.63	0.94	>1.3	0.31	0.31	0.63	0.31
(1)	0.63	0.15	0.31	0.15	0.15	0.15	**0.080**	>1.3
$F2a_1$	>1.3	>1.3	>1.3	>1.3	>1.3	>1.3	>1.3	>1.3
(2)	>1.3	>1.3	1.3	>1.3	>1.3	>1.3	>1.3	>1.3
$F2a_2$	>1.3	>1.3	1.3	>1.3	>1.3	>1.3	>1.3	>1.3
(3)	>1.3	>1.3	>1.3	>1.3	>1.3	>1.3	>1.3	>1.3
Cipro*	0.31	0.63	0.040	0.63	0.63	0.63	0.63	0.31

***B. c*: *Bacillus cereus*, *E. c*: *Escherichia coli*, *K. p*: *Klebsiella pneumoniae*, *P. a*: *Pseudomonas aeruginosa*, *S. a*: *Staphylococcus aureus*, *S. e*: *Staphylococcus epidermidis*, *S. s*: *Shigella sonnei*, *S.t*: *Salmonella typhimurium*. CE: Crude extract, F: fraction, (1): Sericic acid, (2): Resveratrol-3-rutinoside, (3): Sericoside, Cipro: Ciprofloxacin positive control (µg/mL)**

Resveratrol-3-rutinoside only exhibited some degree of antibacterial activity against *K. pneumoniae* (MIC = 1.3 mg/mL). Sericoside was not active against any of the selected pathogens either. Unfortunately, the antibacterial activity of arjunglucoside I could not be assessed, because of an insufficient quantity. Although anolignan b, termilignan b and arjunic acids, previously isolated from *T. sericea* root, have been reported to possess antibacterial activity against *Bacillus subtilis*, *E. coli*, *K. pneumoniae* and *S. aureus*, these compounds are not major constituents of the root, the organ with the best overall antibacterial activity of all the plant parts. So far, sericic acid, a major constituent of the roots, can be described as the major antibacterial constituent of the root. An MIC value of 1.0 mg/mL was reported by Hess et al. (1995) for sericic acid against *S. aureus.* There are no other reports available concerning the activity of sericic acid against bacterial pathogens. Although resveratrol-3-rutinoside and sericoside are major root metabolites, they do not seem to contribute to the antibacterial activity of the root.

Parker (2016) reported that *T. sericea* roots provide protection against oxidative damage and microbial infection of wounds. In the current study, sericic acid was the only isolated compound that inhibited the growth of *S. epidermidis* and *P. aeruginosa*, pathogens implicated in wound infections. This implies that sericic acid is the major compound responsible for the

inhibition of wound-infecting pathogens. The activity of sericic acid against *S. sonnei* demonstrates that it has good inhibitory activity against *Shigella* spp., which is associated with serious intestinal diseases, including dysentery and diarrhoea (Tekwu et al., 2012). A previous study has revealed good inhibitory activity of *T. sericea* bark against *Shigella flexneri* (Van Vuuren et al., 2015). In the current study, root crude extracts (ethyl acetate and acetone) and sericic acid displayed good activity against *K. pneumoniae*, *P. aeruginosa* and *S. sonnei*. This indicates that the roots can be used for the treatment of infections associated with these pathogens and confirms the traditional use of *T. sericea* for treatment of infections, diarrhoea and skin wounds.

5.3.4 Effect of geographical variation on antibacterial activities of *T. sericea* root crude extract

Dichloromethane:methanol (1:1) was used for the isolation of arjunglucoside I, resveratrol- 3-rutinoside, sericic acid and sericoside, because the solvent mixture has an intermediate polarity and also had good extract yield. In addition, the solvent mixture was used to prepare extracts for UPLC-MS analysis. The same extracts were used for evaluating the effect of geographical variation on the antibacterial activities of the roots, thereby also limiting solvent waste and saving time. Furthermore, using the same extract for quantification and activity determination provided the opportunity to link the antibacterial activities to the chemistry.

The dichloromethane:methanol crude root extracts displayed variations within and between populations (Table 5.4) against *B. cereus*, *S. sonnei,* and *S. typhi*. Populations 1, 2, 3, 7, 8, 9 and 10 displayed noteworthy activities against *S. typhi,* with values ranging from 0.25-0.50 mg/mL. The variations in the antibacterial activities against *S. sonnei* and *S. typhi* could be attributed to the variation in the chemical constituents (Section 4.3.2). The efficacy of herbal products is dependent on the chemical variability of the raw material (Folashade et al., 2012; Govindaraghavan and Sucher, 2015). Sericic acid was identified as the main antibacterial compound in the roots, however, it was not active against *S. typhi* and therefore not responsible for the variations in activity observed within and between populations against *S. typhi*. The average concentration of

sericic acid across all of the populations ranged from 1.59 – 8.50 mg/g (Section 4.3.2).

Population P3 exhibited the best activity against *S. typhi*, but contained a low concentration of sericic acid (1.62 mg/g) when quantified. Similarly, Populations P2, P7 and P8 displayed good activity against *S. typhi*. Sericic acid was not detected in Samples P2.1, P2.2 and P2.4, or in Samples P7.1-P7.5 and P8.2, P8.3, which all displayed good activity against the organism, confirming that the compound was not responsible for the activity. In this study it was found that the lower the concentration of sericic acid, the higher the concentration of other antibacterial compound(s), and the higher the activity of the extracts against *S. typhi*.

The quantification of the isolated compounds (Chapter 4) indicates that resveratrol-3- rutinoside is the most abundant compound in most of the extracts with an average concentration range of 2.17-23.16 mg/g. However, the pure compound did not display good antibacterial activities against the tested pathogens. A few individual samples (P2.1, P2.2, and P9.5), containing low concentrations of resveratrol-3- rutinoside, displayed better antibacterial activity against *S. typhi* compared to the other individual samples from the same population. In contrast, populations (P4, P5, and P6), with high average resveratrol-3-rutinoside concentrations, exhibited poorer antibacterial activity (MIC values of 1.00 mg/mL). This observation suggests that other compounds present are responsible for the antibacterial activity of the roots against *S. typhi*. The variation in the concentration of resveratrol-3-rutinoside also affected the activity against *S. sonnei*. Population 4 contained the most resveratrol-3-rutinoside, but was not active against *S. sonnei* at the tested concentration. Some individual plants with higher resveratrol-3-rutinoside concentrations within individual populations (P5.1, P5.2, P9.3 and P9.4) were also not active against *S. sonnei*. This implies that the compounds responsible for the activity of *S. sonnei* are in lower concentration in the populations with higher concentrations of resveratrol-3- rutinoside, resulting in a decrease the activity of the plant extracts against *S. typhi* and *S. sonnei*. The study has demonstrated that variation in the concentrations of sericic acid and resveratrol-3-rutinoside and

other active compounds (not yet identified) affect the antibacterial activities of crude extracts against some of the pathogens tested.

Table 5.4 Variation in antibacterial activities of dichloromethane:methanol extracts of *T. sericea* roots collected from different populations. Boldface indicates samples with noteworthy activities

Population	Code	*B. c*	*P. a*	*S. s*	*S. t*
P1.1	BP1	1.00	2.00	8.00	**0.50**
P1.2	BP2	1.00	2.00	8.00	**0.25**
P1.3	BP3	1.00	2.00	8.00	**0.25**
P2.1	G1	1.00	2.00	8.00	**0.25**
P2.2	G2	1.00	2.00	8.00	**0.25**
P2.3	G3	1.00	2.00	8.00	**0.50**
P2.4	G4	1.00	2.00	>8.00	**0.50**
P3.1	J1	1.00	2.00	8.00	**0.25**
P3.2	J2	1.00	2.00	8.00	**0.25**
P3.3	J4	1.00	2.00	8.00	**0.25**
P3.4	J5	1.00	2.00	8.00	**0.25**
P4.1	K1	1.00	2.00	>8.00	1.00
P4.2	K2	1.00	2.00	>8.00	1.00
P4.3	K3	1.00	2.00	>8.00	1.00
P4.4	K4	1.00	2.00	>8.00	1.00
P4.5	K5	1.00	2.00	>8.00	1.00
P5.1	MMR1	1.00	2.00	>8.00	1.00
P5.2	MMR3	1.00	2.00	>8.00	1.00
P5.3	MMR4	1.00	2.00	8.00	1.00
P5.4	MMR5	1.00	2.00	8.00	1.00
P6.1	MP1	1.00	2.00	8.00	1.00
P6.2	MP2	1.00	2.00	8.00	1.00
P6.3	MP3	1.00	2.00	8.00	1.00
P6.4	MP4	1.00	2.00	8.00	1.00
P6.5	MP5	1.00	2.00	8.00	1.00
P7.1	TSA1	1.00	2.00	8.00	1.00
P7.2	TSA2	**0.75**	2.00	8.00	**0.50**
P7.3	TSA3	1.00	2.00	8.00	**0.50**
P7.4	TSA4	1.00	2.00	8.00	**0.50**
P7.5	TSA5	1.00	2.00	8.00	**0.50**
P8.1	TSH1	1.00	2.00	8.00	**0.50**
P8.2	TSH2	1.00	2.00	8.00	**0.50**
P8.3	TSH3	1.00	2.00	8.00	**0.50**
P8.4	TSH4	1.00	2.00	8.00	**0.50**
P8.5	TSH5	1.00	2.00	8.00	**0.50**
P9.1	TZ1	1.00	2.00	8.00	**0.50**
P9.2	TZ2	1.00	2.00	>8.00	**0.50**
P9.3	TZ3	1.00	2.00	>8.00	**0.50**
P9.4	TZ4	1.00	2.00	>8.00	**0.50**
P9.5	TZ5	1.00	2.00	8.00	**0.25**
P10.1	V1	1.00	2.00	>8.00	**0.50**
P10.2	V2	1.00	2.00	>8.00	**0.50**

B. c: Bacillus cereus*, *P. a*: *Pseudomonas aeruginosa, S. e: Staphylococcus epidermidis, S. s*: *Shigella sonnei, S.t*: *Salmonella typhimurium

5.3.5 Anti-oxidant activity and flavonoid contents of *T. sericea* parts

The anti-oxidant activities of the aqueous (cold infusion), acetone, ethyl acetate, acetone:methanol, dichloromethane:methanol and methanol leaf, stem and root extracts from one sample in each of the populations as determined by the free radical scavenging activity and the reducing power activity assays are presented in Table 5.5. The highest free radical scavenging (DPPH) anti-oxidant activity was observed for the methanol:acetone extract (MAE) of the root, but the activity was not significantly different ($p < 0.05$) from that of the methanol:acetone extract of the stems and the hot water extract of the leaves.

In the reducing power assay, the highest activities were displayed by the hot water extract of the stems and roots, and were significantly different from the hot water extract of the leaves and the reference standards (ascorbic acid and gallic acid). The result of the reducing power activity showed that compounds with reducing power activity in *T. sericea* are best extracted with hot water, regardless of the organ.

Table 5.5 Anti-oxidant activity (IC_{50} µg/mL) of parts of *T. sericea*

	Leaves	Stem	Root	Vit.C	Gallic Acid
DPPH					
CE	12.57±1.65^{a}	8.14±0.2^{a}	23.17±0.3^{f}		
HE	4.25±0.49^{b}	16.91±0.17^{e}	17.89±0.76^{g}		
MAE	57.68±1.56^{c}	4.51±0.62^{b}	2.4±0.06^{b}	4.63±0.15^{b}	3.04±0.14^{b}
RP					
CE	43.69±0.83^{a}	9.08±0.03^{a}	47.53±0.21^{f}		
HE	2.95±0.22^{b}	0.31±0.03^{e}	0.86±0.02^{e}		
MAE	59.88±1.27^{c}	8.63±0.06^{a}	58.71±0.74^{c}	76.03±0.64^{g}	49.72±1.91^{f}
TFC					
CE	15.98±0.20^{a}	65.62±1.16^{a}	67.37±0.22^{a}		
HE	25.22±0.74^{b}	56.52±2.86^{e}	78.46±0.07^{f}		
MAE	39.62±1.69^{c}	78.46±0.07^{f}	78.46±0.07^{f}		

Data are the mean of three triplicates. Values in rows and columns of each anti-oxidant activity indicated by the same alphabetical letter are not significantly different ($P < 0.05$). CE: Cold water extract; HE: Hot water extract; MAE: Methanol/Acetone extract. Vit.C: Vitamin C

5. 3. 6 Effect of solvent on anti-oxidant activity of *T. sericea* root

The evaluation of the anti-oxidant activities of the plant parts (leaf, stem and root) indicated that the root (methanol:acetone extract) exhibited higher free

radical scavenging activity compared to the methanol:acetone extracts of the stem and leaves. The hot water extract also indicated a comparable reducing power activity when compared to the hot water extract of the stem. Considering the effect of solvents on the antibacterial activity of the root, and the anti-oxidant activities of the organic extracts of the plant parts, the effect of other solvents (acetone, ethyl acetate, and dichloromethane/methanol) on the anti-oxidant activities of the root was evaluated. Therefore, crude acetone, aqueous, ethyl acetate, dichloromethane/methanol and methanol extracts were evaluated for anti-oxidant activity. The anti-oxidant activities of these crude extracts are presented in Table 5.6. The highest DPPH free radical scavenging activity was observed with the acetone extract (7.09 ± 0.37 µg/mL), which was significantly better ($p < 0.05$) than the activity of aqueous, dichloromethane:methanol, ethyl acetate, methanol extracts and ascorbic acid. Poor activity was obtained with the aqueous extract (36.1 ± 3.7µg/mL). The acetone extract also exhibited the highest reducing power activity, which was significantly better than for other crude extracts and ascorbic acid. The DME extract exhibited the least activity, but it was not significantly different from that of the aqueous extract.

Table 5.6 Effect of the extraction solvent on the anti-oxidant activities (µg/mL) of *Terminalia sericea* crude root extracts as reflected by the DPPH and reducing power assays

Extract	DPPH	RP
Acetone	7.09 ± 0.64^{a}	14.1 ± 0.3^{a}
Aqueous	36.1 ± 3.7^{b}	36.0 ± 1.0^{b}
Dichloromethane:methanol	16.0 ± 1.0^{c}	37.5 ± 0.2^{b}
Ethyl acetate	14.0 ± 0.1^{c}	26.1 ± 2.7^{c}
Methanol	12.6 ± 0.6^{c}	25.4 ± 2.3^{c}
Ascorbic acid	11.3 ± 0.1^{ac}	144 ± 4^{d}

Mean (n = 6) ± standard deviation (SD). A different lowercase alphabetical letter in each column indicates a significant difference at $p < 0.05$

Methanol has been reported to be a suitable extractant for anti-oxidants, particularly for low molecular weight phenolic compounds, from plant matrices (Do et al., 2014; Khoo et al., 2015). However, this study has demonstrated that acetone is the most suitable solvent for extracting anti-oxidant constituents from *T. sericea* root. Acetone and ethyl acetate extracts displayed both good anti-oxidant and antibacterial activities. 5.3.5 Effect of geographical locations on the

anti-oxidant activity (AA) and total flavonoid contents (TFC) of *T. sericea* root dichloromethane/methanol extracts.

The anti-oxidant activity (Table 5.7) was evaluated using the DPPH free radical scavenging assay. The range of anti-oxidant activity observed in this study was 4.58 to 25.97 (µg/mL). The highest activity was observed for P3 and P7, while P10 exhibited the least activity. There was a significant variation ($p < 0.05$) in the anti- oxidant activity between P1-P9 and P10. The anti-oxidant activity of ascorbic acid was also significantly lower ($p < 0.05$) than that of P10. Populations P1, P4 and P9 displayed the largest variation in activity within the population, with standard deviations of 3.02, 10.83 and 4.95 (µg/mL), respectively, suggesting considerable chemical variation.

Table 5.7 Average values of anti-oxidant activities using the DPPH inhibition assay (µg/mL) and total flavonoid contents (mgQE/g) of dichloromethane/methanol extracts of samples from 10 populations

Population	DPPH	TFC
P1	9.78 ± 3.02^{a}	5.27 ± 0.44^{a}
P2	6.31 ± 0.82^{a}	5.48 ± 0.14^{a}
P3	5.17 ± 0.74^{a}	5.69 ± 0.10^{ac}
P4	12.2 ± 10.8^{a}	4.65 ± 0.29^{b}
P5	6.74 ± 0.79^{a}	5.40 ± 0.14^{a}
P6	7.33 ± 1.02^{a}	4.82 ± 0.45^{b}
P7	4.58 ± 1.71^{ac}	5.24 ± 0.22^{a}
P8	5.66 ± 0.56^{a}	5.28 ± 0.11^{a}
P9	14.4 ± 5.0^{ad}	5.11 ± 0.27^{ad}
P10	26.0 ± 1.9^{b}	5.07 ± 0.11^{ae}
Ascorbic acid	$11.\ 3 \pm 0.1^{a}$	

mgQE/g: milligram quercetin equivalent per gram. P: population. Values are expressed in mean ± SD. Different lowercase alphabetical letters in each column indicate significant difference at $p < 0.05$

The total flavonoid contents of *T. sericea* root samples from different populations are presented in Table 5.6. Population 3 (P3) contained the highest total flavonoid content (TFC) of 5.69 mgQE/g, which was significantly higher ($p < 0.05$) than those of P4, P6, P9 and P10. The lowest TFC was present in P4 and was significantly lower (4.65 mgQE/g) than that of the other populations, with the exception of P6, P9 and P10. In this study, P3 exhibited the best antibacterial activities against *S. typhi*, and the highest TFC. It also displayed a high anti-oxidant activity compared to the other populations. The phenolic compounds

present in *T. sericea* have not been studied sufficiently to understand the relationship between the anti-oxidant capacity of *T. sericea* and the compounds responsible.

5.3.7 Anti-oxidant activity of the column fractions and isolated compounds

Three of the four compounds isolated from *T. sericea* root were tested for anti- oxidant activity using the DPPH free radical and reducing power assays. The results of the anti-oxidant activity of only resveratrol-3-rutinoside are presented in Table 5.8. The anti-oxidant activities of sericic acid and sericoside were not included in the table, because they were inactive. Resveratrol-3-rutinoside displayed weak anti- oxidant activity in both assays and the activity was significantly lower ($p < 0.05$) than the anti-oxidant activity of the fractions and ascorbic acid. In the DPPH assay. Fraction F2 displayed the best anti-oxidant activity, but it was not significantly different ($p < 0.05$) from that of F1. Fraction 2 also exhibited the best anti-oxidant activity in the reducing power assay and it was significantly higher ($p < 0.05$) than that of the other fractions and ascorbic acid.

Table 5.8 Anti-oxidant activity of resveratrol-3-rutinoside as determined in both the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (µg/mL) and reducing power (µg/mL) assays

Sample	DPPH	RP
Resveratrol-3-rutinoside	186 ± 3^{a}	184 ± 3^{a}
CE	22.3 ± 1.7^{b}	24.4 ± 0.2^{bc}
F1	14.8 ± 0.5^{cde}	28.8 ± 1.0^{bd}
F2	9.36 ± 0.28^{cd}	17.0 ± 0.2^{e}
F3	22.3 ± 0.4^{ce}	33.7 ± 4.2^{bd}
F4	20.3 ± 1.4^{ce}	31.5 ± 2.6^{bd}
Ascorbic acid	11.3 ± 0.1^{f}	145 ± 4^{f}

CE: Crude extract, F1: fraction1, F2: Fraction 2, F3: Fraction 3, F4: Fraction 4. Different lowercase alphabetical letters in each column indicate significant difference at $p < 0.05$

This result suggests that the major anti-oxidant constituents are present in F2. It also confirms that resveratrol-3-rutinoside is not the major contributor to the anti-oxidant activity, since it exhibited a weak anti-oxidant activity. The high anti-oxidant activity also observed for F1, compared to those of F3 and F4, is an

indication of other anti- oxidant constituents present in the crude extract that are of intermediate polarity, since F1 was eluted from the column with ethyl acetate (100%). Concerning the antibacterial activity of the fractions and isolated compounds, F1 exhibited the tighest activity when compared to other fractions, while Fraction F2 exhibited the best anti-oxidant activity. Furthermore, sericic acid isolated from F1 exhibited the best antibacterial activity, while resveratrol-3-rutinoside, isolated from F2 displayed the highest anti-oxidant activity. Although this suggests that terpenoids are responsible for the antibacterial activities while stilbenes are responsible for the for the anti-oxidant activities, other studies have reported compounds of the same class possessing both antibacterial and anti-oxidant activities (Sivasothy et al., 2013; Benmerache et al., 2017).

5.3.8 Summary

The following conclusions can be made:

- The root of *T. sericea* was the most active against the pathogens associated with stomach and skin infections compared to the stem or leaf.
- Ethyl acetate and acetone are the best solvents for the extraction of antibacterial and anti-oxidant constituents from *T. sericea* root.
- For the first time, sericic acid was identified as the main antibacterial agent in the root of *T. sericea,* particularly against Gram-negative bacteria.
- Resveratrol-3-rutinoside displayed anti-oxidant activity while sericic acid and sericoside did not exhibit anti-oxidant activity.
- The variation in the antibacterial and anti-oxidant activities in the different populations was due to the chemical variability of the root samples.

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CHAPTER 6

TOXICOLOGY STUDIES OF *TERMINALIA SERICEA* USING VERVET MONKEYS

6.1 INTRODUCTION

Toxicology is concerned with the chemical and physical properties of potentially toxic compounds, their physiological or behavioural effects on living organisms, the qualitative and quantitative methods for their analysis in biological and non-biological materials, and the development of procedures for treatment of adverse effects (Langman and Kapur, 2006). Drugs are constantly withdrawn from the market because of reported unacceptable toxicity in humans. A lack of adequate and reliable toxicology screening methods is one of the challenges responsible for the inability of *in vitro* and *in vivo* assays to accurately predict the toxicity of compounds and mixtures towards humans. This has led in the continous search for better assays that are good predictors of human toxicity (Astashkina and Grainger, 2014; Greene et al., 2010).

The importance of accurate prediction of the side effects of compounds in the early stage of drug development can be illustrated using the example of terfenadine. This antihistamine was withdrawn from the market because it caused cardiotoxicity at therapeutic concentrations in both animals and humans (Park et al., 2013). A better understanding of biology, species differences in xenobiotic handling and molecular toxicology has gradually resulted in an improvement in the design of reliable toxicological assays that can predict toxicity in humans (Jennings, 2015).

Traditionally, animals have been used to evaluate human safety. These studies were mostly conducted on rodents. However, the finding that rodents are poor predictors of certain mechanisms of toxicity in humans, led to the inclusion of non-rodent species, such as rabbits. For example, rats and mice did not show any foetal abnormalities when treated with thalidomide during pregnancy, but this compound is a known teratogenic agent in humans. Rabbits were subsequently introduced into the predictive testing guideline (Chapman et al., 2013; Jennings,

2015). It is therefore important to review regularly the appropriate use of animals in toxicology studies so as to improve predictive values (Chapman et al., 2013). Non human primates have been reported (Dalgaard, 2015) to be the closest to humans in terms of genetic homology, compared to dogs and pigs, and are therefore a more reliable species for the study of human toxicology.

During drug development, lead compounds with desired pharmacological effects are synthesized. These compounds are screened for toxicity before clinical trials (Bonifas et al., 2010). Stringent regulatory demands have also put pressure on drug manufacturing companies to shift from *in vivo* to *in vitro* systems for drug screening. The screening of these potential drugs on animals become cumbersome, costly and time consuming. In some cases, toxic effects only become apparent once the drugs have been in the market. Some side effects are not detected during the pre-clinical testing phase, but only become evident after extensive use. The use of *in vitro* assays early in the process of drug development helps to eliminate potentially toxic compounds before pre-clinical trials in animal models, using only a few of the promising candidatesidentified for screening (Bonifas et al., 2010; Kramer et al., 2015).

Traditional medicine (TM) is commonly used in many developing countries, because it is accessible and affordable (Bussmann et al., 2011). In Africa and other parts of the world, herbal medicine is an important part of the culture and tradition of the people. Medicinal plants exert broad actions on physiological systems due to the complexity of their chemical structures. A pharmacological effect may be observed through complementary or synergistic actions, which are usually non-specific and directed towards aiding the host's healing processes. These activities provide new and important leads against pharmacological targets. Medicinal plants are usually assumed safe, based on their traditional use over long periods of time. However, research has indicated that many plants used as food or medicine can lead to potentially toxic, mutagenic and carcinogenic effects (Carvalho et al., 2011; Edziri et al., 2011; Fennell et al., 2004; Nasri and Shirzad, 2013). The liver and kidneys are prime targets, because they are involved in the degradation and excretion of a myriad of chemical compounds (De Oliveira et al., 2011). Toxicants interfere with the central functions of an organism e.g. neurotoxins affect the brain and

nervous system, while cytotoxins and metabolic poisons damage the liver, kidneys, heart or the respiratory system (Mulaudzi et al., 2013). Toxicology studies have revealed that *T. sericea* crude extracts are cytotoxic to both normal and cancerous cells (Bessong et al., 2004; Fyhrquist et al., 2006; Tshikalange et al., 2005) in *in vitro* assays. The aim of this study is to investigate the toxicity of *T. sericea* in an *in vivo* model using vervet monkeys (*Chlorocebus pygerythrus*).

6.2 MATERIALS AND METHODS

6.2.1 Ethical approval

The study was approved by the Ethics Committee for Research on Animals (ECRA) of the South African Medical Research Council (Project No.09/13).

6.2.2 Acquisition and preparation of plant materials

A root sample of *T. sericea* was collected from the Vuwani area of the Limpopo Province, South Africa, in December 2014. Aerial parts of the tree were identified at the Department of Botany, University of Venda and the voucher number CPA010 was assigned. After drying, the samples were ground to powder using an industrial grinder. The milled powder was sterilized by irradiation at 18 kGy for 24 h. The powders were tested before and after irradiation for bacterial and fungal contamination by Swift Silliker (Claremont, Cape Town) (Appendix 7). The same homogenous batch of plant material was used for the entire study.

6.2.3 Experimental animals and feeding

The vervet monkeys (*Chlorocebus pygerythrus*) used for the study were housed and maintained at the Primate Unit and Delft Animal Centre (PUDAC), Medical Research Council, Tygerberg, South Africa, according to the guidelines of the South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008).

Eight male captive-bred vervet monkeys used for this study were identified with numbers in ink tattoo. A maintenance diet consisting of 100 g (containing 2.14 g/kg of the powdered samples) stiff maize porridge with micro- and macronutrient supplementation was fed throughout the study, and water was available *ad libitum*. The individual animals were maintained under identical housing conditions in single cages and had also regular access to exercise cages and environmental enrichment. In addition, all cages were marked according to individual, group designation, and experiment number. The closed indoor environment was maintained at 25 – 27 °C, a humidity of 45%, about 15-20 air changes/hour and a photoperiod of 12 h per day. Adult vervet males (n=8) were randomly assigned into two groups of four each. The first group received the experimental diet containing 2.14 g/kg of the plant powder (which is 25 times higher than the recommended daily dose of *T. sericea*) for 12 weeks. The feed was then replaced with normal feed for another 4 weeks (washout). The second group received only the maintenance diet throughout the 16 weeks (90 days). The animals received the food bolus once per day (at 7:00). The food bolus was consumed voluntarily by animals and food intake was recorded daily, according to the standard protocol used at PUDAC.

6.2.4 Blood collection

All animals were fasted overnight before clinical evaluation and blood sampling for biochemical, haematochemical, clinical and behavioural parameters. Blood samples (6 mL) were drawn from each animal (2 mL sodium fluoride/potassium oxalate tubes, 2 mL EDTA tubes and 2 mL in SST) at baseline, then once every four weeks during the 90-day experimental phase, and at the end of the washout period for both treated and control group animals. Blood was obtained *via* femoral venipuncture after ketamine anaesthesia at 10 mg/kg bodyweight. The volume of blood collected at each time point per individual was below 10% of the circulating blood volume, which is considered the maximum permissible sample volume within a four-week blood collection frequency.

6.2.5 Clinical biochemistry analysis

The clinical chemistry included tests for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total protein, bilirubin (total and direct), glucose, albumin, globulin, cholesterol (total, LDL-C, HDL-C), urea, creatinine, triglycerides, chloride, potassium, calcium and sodium, anion gap, creatine kinase (CK), total bicarbonate (CO_3^{2-}) and glucose. Complete blood count examination included total red blood cells (RBC), total white blood cells (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH); mean corpuscular haemoglobin concentration (MCHC), haematocrit (HCT), red blood cell distribution (RDW), haemoglobin (Hb), neutrophil, eosinophil, basophil, lymphocyte, monocyte and platelet count.

6.2.6 Observations

At the time of blood sampling (baseline-washout) the weight of each animal, body temperature, pulse, respiration rate, and blood pressure was recorded using a Dinamap XL vital signs monitor with a neonatal blood pressure cuff #4. Food and water intake was observed daily and recorded, while general well-being was observed daily and recorded weekly. These included posture, coordination, locomotion, activity, behaviour (alert, fearful, aggressive, confused, depressed, vocalisation), discharge from orifices, appetite, condition of faeces and urine.

6.2.7 Statistical analysis

All variables were analyzed by the MRC Biostatistics Unit utilizing the STATA statistical package with Repeated Measures Analysis of Variance; $p<0.05$ was considered significant. Statistics for changes from baseline were generated for time effect, group effect, group-time interactions and differences between each treatment group and the controls. Graphs were constructed using Graphpad 6 prism (Graphpad Software Inc.).

6.3 RESULTS AND DISCUSSION

6.3.1 Average food intake

The results of the average food intake (%) of the vervet monkeys (Figure 6.1) indicate that there was a reduction in the food intake in the group treated with *T. sericea* after Week 4 (87.4 ± 1.13%) and Week 8 (84.7 ± 1.08%).

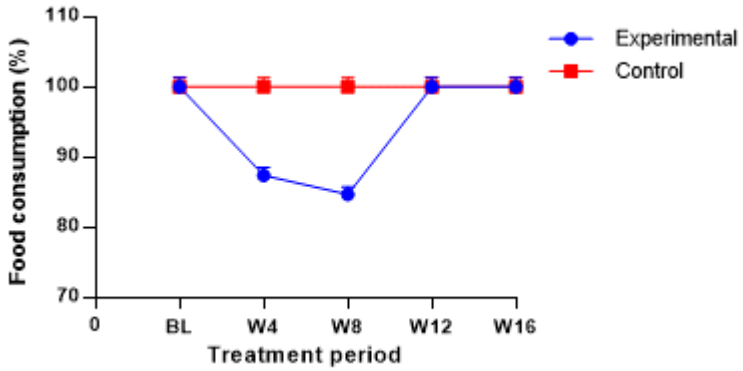

Figure 6.1 Graph indicating the average food intake of vervet monkeys fed a control diet, or a diet supplemented with powdered root of *T. sericea* (experimental) for 12 weeks and then washout (Week 16). BL: baseline, W: week

6.3.2 Liver function biomarkers

6.3.2.1Drug metabolism in the liver

The liver is one of the largest organs in the human body and the primary site of metabolism and excretion (Hamza and Al-Harbi, 2015). It is the site for detoxification, protein synthesis, bile secretion, glycogen storage, and production of hormones and decomposition of red blood cells (Yazdani et al., 2013). Any injury to the liver therefore, attenuates the metabolic functions regulated by the liver (Al-Attar and Shawush, 2015). Liver disease is a major threat to human health worldwide and drug-induced liver injury is one of the major causes of withdrawal of drugs from the market (Saito et al., 2016; Wang et al., 2016; Zhang et al., 2016).

Drugs are primarily metabolized in the liver by a network of enzymes known as “drug metabolizing enzymes” (Hussein et al., 2013). Drug metabolism occurs in

two phases. Phase I is known as the "oxidation stage" and involves the oxidation of drugs by the cytochrome P450 enzyme complex to more water soluble metabolites, which can be potentially toxic. In Phase II – also known as the conjugation phase- metabolites from Phase I (including electrophiles and ROS) and other foreign compounds, are enzymatically conjugated with polar groups, such as glucoronate and gluthathiones, for easy excretion (Xing-Hua et al., 2014; Yao et al., 2012). Liver injury occurs as a result of oxidative stress, when metabolites and ROS from the Phase I reaction disrupt the integrity of the hepatic membrane. This leads to an increase in permeability or leakage of the enzymes into the blood (Nwachukwu et al., 2015; Seif et al., 2016). Liver damage is estimated in the serum when there are elevated levels of liver enzymes (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyl transferase), which are used as markers for hepatotoxicity (Hussein et al., 2013). Other non-enzyme markers include: proteins (albumin and glutamine), bilirubin, triglycerides and cholesterol (Nasir et al., 2013).

6.3.2.2Effects of *T. sericea* roots on liver function enzymes

Liver function enzymes, i.e. alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT), were used to evaluate liver toxicity (Figure 6.2). There was a significant ($p < 0.05$) increase (87%) in the average serum AST levels (Figure 6.2A) from baseline (71.3 ± 5.06 U/L) to Week 4 (133 ± 55 U/L) in the experimental group treated with *T. sericea*. Treatment over subsequent weeks indicated a decline in the serum AST levels, so that these were no longer significantly ($p < 0.05$) different from the baseline values. After Week 4, there was no significant difference ($p < 0.05$) in the AST levels between the treated group and the controls. The control group did not display any changes in their serum AST levels during the intervention.

A tremendous spike (130%) in serum ALT levels (Figure 6.2B) was observed in the experimental group after Week 4. This increase in serum ALT was significantly different ($p < 0.05$) from the baseline. However, the increase in average serum ALT levels was not sustained after Week 8. There was a

significant ($p < 0.05$) reduction in the average ALT levels from Week 4 (109 ± 59 U/L) to Week 8 (54.0 ± 29.9 U/L). The reduction in the ALT levels continued through Weeks 12 and 16. The control group displayed elevated average serum ALT levels (120 ± 54 U/L) after Week 8 and these levels were significantly higher than those of the experimental group (Week 8). There was an increase in the average serum ALP levels (Figure 6.2C) from the baseline (156 ± 48.1 U/L) to Week 4 (248 ± 89.6 U/L) of the experimental group, but this increase was not significantly higher ($p < 0.05$). There was also no significant difference in the average serum ALP levels between the experimental and control groups.

In the experimental group, there was a significant increase (82.5%) in the serum GGT levels (Figure 6.2D) from the baseline (71.5 ± 16.8 U/L) to Week 4 (130 ± 36 U/L). The elevated serum GGT levels were sustained after Week 8, before declining (not significantly different at $p < 0.05$) after Week 12 (108 ± 36 U/L) and 16 (85.0 ± 33 U/L). There was a significant difference in the serum GGT levels between the experimental and the control after Week 4. The control did not display any significant difference in the serum GGT levels during the study period.

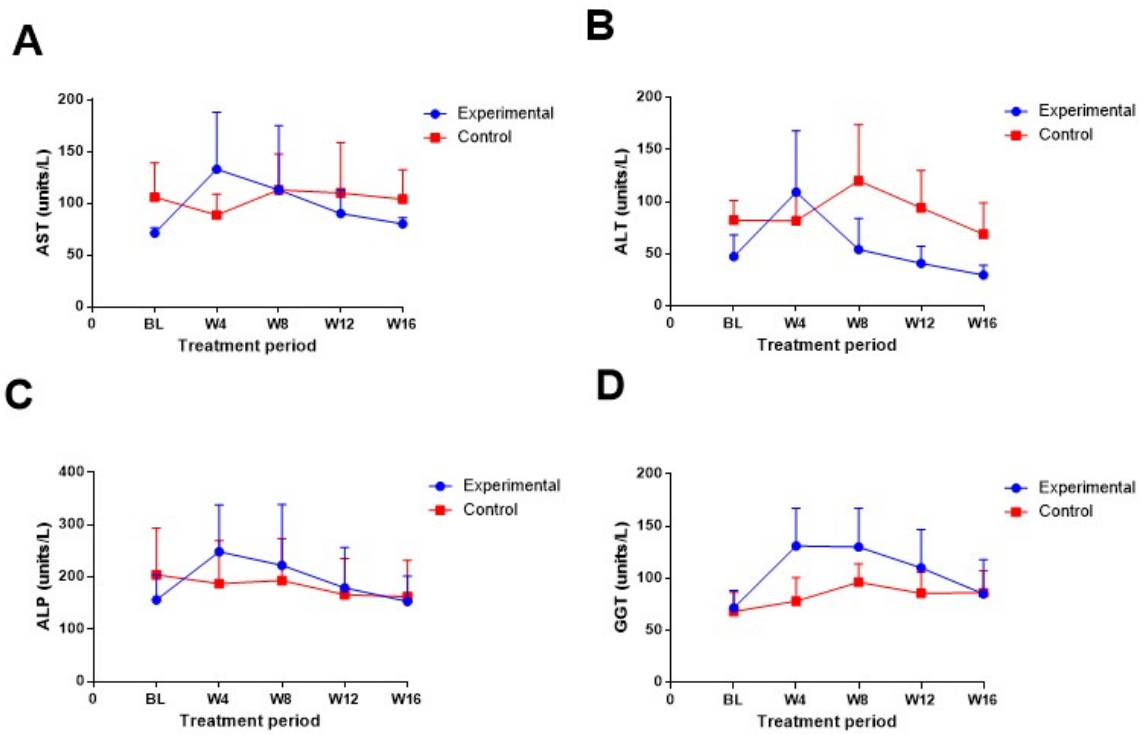

Figure 6.2 Concentrations of average serum A) AST, B) ALT, C) ALP, D) GGTin vervet monkeys after 12 weeks of exposure to a diet supplemented with *T. sericea* root (experimental) and an additional 4 weeks washout period. BL: baseline, W: week

In the current study, the experimental group presented with elevated serum levels of AST, ALT ALP and GGT. The increase in the serum enzyme levels during this period of the study is an indication of hepatic injury, particularly after four weeks. The liver enzymes, AST and ALT, are present in the liver, kidney, heart and skeletal muscles. However, ALT is more abundant in the liver compared to AST (Nayanna Brunna et al., 2013; Kanife et al., 2012; Narasimhulu et al., 2014; Shehab et al., 2015). Compared to AST, ALT is a more specific biomarker for liver injury, because it is exclusively a cytoplasmic (80% sub-cellular concentration) enzyme, while the sub- cellular concentration of AST is higher in the mitochondria (80%) than in the cytosol (20%). Therefore, when hepatic membrane damage occurs, there is an initial increase in the levels of ALT in the serum due to the abundance of ALT in the cytosol. Severe damage in the liver results in a subsequent increase in the level of AST in the serum (ArAGon and Younossi, 2010; Borlak et al., 2014).

Alkaline phosphatase (ALT), is a globulin enzyme that is present in high concentrations in the bones, hepatobiliary tract and in the kidneys (Nagaraja and Krishna, 2016). In contrast, GGT is not a specific liver enzyme, but is widely distributed in tissues involved in secretory and absorption processes (Akpanabiatu et al., 2009; ArAGon and Younossi, 2010). In the present study, the elevated levels of ALP and GGT in the serum is an indication of biliary excretory dysfunction (Nazari et al., 2014; Nielsen et al., 2016; Zaidi et al., 2015).

This study has demonstrated that the roots of *T. sericea* are toxic to both the hepatocytes and the bile system after 4 weeks of intake. It therefore could mean that the mechanism of hepatotoxicity exerted by *T. sericea* is through hepatocellular and cholestatic injury. However, the levels of these enzymes reduced gradually during the intervention. All the enzyme activities returned to normal during the washout period (Week 16). This could mean that the liver was able to cope with the initial toxicity caused by the plant by increasing the production of endogenous anti-oxidants. It could also imply that the exogenous anti-oxidants, present in *T. sericea* roots, were able to compensate for the initial toxicity during Phase I metabolism. Resveratrol-3-rutinoside, isolated from the root, displays moderate anti- oxidant activity (Section 5.3.7). The hydrolysis of resveratrol-3-rutinoside yields resveratrol (Bombardelli, 1975), a known anti-oxidant that is present in wine, fruits and vegetables (Gülçin, 2010; Kasiotis et

al., 2013). Hydrolysis of resveratrol-3-rutinoside (during Phase 1 metabolism) could increase the levels of resveratrol, thereby increasing the hepatoprotective effect of *T. sericea*.

6.3.2.3 Effects of T. *sericea* root on total and direct bilirubin

The effects of *T. sericea* roots on total bilirubin serum levels are illustrated in Figure 6.3A. The experimental group exhibited a significant ($p < 0.05$) increase (146%) in the average serum total bilirubin levels from the baseline (6.00 ± 2.16 μmol/L) to Week 4 (14.8 ± 3.50 μmol/L). There was a decline in the average total bilirubin level after Week 8 through Week 16. However, the levels after Weeks 8 (13.3 ± 5.50 μmol/L) and 12 (11.5 ± 3.51 μmol/L) were significantly higher ($p < 0.05$) than the baseline values (6.00 ± 2.16 μmol/L). Furthermore, there were significant differences ($p < 0.05$) between the average serum total bilirubin of the experimental group and the controls after Weeks 4, 8, and 12.

The effects of the roots on direct bilirubin serum levels are presented in Figure 6.3B. The average concentrations of the experimental group was elevated 2.4 fold from the baseline (1.25 ± 0.50 μmol/L) to Week 4 (3.00 ± 0.82 μmol/L) and Week 8 (3.00 ±1.83 μmol/L). A decline in the average direct bilirubin concentration was observed after Week 12 (2.25 ± 0.50 μmol/L) and Week 16 (1.25 ± 0.50 μmol/L). There was a significant difference ($p < 0.05$) between the experimental and control groups after Week 4 and Week 8. The control group did not display any significant changes ($p < 0.05$) over the study period (16 weeks). At the end of Week 16, the average serum total bilirubin of the experimental (5.00 ± 2.00 μmol/L) and the control groups (5.75 ± 2.22 μmol/L) were comparable.

Bilirubin is the main bile pigment that is formed after the breakdown of heme in worn out red blood cells. The bilirubin then binds to albumin and is transported to the liver for conjugation with glucuronic acid, a step regulated by glucuronosyl transferase. The conjugated product is secreted into the bile by hepatocytes (Nagaraja and Krishna, 2016; Raju et al., 2011; Udo et al., 2014). According to Seif, (2016), an increase in the bilirubin levels in the blood could result from gall stones, liver disease or the excessive breakdown of red blood cells. This result indicated that *T. sericea* root elevated the average serum total and direct bilirubin in the vervet monkeys. However, after the washout period,

(Week 16), the level of the serum total bilirubin of the experimental group was comparable with that of the control group.

The elevation of total bilirubin (unconjugated) in the serum suggests that after the breakdown of heme, there was a decrease in the conjugation of the bilirubin in the liver by Phase II enzymes. This resulted in the inability of the hepatocytes to secrete unconjugated bilirubin into the bile. The increase in the levels of direct bilirubin in the blood suggests that, despite the conjugation of the bilirubin, there was a problem with the secretion of the conjugated bilirubin into the bile or that a blockage of the bile duct occurred (Anosike et al., 2008). In other words, the metabolites of *T. sericea* may have increased the bilirubin content in the blood by either reducing the ability of the hepatocytes to conjugate the bilirubin, or by interfering with the ability of the hepatocytes to secrete the conjugated bilirubin into the bile.

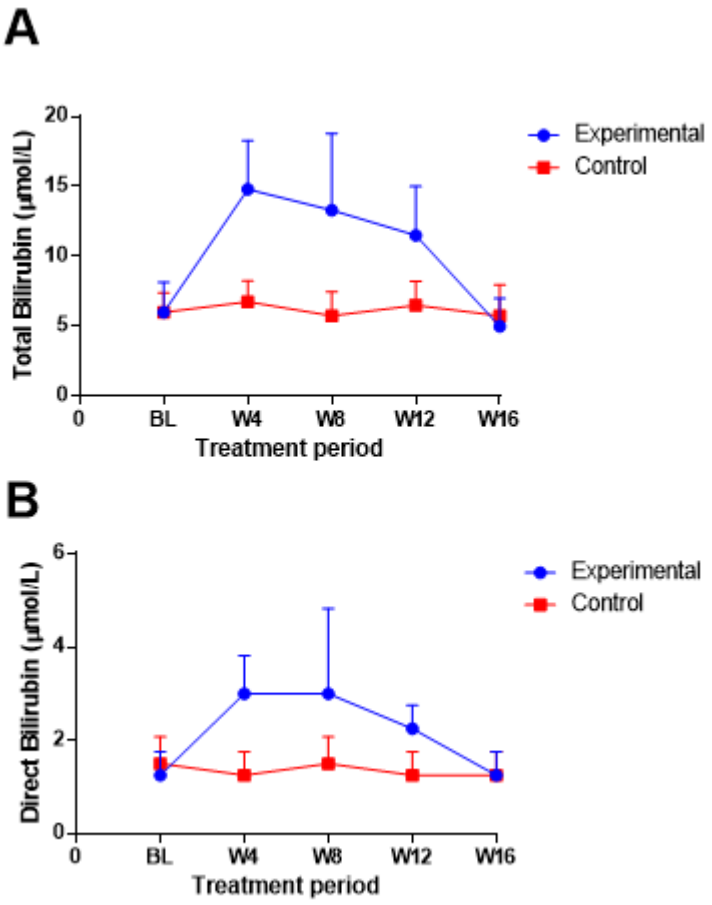

Figure 6.3 Concentrations of average serum A) Total bilirubin, B) Direct bilirubin in vervet monkeys after 12 weeks of exposure to a diet supplemented with *T. sericea* root and an additional 4 weeks washout period. BL: baseline, W: week

6.3.2.3 Effects of T. *sericea* root on total serum protein and albumin

The results of the effect of *T. sericea* root on serum protein content are presented in Figure 6.4A. There was a significant reduction ($p < 0.05$) in the average serum protein content between the baseline and Weeks 4, 8, and 12 of the experimental group. The experimental group also displayed a significant reduction ($p < 0.05$) in the average serum protein content compared to the control group after Weeks 8 and 12. The serum protein content of the control group did not change significantly throughout the study period.

The effects of the roots on the serum albumin content are illustrated in Figure 6.4B. There was a significant reduction ($p < 0.05$) in the average serum albumin content of the experimental group between the baseline (58.3 ± 2.99 g/L) and Weeks 4 (53.0 ±4.90 g/L), 8 (51.5 ± 3.70 g/L) and 12 (51.3 ± 3.59 g/L). The serum albumin content of the experimental group also differed significantly from that of the control group after Weeks 4, 8. 12, and 16. There were no significant changes in the serum albumin content of the control group throughout the study period. There were also no significant differences in the serum globulin content between the control group and the experimental group (Appendix 8). This study indicated a significant decrease in the total protein and albumin levels of the group treated with *T. serica* roots. The reduction in the total protein content was as a result of a reduction in the albumin levels. Although an increase in globulin levels were measured, they were not significantly higher than the values measured at baseline.

Albumin and globulin are two major components of human serum protein that are synthesized by the parenchyma cells in the liver (Hamza and Al-Harbi, 2015; Liu et al., 2016; Nwangwu et al., 2011). Albumin is the main blood protein and accounts for 60% of serum protein (Hamza and Al-Harbi, 2015). It is associated with nutritional status and severity of disease. Globulin functions as a carrier of sex hormones and plays a major role in immunity and inflammation (Liu et al., 2016; Singh et al., 2011). The decrease in serum proteins could be the result of a decrease in the synthesis of the albumin in the parenchyma cells. Specific metabolites present in the roots of *T. sericea* possibly reduce the ability of the parenchyma cells to synthesize albumin. The decrease in the average

serum protein could also be an indication of malnutrition, since the amount of food consumed after Week 4 (Figure 6.1) was the lowest during the experimental period. It is possible that the monkeys ate less of the food initially, because the food was not palatable. However, they gradually adjusted to the food or were hungry, and had no other option. According to Liu et al. (2016), a decrease in albumin levels and a n increase in globulin levels suggest chronic inflammation, indicating that the roots possibly exert a pro-inflammatory effect. It is not clear if the reduction of the total protein was due to the reduction of food intake, which could affect the synthesis of protein, or as a result of toxicity of the roots towards the parenchyma cells.

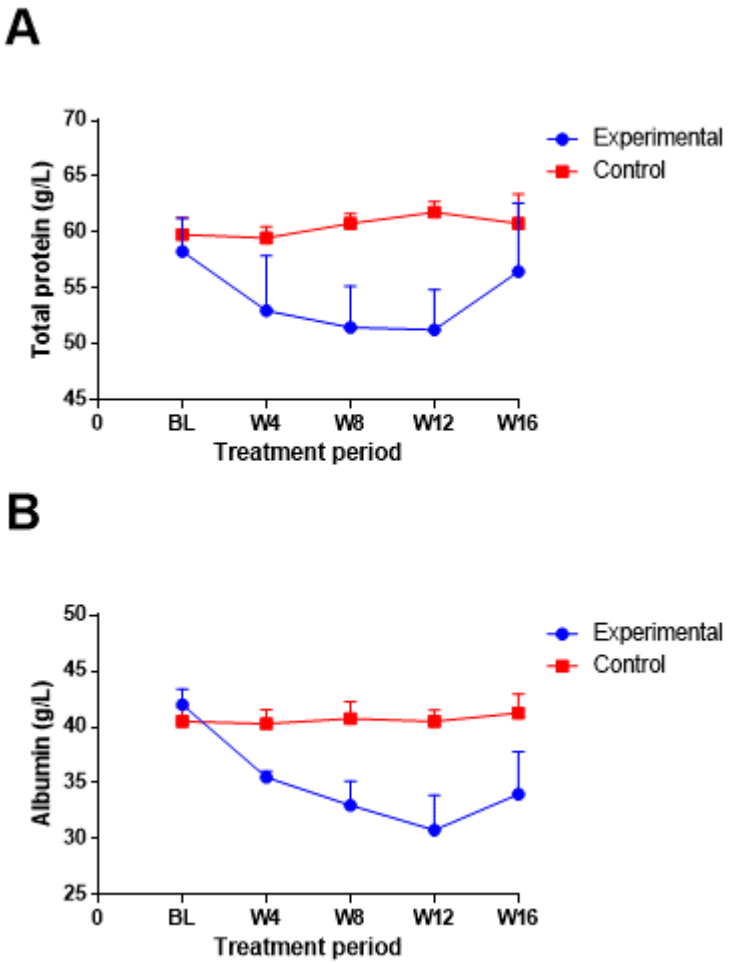

Figure 6.4 Concentrations of average serum A) Protein, B) Albumin in vervet monkeys after 12 weeks of exposure to a diet supplemented with *T. sericea* root and an additional 4 weeks washout period. BL: baseline, W: week

6.3.2.4 Effects of *T. sericea* roots on serum glucose and lipid levels in vervet monkeys

The effects of the roots on the serum glucose levels are presented in Figure 6.5. The experimental group displayed lower average serum glucose levels compared to the controls with significant differences ($p < 0.05$) observed after Week 4 (experimental,
2.66 ± 0.48; control, 3.75 ± 0.75 mmol/L) and Week 12 (experimental, 3.38 ± 0.62; control, 4.75 ± 1.11 mmol/L). The highest reduction was observed in the experimental group, after Week 4 (2.66 ± 0.48 mmol/L) and it was significantly lower ($p < 0.05$) than the baseline (4.13 ± 0.29 mmol/L). After the washout (Week 16), the serum glucose levels of the experimental group returned to normal (4.18 ± 0.36 mmol/L). The average serum glucose levels of the control group remained unchanged throughout the study period. The roots had no effect on the serum HDL, LDL, and triglyceride levels of the vervet monkeys (Appendix 9).

The reduction in the serum glucose observed in the group treated with *T. sericea* root could be an indication of hypoglycermic potential. A reduction in food intake could also affect glucose availability in the blood, especially after Week 4. As the food intake increased in the subsequent weeks, there was a corresponding increase in serum glucose concentrations. However, the root did not influence the serum lipid levels of the monkeys. This finding indicates that the metabolism of lipids was not affected by the intake of *T. sericea* root.

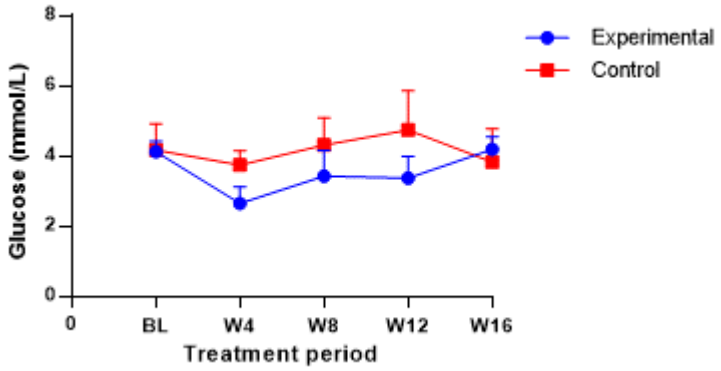

Figure 6.5 Concentrations of average serum glucose in vervet monkeys after 12 weeks of exposure to a diet supplemented with *T. sericea* root and an additional 4 weeks washout period. BL: baseline, W: week

6.3.3 Kidney function biomarkers

The serum creatinine levels after exposing the monkeys to *T. sericea* roots are presented in Figure 6.6. The average serum creatinine concentrations of the experimental group were elevated compared to the controls. Higher concentrations were measured at Week 8 (117 ± 21.1 U/L) and Week 12 (110 ± 23.7 U/L) and these were significantly different ($p < 0.05$) from those of the control group (Week 8, 87.3 ± 20.8; Week 12, 79.8 ± 13.3 U/L). The serum levels of the experimental group after Weeks 8 and 12 were also significantly higher than the baseline values (79.5 ± 17.1). The control group did not display any significant fluctuation in average serum creatinine levels during the study period. There were no significant changes in the serum urea (Appendix 10), creatine kinase (Appendix 10) and electrolytes (Appendix 11) in the experimental group.

The kidney is a vital organ for the removal of metabolic waste products. It also regulates the extracellular fluid volume, concentrations of inorganic electrolytes, osmolality, acid-base balance and blood pressure (Vranješ et al., 2016). Creatinine is generated from the metabolism of creatine in the muscles. An increase in the levels of serum creatinine for the group treated with *T. sericea* indicates poor glomeruli filtration, resulting from damage to functional nephrons (Afolabi et al., 2014; Arfat et al., 2014; Gad and Zaghloul, 2013; Okechukwu et al., 2013). In the current study, the increase in the serum creatinine level could be the result of poor creatinine clearance by the kidney. The effect of the damage on the kidney was reduced at Week 16, once the treatment feed had been replaced with normal feed. The effect of *T. sericea* on the liver was pronounced after four weeks of exposure, while the effects on the kidney was pronounced after eight weeks and sustained to Week 12. This is understandable since the liver is the first site of metabolism and therefore the effects are observed earlier.

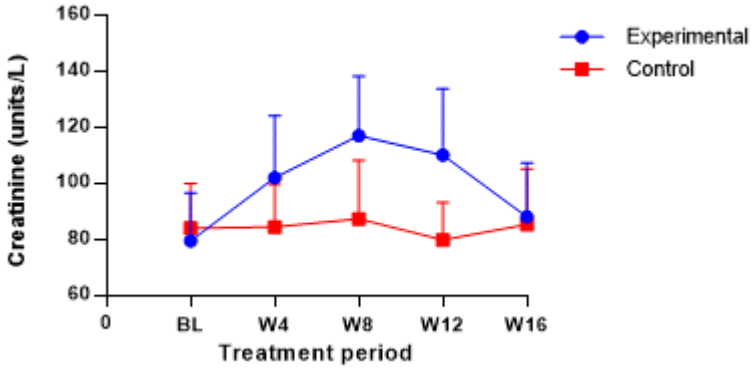

Figure 6.6 Concentrations of average serum creatinine in vervet monkeys after 12 weeks of exposure to a diet supplemented with *T. sericea* root and an additional 4 weeks washout period

6.3.4 Hematological parameters

The effect of *T. sericea* root on haematological parameters is presented in Figure 6.7. The experimental group exhibited a significant reduction ($p < 0.05$) in the average RBC (Figure 6.7A) after Week 8 (experimental 5.99 ± 0.45 $x10^9$/L; control 6.70 ± 0.36 $x10^9$/L), Week 12 (experimental 5.47 ± 0.36 $x10^9$/L; control 6.61 ± 0.32 $x10^9$/L) and Week 16 (experimental 5.40 ± 0.73 $x10^9$/L; control 6.53 ± 0.34 x 10^9/L) compared to the control. In the experimental group, the RBC levels after Week 12 (5.47 ± 0.36 $x10^9$/L) and Week 16 (5.40 ± 0.73 $x10^9$/L) were significantly lower ($p < 0.05$) than the baseline (6.10 ± 0.37 $x10^9$/L). The decrease in the RBC could be as a result of reduction in the hemoglobin content which also affected the hematocrit. There was an increase in the white blood cell count and neutrophils in the group treated with *T. sericea*, but the values were not statistically significant and therefore cannot be attributed to the effect of *T. sericea*. There was no significant fluctuation in the RBC levels of the control during the study. The experimental group displayed significantly lower ($p < 0.05$) Hb levels (Figure 6.7B) compared to the control after Week 8 (experimental 14 ± 0.75; control 16.3 ± 0.73 g/dL) Week 12 (experimental 12.9 ± 0.75; control 16.3 ± 0.93 g/dL) and 16 (experimental 13.1 ± 1.77; control 16.5 ± 1.06 g/dL). In the experimental group, the average Hb levels at Week 12

(12.9 ± 0.75 g/dL) and Week 16 (13.1 ± 1.77 g/dL) were significantly lower ($p < 0.05$) lower than the baseline (15 ± 0.65 g/dL). The control group did not display any significant difference ($p < 0.05$) in the average Hb levels during the study period.

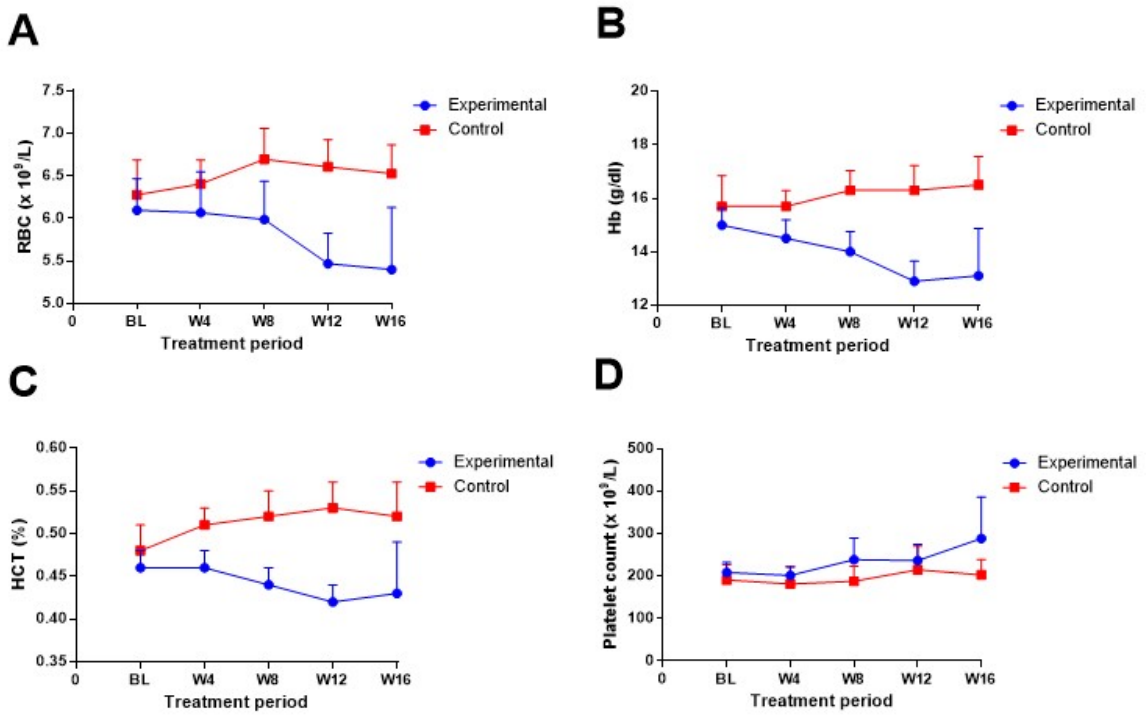

Figure 6.7 Concentrations of average serum A) Red blood cell count, B) Haemoglobin, C) Haematocrit, D) Platelet count in vervet monkeys after 12 weeks of exposure to a diet supplemented with *T. sericea* root and an additional 4 weeks washout period

The average haematocrit concentrations of the experimental group were significantly lower ($p < 0.05$) (Figure 6.7C) than those of the control group after Week 4 (experimental 0.46 ± 0.02%; control 0.51 ± 0.02%), Week 8 (experimental 0.44 ± 0.02%; control 0.52 ± 0.03%), Week 12 (experimental 0.42 ± 0.02%; control 0.53 ± 0.03%) and Week 16 (experimental 0.43 ± 0.06%; control 0.52 ± 0.04%). However, for the experimental group, the concentrations after Week 12 (experimental 0.42 ± 0.02%) were the only ones that were significantly lower ($p < 0.05$) compared to the baseline (0.46 ± 0.02%).

Blood consists of a protein-rich plasma, which constitutes 55% of blood volume, and blood cells (red and white blood cells and platelets) that make up the

remaining 45% of the blood volume. Hemoglobin is the main component of the red blood cell (RBC) and facilitates the transportation of oxygen and other respiratory gases to tissues. Hematocrit refers to the relative volume of the blood cells to the total blood volume (Samuel et al., 2015). Hematological parameters are regarded as good indicators of plant toxicity (Olusola et al., 2015). This study has indicated that *T. sericea* caused a decrease in the levels of RBC, hemoglobin and hematocrit.

Unlike the liver function indicators that reflect profound toxicity after Week 4, the effect on the kidney and blood parameters suggest that the metabolites from the liver, while being transported in the blood, gradually exerted toxicity in the blood and kidney. The decrease in the blood parameters (anaemia) could be the result of oxidative stress caused by the generation of ROS during Phase 1 oxidation in the liver (Miikue-Yobe et al., 2015). These radicals may have led to the shrinkage and destruction of red blood cells through oxidation of the sulfydryl groups of the erythrocyte membrane, causing injury to the membrane and making the cells prone to lysis (Eshak et al., 2015). The reduction could also be caused by inhibition of red blood cell production or by increased stimulation of macrophages, which, in turn, can lead to the increased destruction of red blood cells, ultimately causing anaemia (Ologundudu et al., 2009).

The average platelet count of the experimental group (289 ± 97.7 $x10^9$/L) was only significantly higher ($p < 0.05$) (Figure 6.7D) than that of the control (203 ± 36.2 $x10^9$/L) after Week 16. A similar trend was observed when the average platelet count of the experimental Week 16 was compared to the baseline (209 ± 23.2 $x10^9$/L). The platelet counts of the control group did not change significantly throughout the study. Wound healing is a sequential, but overlapping, complex physiological process that involves tissue repair and regeneration of damaged tissues (Kazemi Mehrjerdi et al., 2008; Zouari et al., 2016). The process of wound healing is initiated by hemostasis, followed by inflammation, cellular proliferation and tissue remodeling (Djemaa et al., 2016; Jee et al., 2016). Hemostasis occurs through a fibrin and platelet plug, which triggers the coagulation cascade (Morton and Philips, 2016). Platelets are rich in naturally occurring growth factors, cytokines and proteases, which are involved in the

process of wound healing (Carter et al., 2003; Nocito et al., 2007). The increase in the platelet count for the treated group indicates that the plant roots stimulate the production of platelets, an effect that aids wound healing. Ethnopharmacological surveys have found that *T. sericea* is used for treating wounds and menorrhagia (Moshi and Mbwambo, 2005). Studies have also revealed that the roots have wound healing ability (Parkar, 2016; Steenkamp et al., 2004). In treating diabetes, *T. sericea* can be used both to reduce blood glucose and to treat wounds associated with diabetes. The plant roots did, however, not affect the concentrations of MCV, MCH, MCHC, RDW, WBC, neutrophils, eosinophils, basophils, lymphocytes, or monocytes (Appendix 12).

6.3.5 Physical and physiological variables

The body weights of the vervet monkeys following exposure to the roots are presented in Figure 6.8. The average body weight of the experimental group decreased significantly ($p < 0.05$), with a loss of 0.65, 0.8 and 1.0 kg mass after Weeks 4 (4.6 ± 0.53 kg), 8 (4.4 ± 0.50 kg) and 12 (4.2 ± 0.49 kg), respectively, compared to the baseline (5.2 ± 0.64 kg). However, there was a gain of weight after Week 16 (4.59 kg). The experimental groups also displayed significantly lower ($p < 0.05$) body weight after Weeks 4, 8, 12 and 16, compared to the controls. There were no significant changes ($p < 0.05$) in the body weights of the control group during the study period. Treatment with *T. sericea* did not significantly affect the pulse, respiration rate, blood pressure or body temperature of the vervet monkeys (Appendix 13). The decrease in the body weight of the treatment group may have resulted from a loss of appetite or their unwillingness to eat due to the taste of the feed. This correlates with a decrease in food consumption after Week 4. The loss of appetite or unwillingness to eat could be due to the tannin content of *T. sericea* (Ajala, 2014).

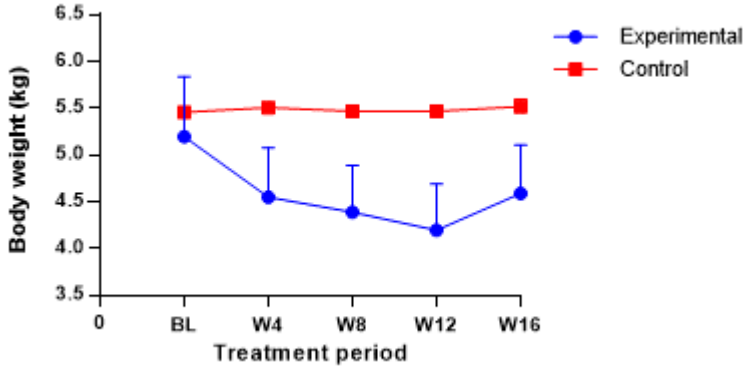

Figure 6.8 Average body weight (kg) of vervet monkeys after 12 weeks of exposure to a diet supplemented with *T. sericea* root and an additional 4 weeks washout period

6.3.6 Summary

The following conclusions can be made after feeding 2.14 mg/kg *T. sericiea* to vervet monkeys for 12 weeks followed by measuring an array of blood parameters:

- Short-term hepatotoxic effects were evident at Week 4, followed by hepatoprotective activity from Weeks 8 to 16.
- Elevated serum creatinine levels indicated possible nephrotoxicity.
- Reduction of the serum glucose at Week 4 suggests hypoglycemic potential.
- Reduction in the serum haematological parameters indicates possible haematotoxicity.
    - Elevated serum platelet count indicates possible wound healing potential.
- Potential nephrotoxicity and hematotoxicity observed at high dosage (25 times higher concentration than would normally be used). The roots therefore seem to have little toxicity.

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CHAPTER 7
CONCLUSIONS AND RECOMMENDATIONS

7.1 OVERVIEW

The main aim of this study was to investigate the phytochemistry, biological activities and toxicity of *Terminalia sericea*. Several objectives were identified to reach the aim.

7.1.1 Phytochemistry

The first objective was to isolate and identify the major constituents of *T. sericea* roots and to investigate the chemical variation of these major compounds within the roots of trees, collected from geographically distant populations. Previously, Bombardelli et al. (1974, 1975) isolated resveratrol-3-rutinoside, sericic acid and sericoside from the root of *T. sericea,* and indicated that sericoside was the major constituents of the root. This current study has further confirmed that sericoside is a major constituent together with sericic acid, resveratrol-3-rutinoside and arjunglucoside I. The current study has also indicated that resveratrol-3-rutinoside and not sericoside, is the most abundant compound in *T. sericea* root from Limpopo Province. Both qualitative and quantitative intra- and inter-population variations of the four major constituents were evident from the data obtained. This finding underlines the need for quality control of *T. sericea* root material for use in herbal products.

The second objective was to use metabolomic profiling to obtain a characteristic chemical fingerprint for the species using UPLC-QToF-MS/PDA. The characteristic chemical fingerprint obtained indicated that resveratrol-3-rutinoside and sericoside were the major constituents in the root samples of all the populations assessed.

The third objective was to identify marker constituents in the root extracts that can be used for the quality control of the roots, using chemometric tools. The models constructed from the UPLC-MS data revealed that resveratrol-3-rutinoside, sericic acid and sericoside can be used as reliable biomarkers for quality control of root material. Furthermore, three groups of T. *Sericea could* be distinguished in the Limpopo Province based on their chemical profiles. The identified biomarkers contributed to the clustering of the populations, based primarily on quantitative, rather than qualitative differences.

The fourth and fifth objectives were to establish rapid, reliable and relatively easy methods for quality control for the roots. Vibrational spectroscopy and HPTLC were selected as the techniques of choice because they are rapid, robust, easy to operate, non-destructive, and are cheaper to purchase and operate when compared to UPLC-MS. For HPTLC, ethyl acetate: methanol: water (81:11:8) was chosen as the developing solvent and 20% sulfuric acid in ethanol as the visualization reagent, following several optimization experiments. The identified biomarkers (arjunglucoside I, resveratrol-3-rutinoside, sericic acid and sericoside) were clearly visible on the plates and the intensities (light or dark) of the bands corresponded to the concentrations determined by UPLC-PDA.

Spectroscopic analysis of the powdered root samples were used to develop calibration models for the quality control of *T. sericea* root material. This was successfully achieved for the quantification of resveratrol-3-rutinoside and sericoside from MIR spectroscopic data. The concentrations of resveratrol-3-rutinoside (RMSEP = 4.37 mg/g) and sericoside (RMSEP = 2.70 mg/g) were predicted with accuracy, indicating that the models can be used to quantify these analytes in powdered root material. Models obtained for sericic acid and arjunglucoside I from the spectral data were inaccurate and could not be used. In addition, models constructed from the NIR spectroscopy data yielded poor model statistics and were discarded.

7.1.2 Biological activity

The sixth objective was to evaluate the antibacterial activities of various plant extracts and the isolated compounds against bacteria associated with stomach disorders and skin conditions. The results indicate that sericic acid is the major antibacterial constituent in the roots with good activities against Gram-negative bacteria. However, sericic acid was not active against *S. typhi* despite noteworthy activities of the crude extracts and several of the fractions, suggesting that one or more other metabolite(s) such as anolignan b, termilignan b and anrjunic acid, previously isolated from the root (Eldeen et al., 2006; 2008) could be responsible for the activity against the pathogen.

The seventh objective was to evaluate the anti-oxidant activities of several of the plant extracts and isolated compounds. The results indicated that resveratrol-3- rutinoside exhibited good anti-oxidant activity, while sericic acid and sericoside did not display any activity when using the DPPH free radical scavenging assay and reducing power assay. However, the anti-oxidant activities of resveratrol-3-rutinoside were lower than that of the crude extract, indicating that other compounds contribute to the anti-oxidant activity.

7.1.3 Toxicology

The final objective was to evaluate the comprehensive *in vivo* pharmacological activity of *T. sericea* root using primates (vervet monkeys). The results revealed that the roots exhibited hepatoprotective activities and hypoglycemic potential. However, it simultaneously displayed nephrotoxic and haematotoxic effects. The hypoglycemic potential supports the use of the plant for the management of diabetes, while the increase in the platelet count is possibly associated with the wound healing ability of *T. sericea.* The monkeys were exposed continuously to a high dose of the plant material (2.14 g/kg) for 12 weeks, before washing out with normal feed over a further four-week period. This exposure is substantially higher than the amount that people would take when using it for a traditional medicine. The major concern arising from this study is the kidney toxicity, which should be further investigated using various concentrations.

7.1.4 Contribution of the study

The efficacy and safety of herbal products is partly dependent on the consistency of the chemical constituents of the raw plant material. Basic research concerning the phytochemistry and chemical variation provide knowledge that assists in the commercialisation of herbal products. Phytochemistry provides necessary information about which metabolites can vary and how this variation affects the efficacy. For the first time, a complete chemical profile indicating the major constituents of *T. sericea* root and their quantitative variability have been reported. This study has also, for the first time, resulted in the development of a validated UPLC-PDA method that can be used to simultaneously determine the biomarker compounds in the roots. . Two relatively simple quality control techniques (HPTLC and vibrational spectroscopy) were developed that can be used by small industries that provide raw material to the herbal drug market.

Many researchers have indicated that the roots have antibacterial activity against stomach and skin pathogens. However, this is the first study where a compound (sericic acid) associated with the activity has been isolated and identified. Sericic acid was identified as the main antibacterial constituent of *T. sericea* and this study has partially elucidated the link between the antimicrobial activity and the chemistry of the roots. Other compounds responsible for the good antibacterial activity against *S. typhi* have not yet been identified. It was established that sericic acid was not active against *S. typhi*. This study has also indicated that Gram-negative bacteria *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Shigella sonnei*, *Salmonella typhimurium,* are highly susceptible to the root extracts. Gram-negative bacteria are generally more difficukt to combat than the Gram-positive ones and these extracts may therefore prove useful in the fight against these organisms.

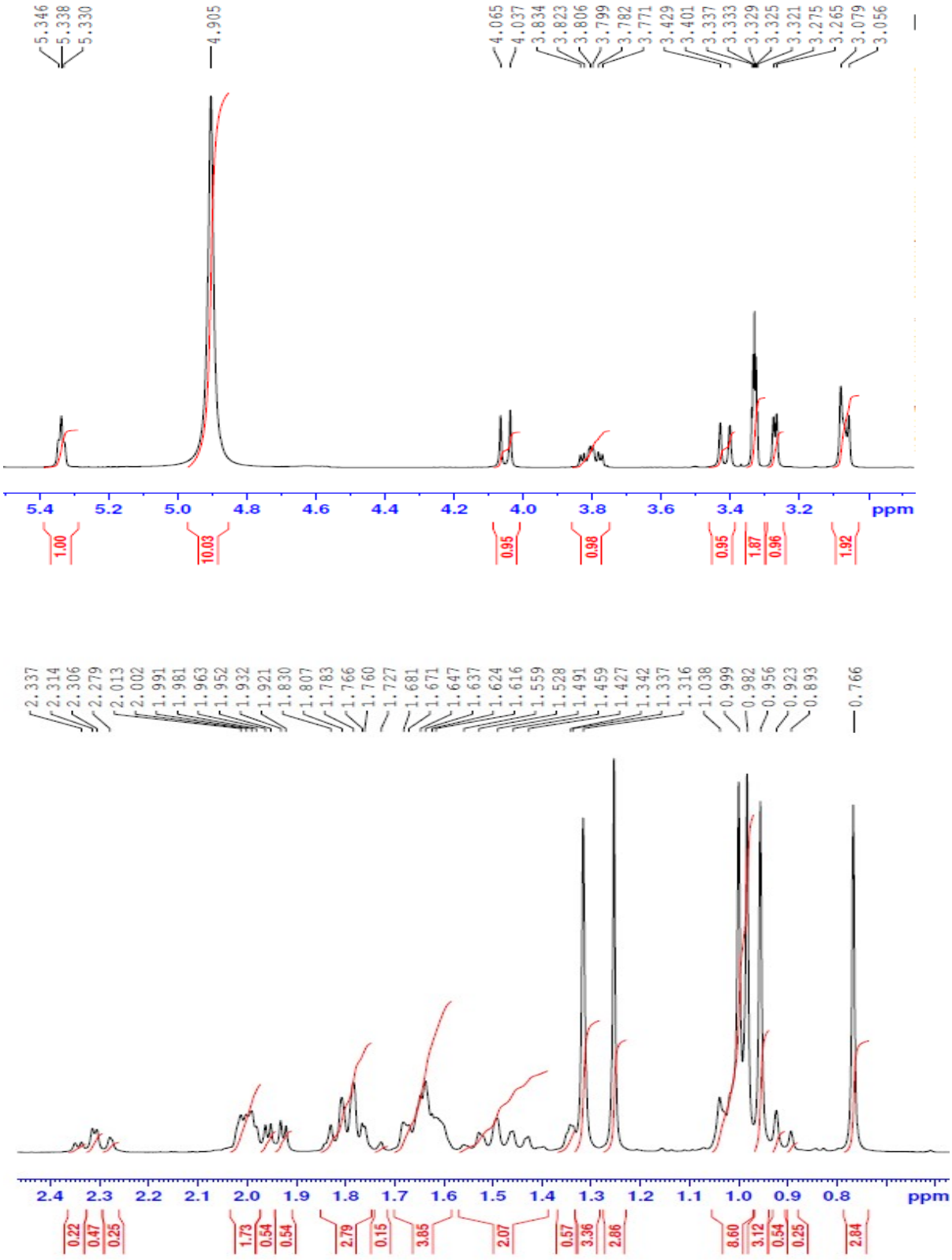
Although the study indicated that the roots have toxic effects, the toxicity was not acute and the plant part is possibly safe at therapeutic concentrations. However, there are indications that people with kidney problems should not use the roots.

7.2 RECOMMENDATIONS

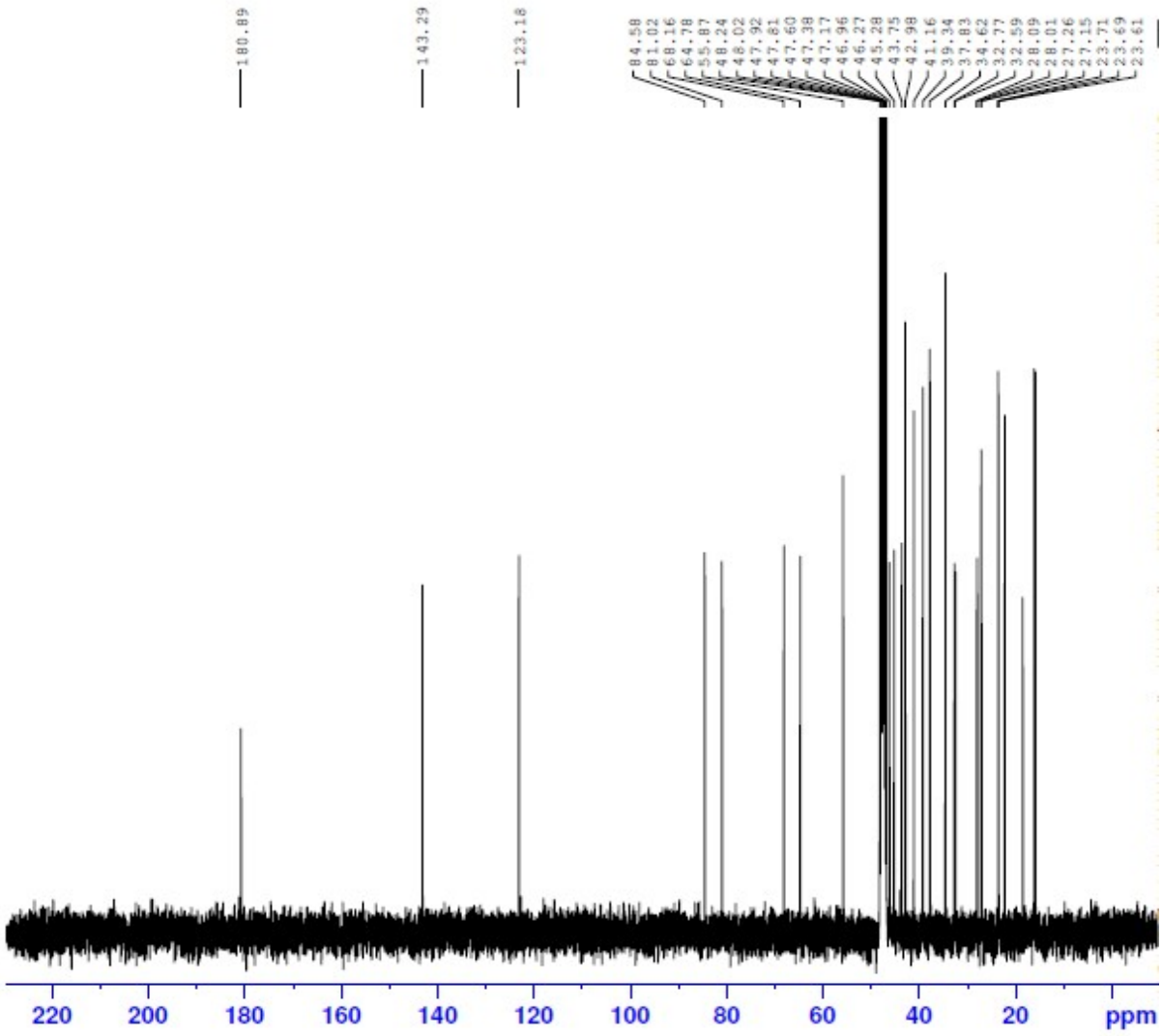
The current study has revealed that resveratrol-3-rutinoside, sericic acid and sericoside are the major constituents of *T. sericea* root and that these compounds can be used as biomarkers for quality control purposes. The study also highlighted that sericic acid is the major antibacterial constituent of the plant. Potential nephrotoxic and hematotoxic effects of the roots were revealed by the toxicity study. The following recommendations are therefore made for further studies and research:

1. Three classes were identified in this study from 10 populations in the Limpopo Province. Further studies are required that encompass a greater diversity of populations, perhaps including samples from trees spread throughout Africa. This would allow a more detailed comparison to be made of the chemical variation within the roots.
2. Ethnomedicinal reports and pharmacological studies have indicated that the roots have antidiabetic, antiHIV and antituberculosis activities. Further studies should investigate how these activities are linked to the presence and concentrations of the various biomarkers.
3. It is important to know if the bioactive compounds or biomarkers are also responsible for the toxicity of the roots. Therefore, biomarkers identified in this study should be investigated for their potential toxicity using the same or an appropriate *in vitro* model.

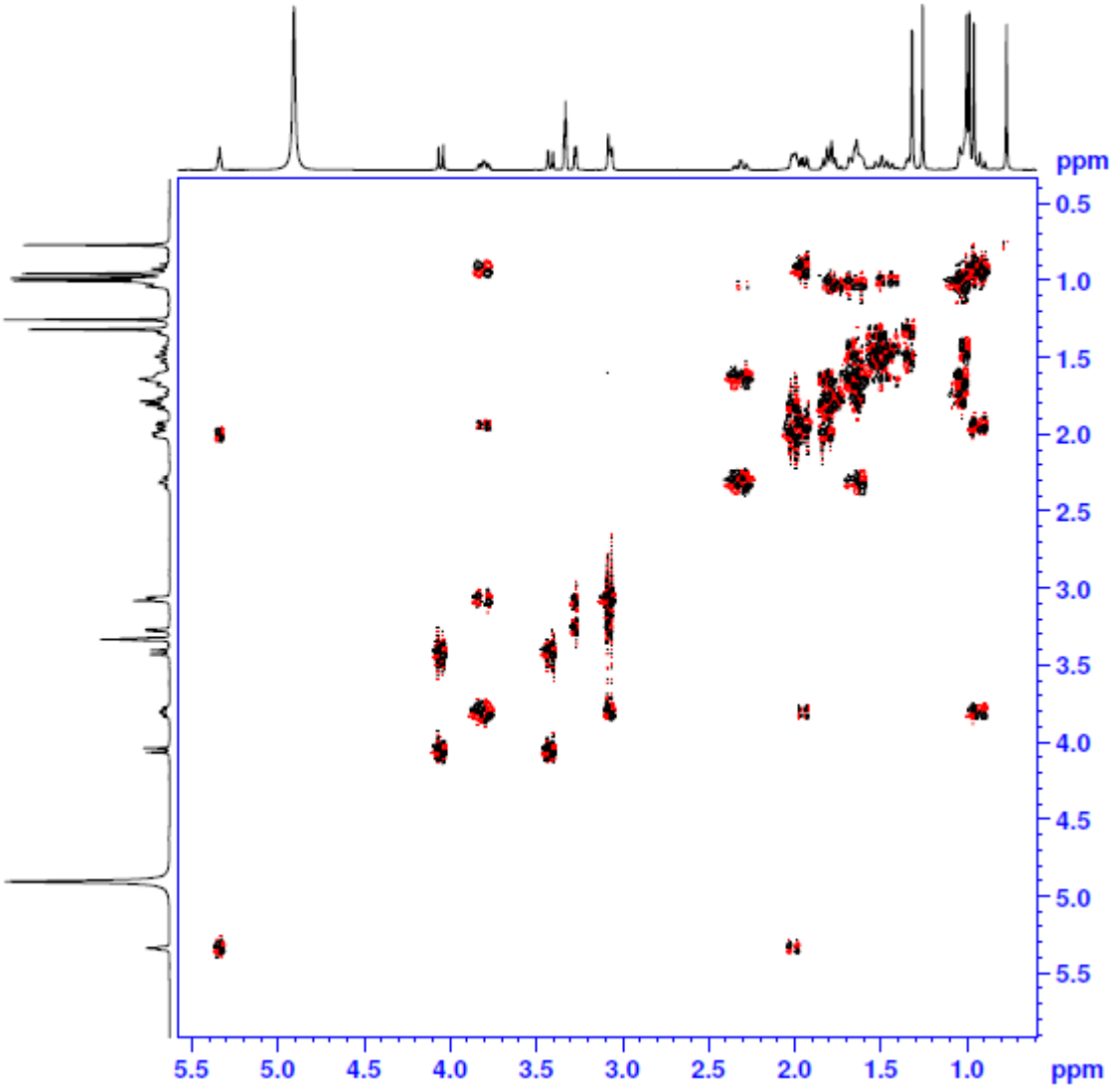
Appendix 1a ^{1}H NMR spectrum of sericic acid in methanol-d_4



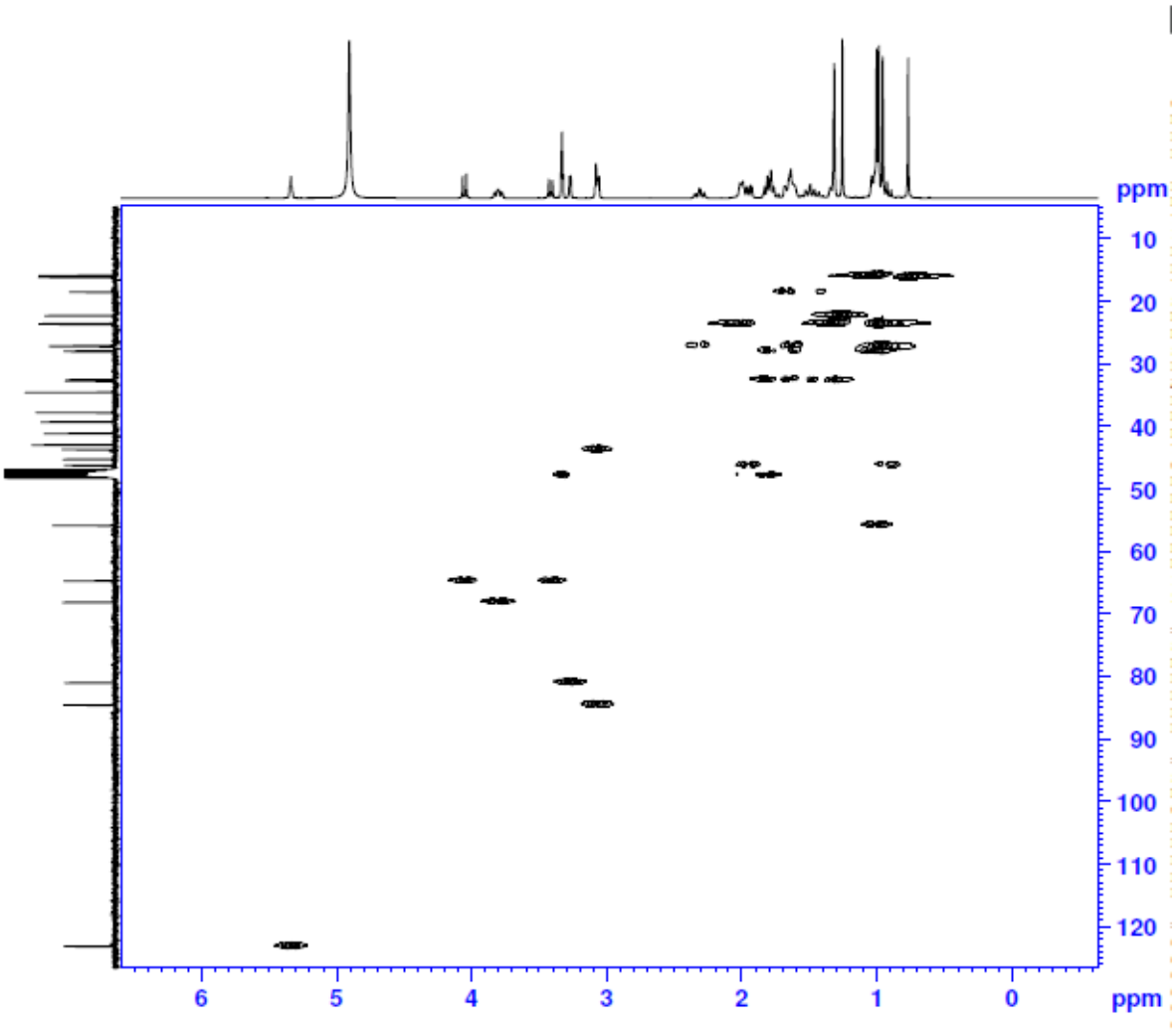
Appendix 1b ^{13}C NMR spectrum of sericic acid in methanol-d_4



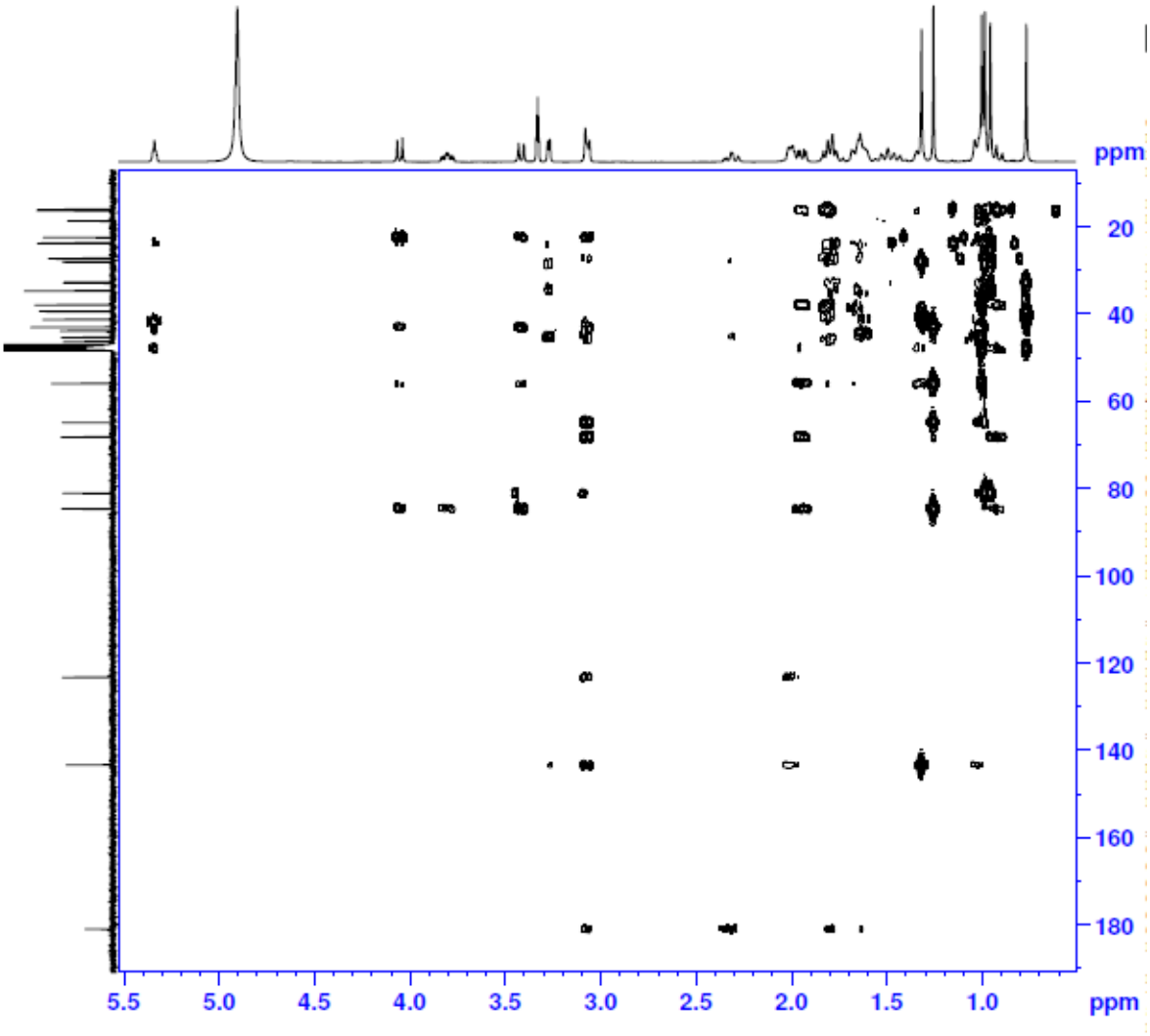
Appendix 1c COSY spectrum of sericic acid in methanol-d_4



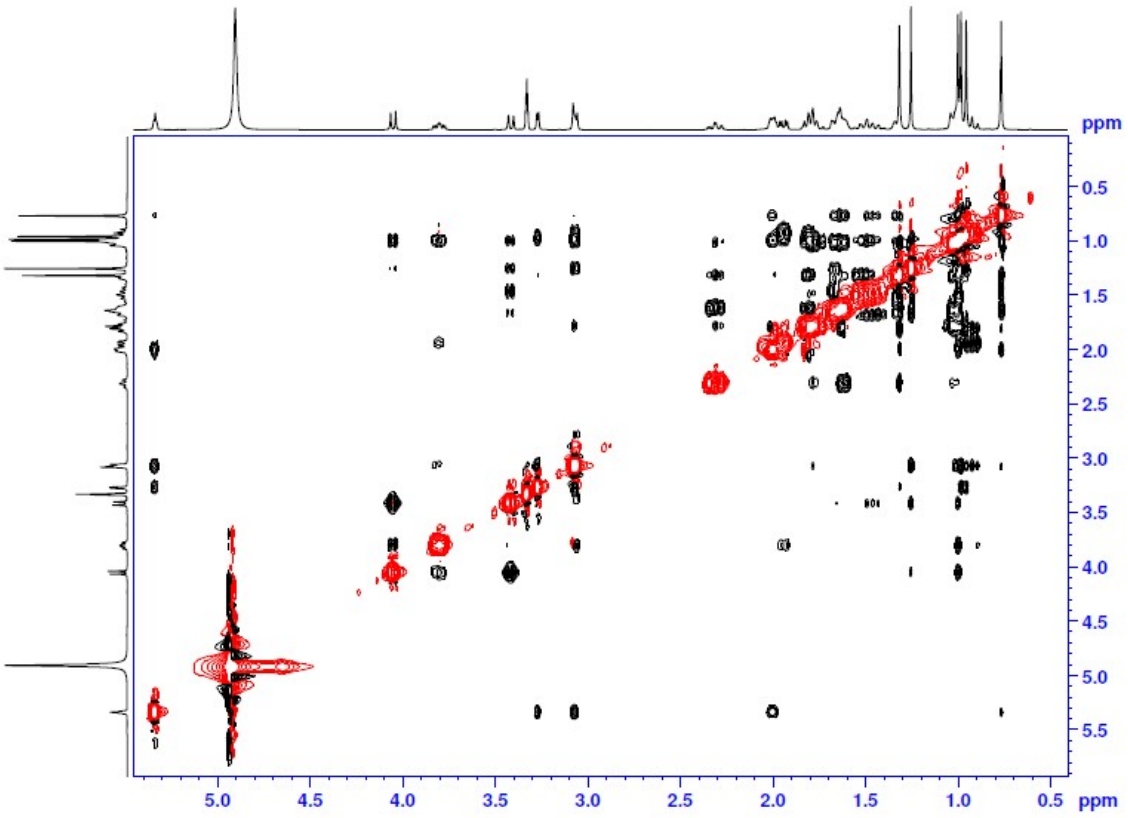
Appendix 1d HSQC spectrum of sericic acid in methanol-d_4



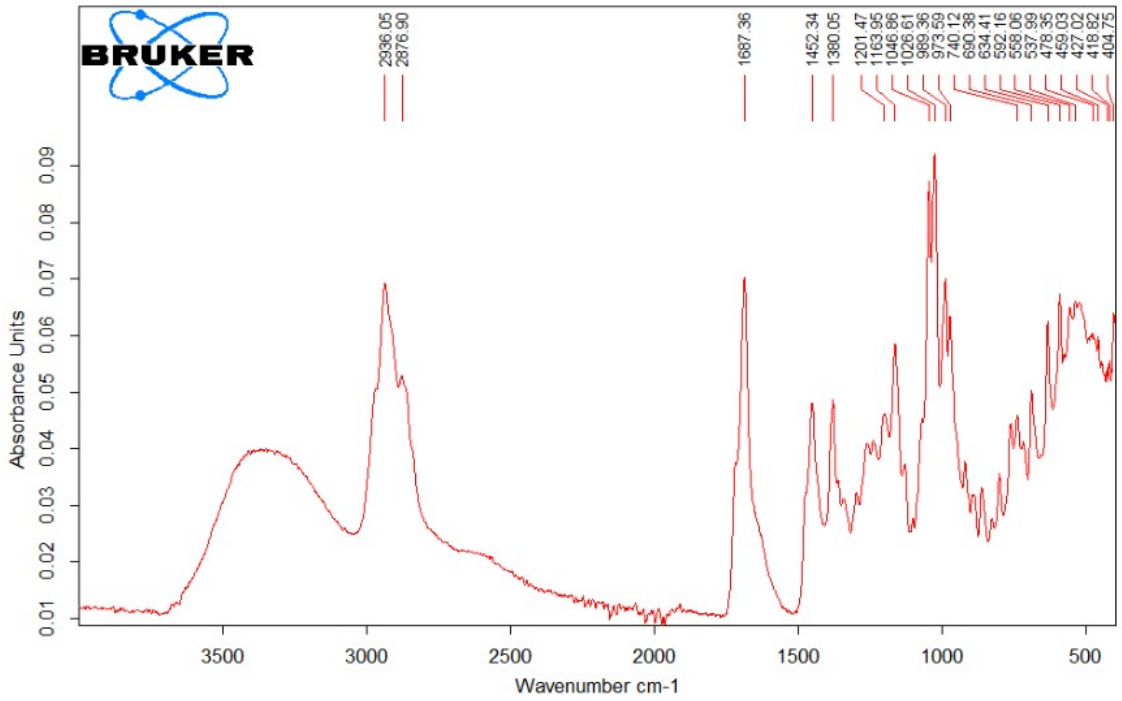
Appendix 1e HMBC spectrum of sericic acid in methanol-d_4



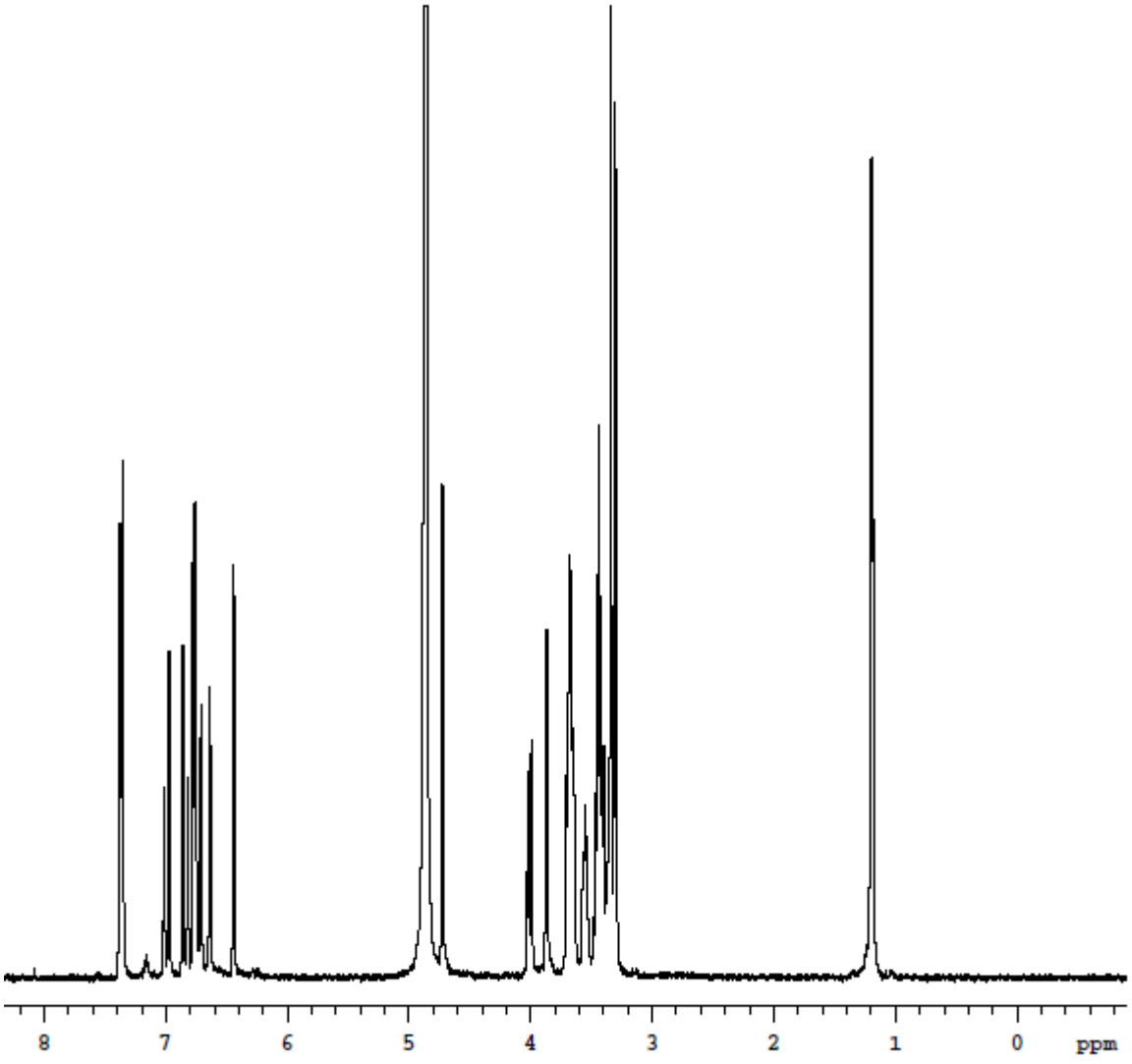
Appendix 1f NOESY spectrum of sericic acid in methanol-d_4



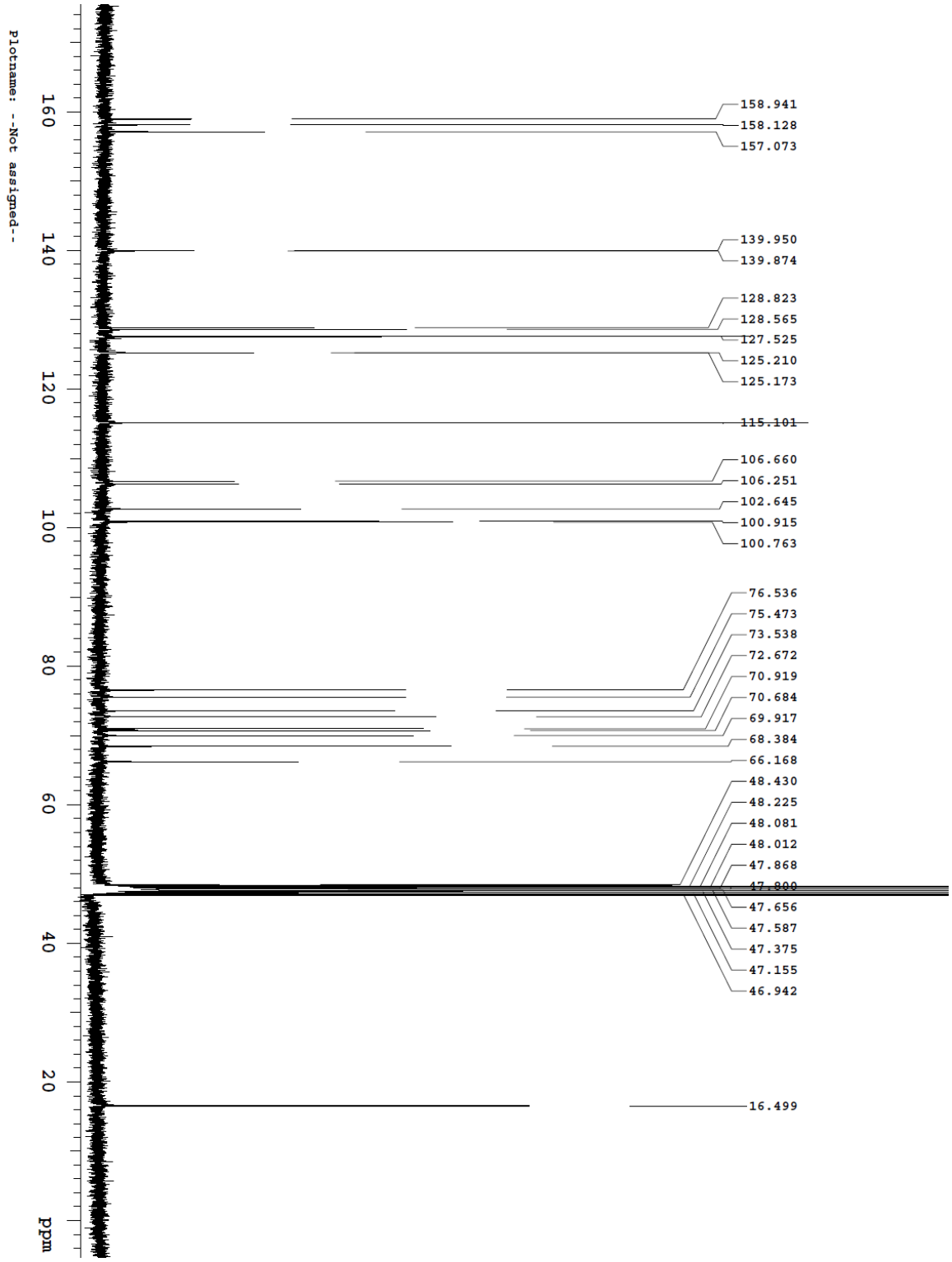
Appendix 1g IR spectrum of sericic acid



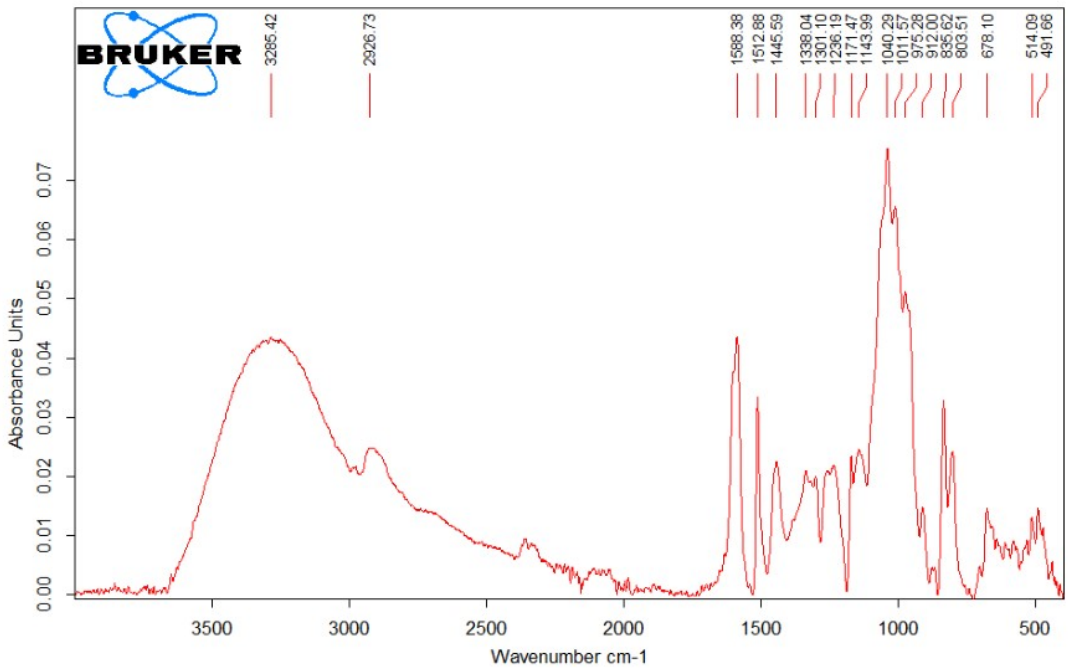
Appendix 2a ^{1}H NMR spectrum of resveratrol-3-rutinoside in methanol-d_4



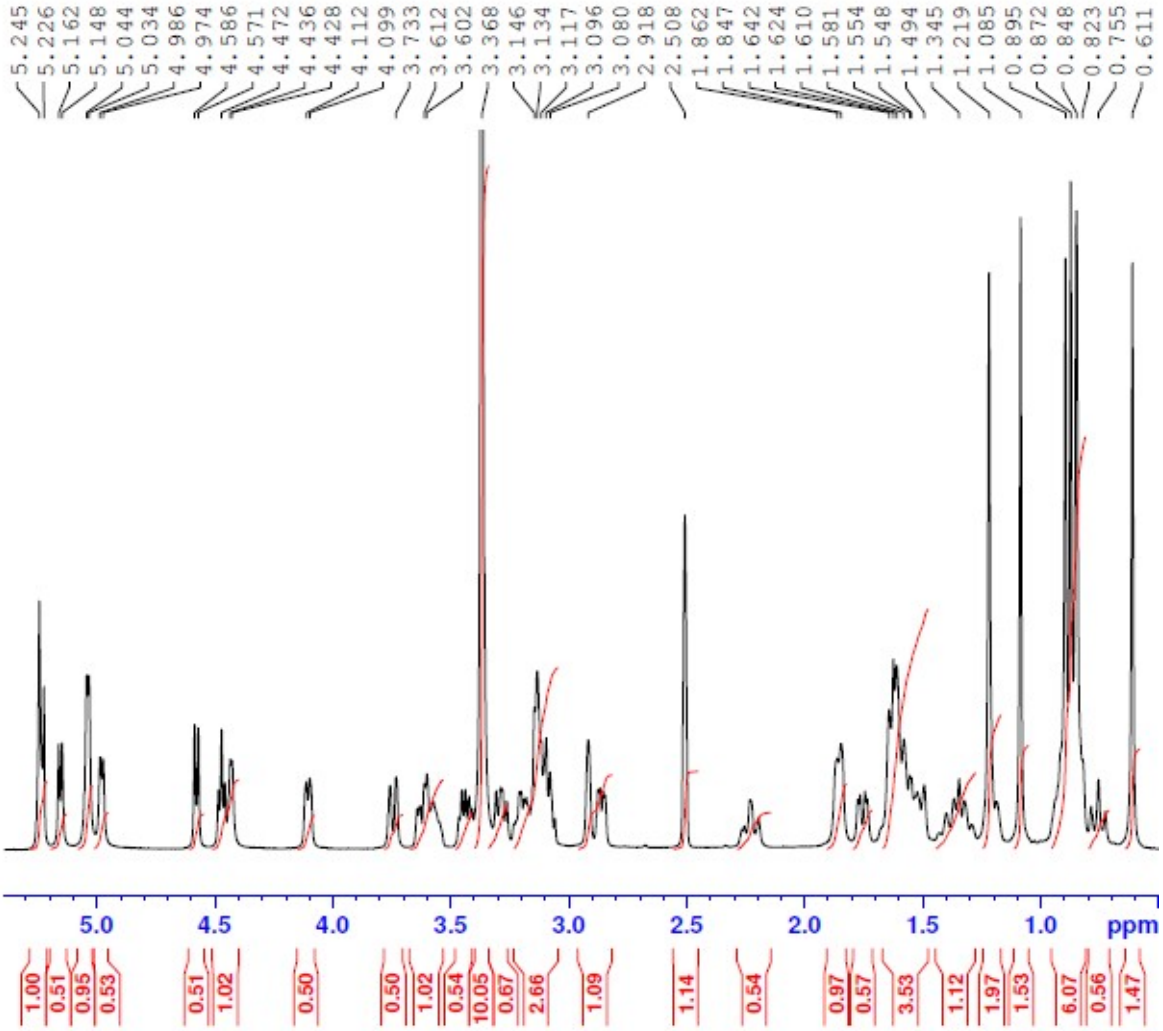
Appendix 2b ^{13}C NMR spectrum of resveratrol-3-rutinoside in methanol-d_4



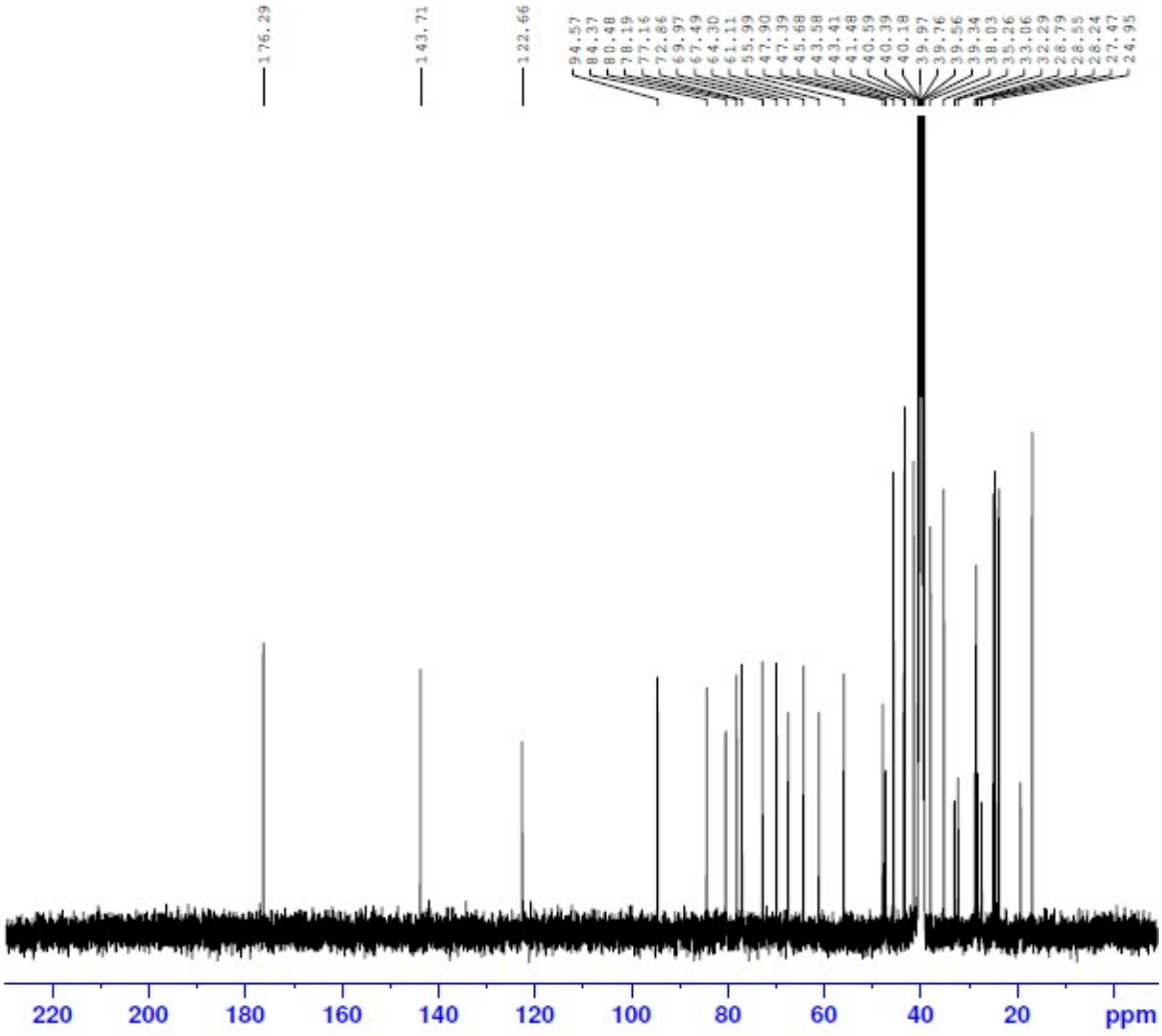
Appendix 2c IR spectrum of resveratrol-3-rutinoside



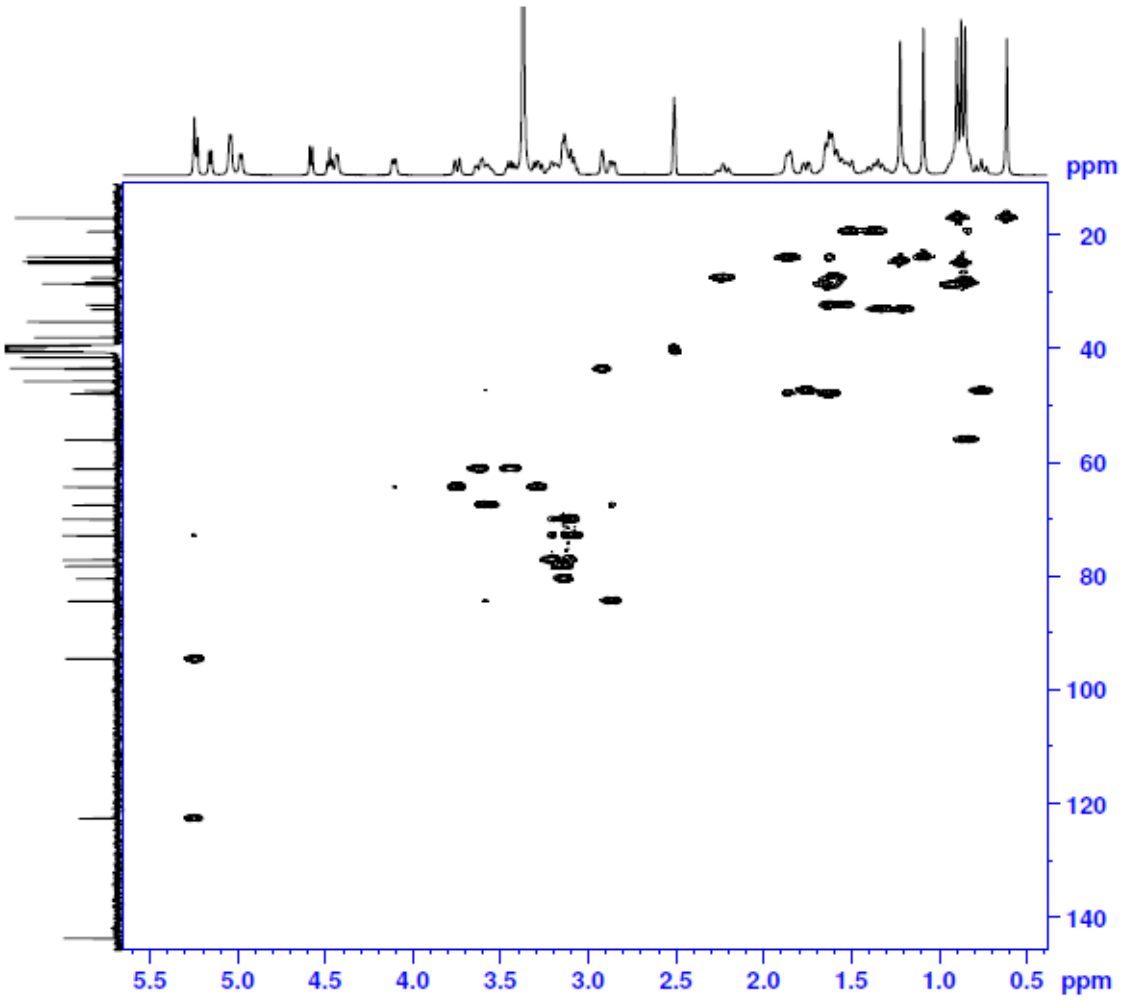
Appendix 3a ^{1}H NMR spectrum of sericoside DMSO-*d*6



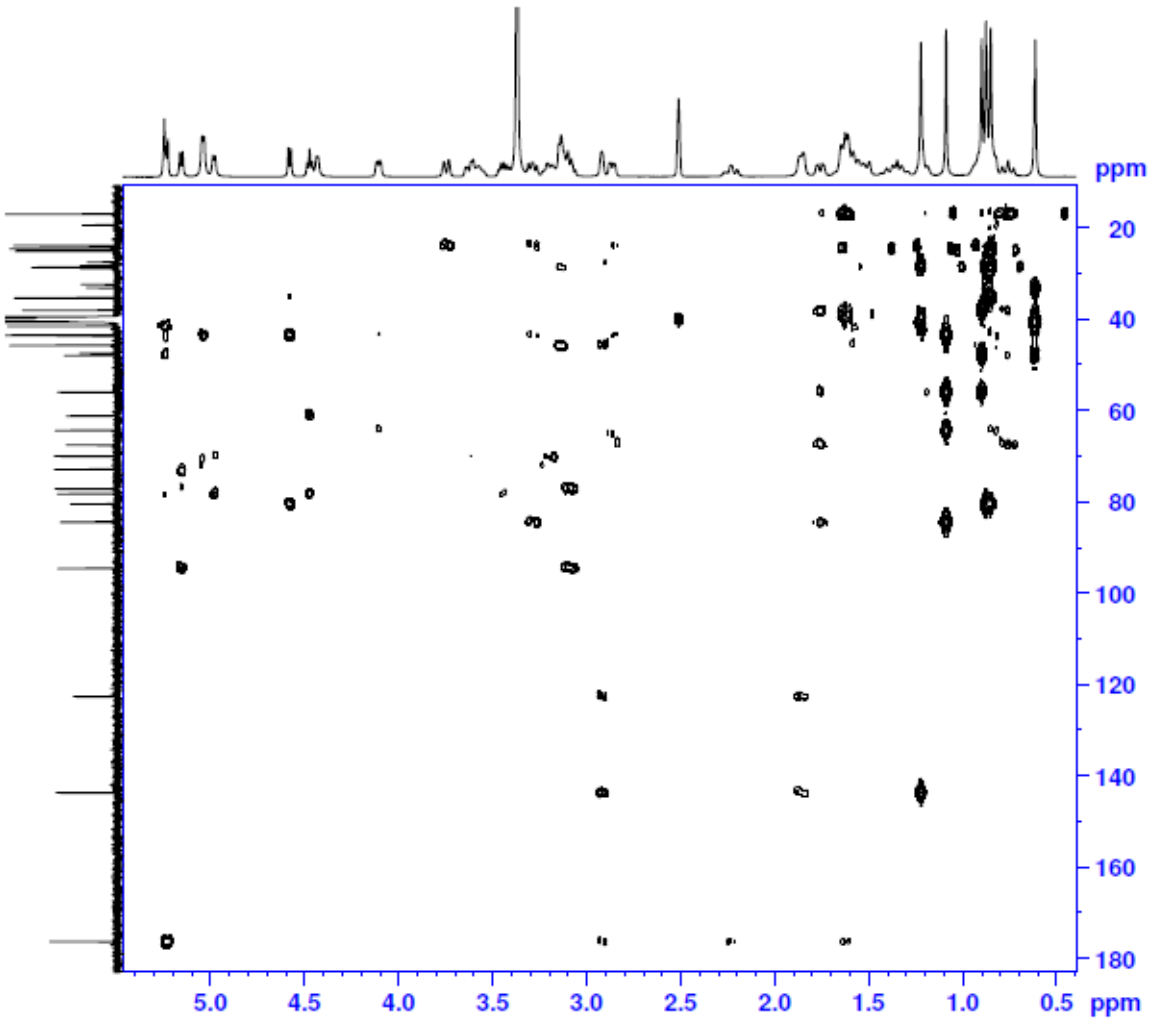
Appendix 3b ^{13}C NMR spectrum of sericoside in DMSO-*d*6



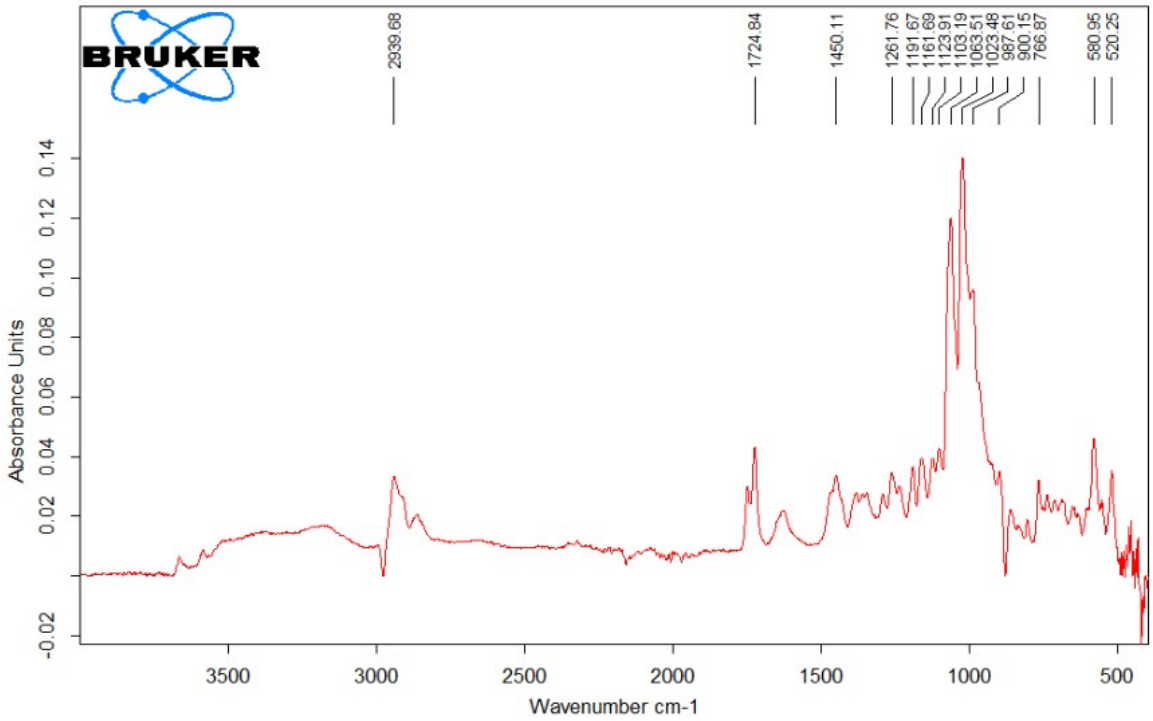
Appendix 3c HSQC spectrum of sericoside in DMSO-*d*6



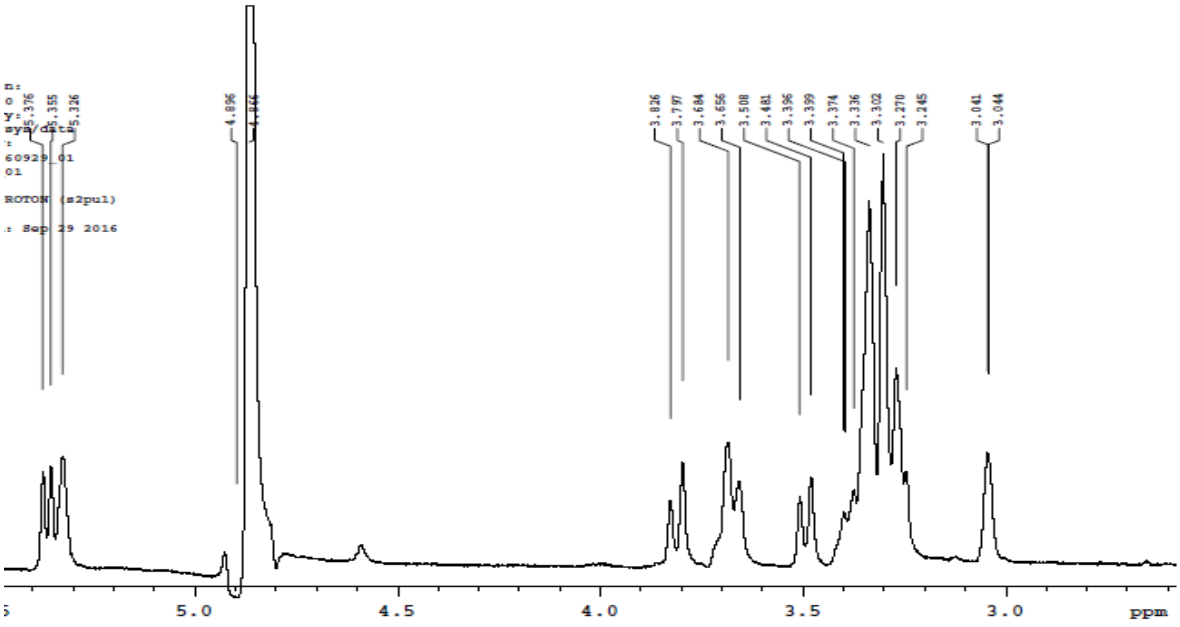
Appendix 3d HMBC spectrum of sericoside in DMSO-*d*6

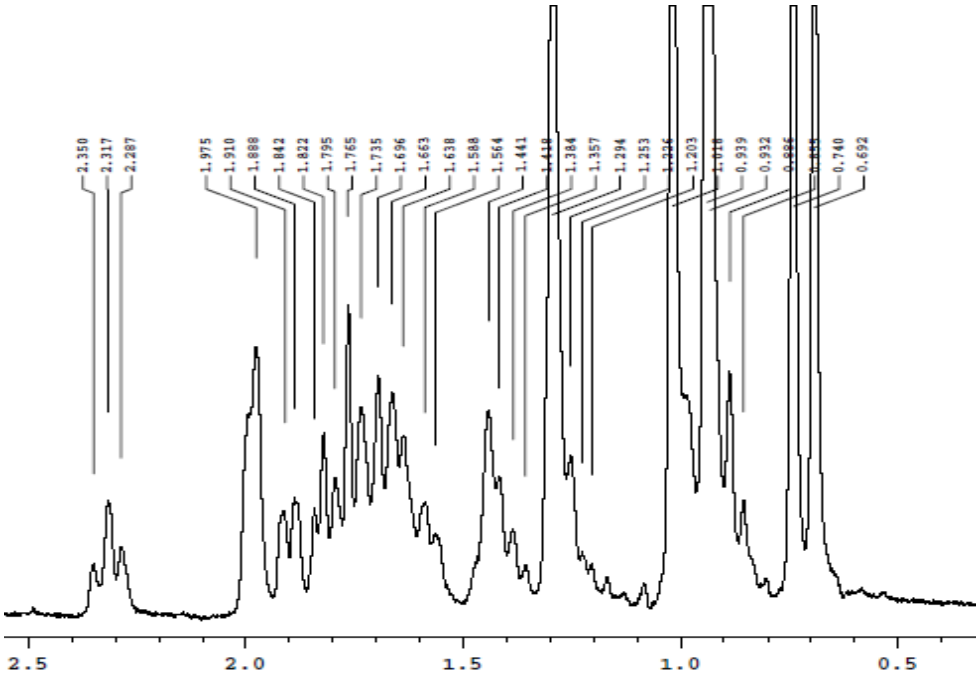


Appendix 3e IR spectrum of sericoside

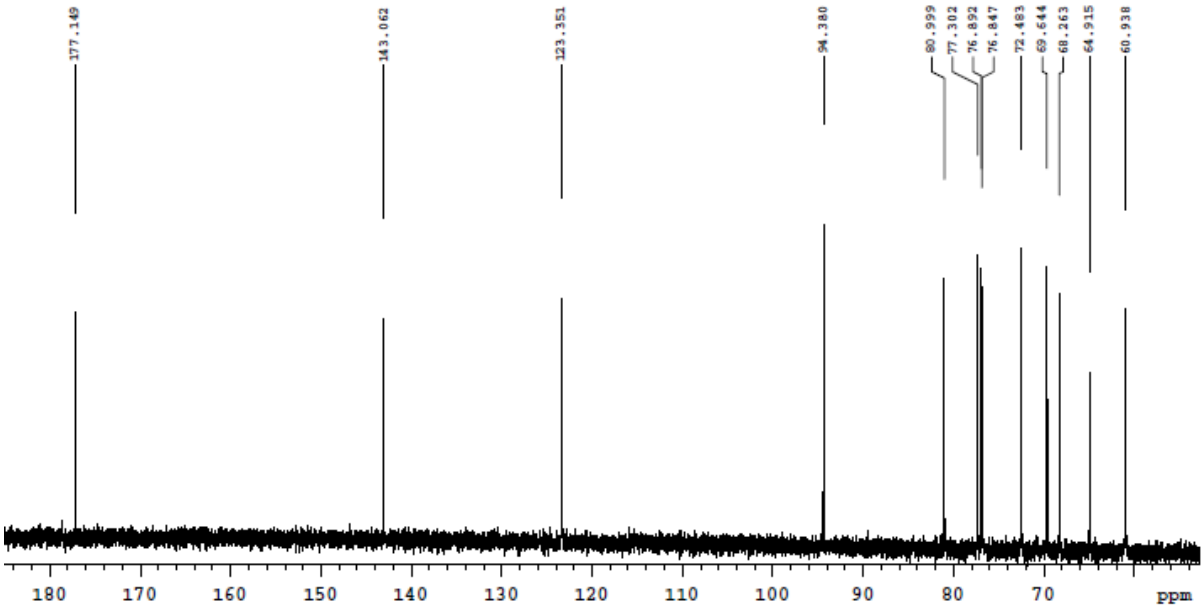


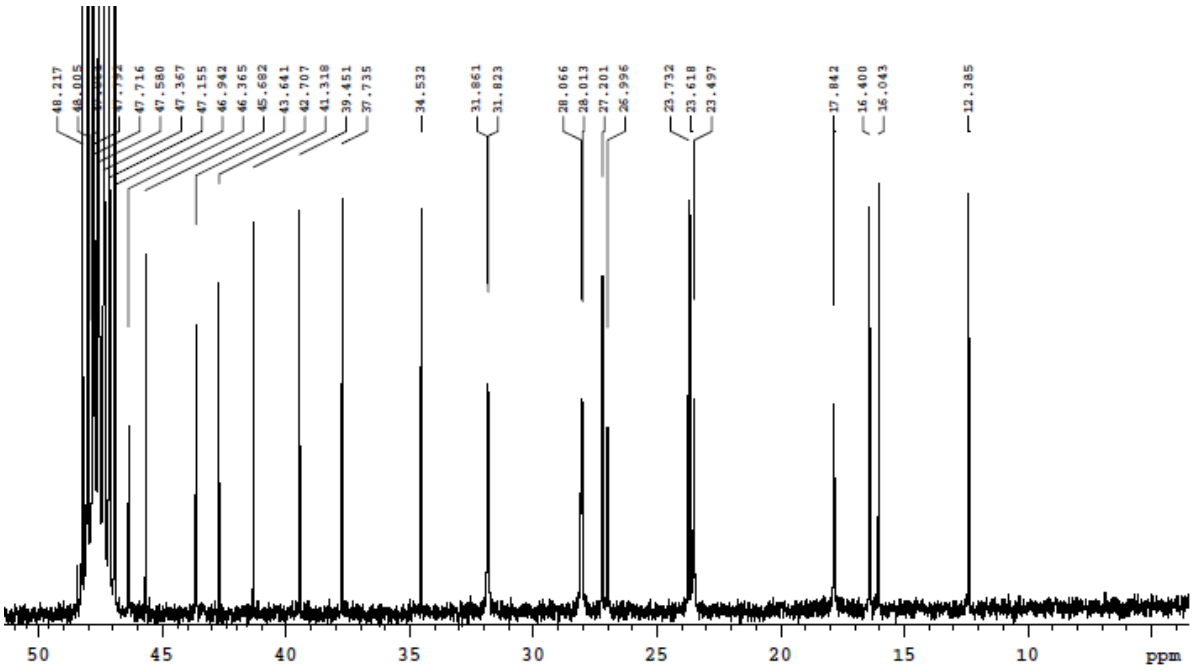
Appendix 4a ^{1}H NMR spectrum of arjunglucoside I in methanol-d_4



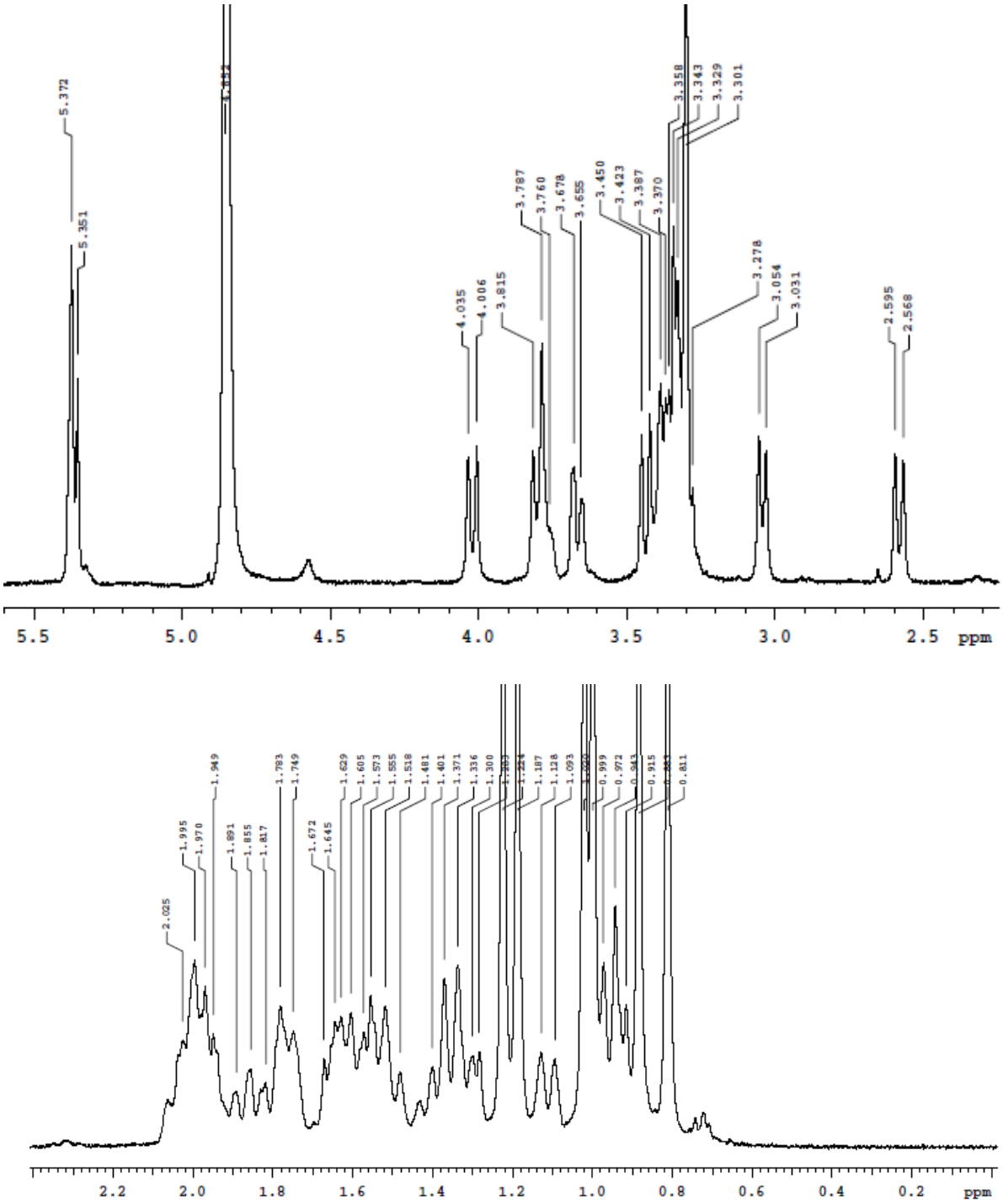


Appendix 4b ^{13}C NMR spectrum of arjunglucoside I in methanol-d_4

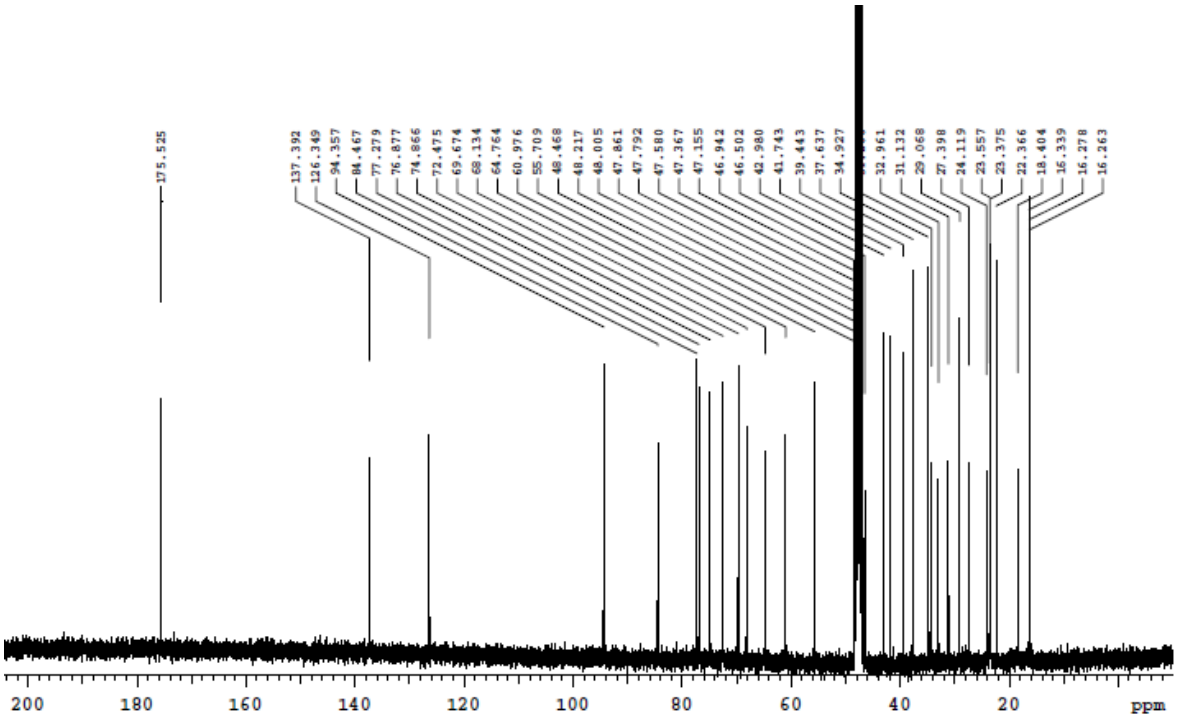




Appendix 5a ^{1}H NMR spectrum of Compound (13) in methanol-d_4



Appendix 5b ^{13}C NMR spectrum of Compound (13) in methanol-d_4



Appendix 6 Quantification of major constituents of *T. sericea* root

Name	Population	C2 (mg/g)	C10 (mg/g)	C3 (mg/g)	C12 (mg/g)
BP1	P1.1	1.82	24.65	6.75	3.56
BP2	P1.2	6.42	17.21	6.12	2.67
BP3	P1.3	8.64	23.51	2.58	1.04
G1	P2.1	ND	17.87	12.22	3.35
G2	P2.2	ND	11.06	11.67	3.44
G3	P2.3	1.78	18.75	9.69	3.18
G4	P2.4	ND	18.13	9.87	5.49
J1	P3.1	0.68	1.54	6.66	7.18
J2	P3.2	0.84	1.43	9.12	6.03
J4	**P3.3**	1.68	5.05	9.57	**8.44**
J5	P3.4	3.28	0.65	9.07	4.41
K1	P4.1	6.64	25.55	16.71	3.79
K2	**P4.2**	**12.55**	24.00	**20.16**	3.60
K3	P4.3	6.14	18.95	12.32	3.56
K4	P4.4	8.17	22.17	18.64	3.12
K5	P4.5	8.72	25.14	19.36	3.21
MM1	P5.1	8.38	**29.82**	7.54	3.09
MM3	P5.2	5.96	20.38	6.51	3.63
MM4	P5.3	ND	16.21	10.33	4.38
MM5	P5.4	2.86	12.35	7.38	5.28
MP1	P6.1	6.78	14.26	4.41	1.53
MP2	P6.2	4.51	10.19	3.78	0.87
MP3	P6.3	4.38	18.86	3.55	2.29
MP4	P6.4	4.212	21.02	3.84	2.41
MP5	P6.5	7.51	19.63	4.87	0.91
TSA1	P7.1	ND	14.58	11.29	5.66
TSA2	P7.2	ND	14.81	12.32	3.99
TSA3	P7.3	ND	12.03	9.01	6.06
TSA4	P7.4	ND	21.63	11.78	6.70
TSA5	P7.5	ND	15.56	11.79	5.55
TSH1	P8.1	1.51	14.31	9.99	4.18
TSH2	P8.2	ND	12.22	9.62	3.67
TSH3	P8.3	ND	10.38	9.30	4.34
TSH4	P8.4	1.68	17.33	12.87	7.11
TSH5	P8.5	1.58	16.05	12.12	3.41
TZ1	P9.1	6.48	3.03	9.45	3.73
TZ2	P9.2	5.23	2.22	4.81	3.84
TZ3	P9.3	1.98	11.56	10.34	3.42
TZ4	P9.4	2.64	14.29	7.77	4.09
TZ5	P9.5	1.77	2.23	2.07	3.28
V1	P10.1	7.78	24.53	7.75	2.14
V2	P10.2	2.79	13.30	3.77	2.30

Appendix 7 Results of analysis of microbial growth present in a *T. sericea* root sample

MICRO FINAL REPORT

Dr Mamello Sekhoacha

3 Sherringham Street
Parklands 7441

REQ. NO.: CT15-027345
Date Received: 01/09/2015
Report Date: 07/09/2015

SWIFT SILLIKER
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Swift Silliker (Pty) Ltd
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South Africa
Phone + 27 (21) 683 8436 / 08613 SWIFT
Fax + 27 (21) 683 8422 / Email info@swift.co.za
www.swift.co.za

ilac-MRA sanas Testing Laboratory T0050

Contact Name: Dr. Mamello Sekhoacha
Your email: mamello.sekhoacha@mrc.ac.za
Report Number: 137521-0

Lab Sample Ref #: CT 15-027345-001 **Test Date:** 01/09/2015 **Date Analysis Started:** 01/09/2015 **Product Description:** Ground plant material
Sample Condition: SEALED
Remark: 2015-08-17 OR 18th

Test Type	Method No.	Result	Limits
TMA	SWJM 35	No Growth cfu/g	N/A
Coliforms	SWJM 48	No Growth cfu/g	N/A
Escherichia coli	SWJM 62	No Growth cfu/g	N/A
Yeast	SWJM 50	No Growth cfu/g	N/A
Mould	SWJM 50	No Growth cfu/g	N/A
Staphylococcus aureus	SWJM 53	No Growth cfu/g	N/A

Lab Sample Ref #: CT 15-027345-002 **Test Date:** 01/09/2015 **Date Analysis Started:** 01/09/2015 **Product Description:** Ground plant material
Sample Condition: SEALED
Remark: 2015-08-17 OR 18th

Test Type	Method No.	Result	Limits
TMA	SWJM 35	No Growth cfu/g	N/A
Coliforms	SWJM 48	No Growth cfu/g	N/A
Escherichia coli	SWJM 62	No Growth cfu/g	N/A
Yeast	SWJM 50	No Growth cfu/g	N/A
Mould	SWJM 50	No Growth cfu/g	N/A
Staphylococcus aureus	SWJM 53	No Growth cfu/g	N/A

Comment:

Technical Signatory: Zubeida Moydien

07/09/2015
Date

Appendix 8 Average concentrations (bold mean ± SD) of globulin (g/L) in vervet monkeys after 12 weeks of exposure to diet supplemented with *T. sericea* root and an additional 4 weeks washout period

	Monkey No	Baseline	Week 4	Week 8	Week 12	Week 16
Experimental	413	13.00	14.00	16.00	18.00	16.00
	254	21.00	20.00	19.00	24.00	32.00
	391	16.00	13.00	15.00	17.00	17.00
	394	15.00	23.00	24.00	23.00	25.00
	Mean	**16.25**	**17.50**	**18.50**	**20.50**	**22.50**
	SD	**3.40**	**4.80**	**4.04**	**3.51**	**7.51**
Control						
	390	20.00	19.00	21.00	22.00	20.00
	389	19.00	19.00	19.00	20.00	16.00
	244	19.00	19.00	19.00	21.00	19.00
	130	19.00	20.00	21.00	22.00	23.00
	Mean	**19.25**	**19.25**	**20.00**	**21.25**	**19.50**
	SD	**0.50**	**0.50**	**1.15**	**0.96**	**2.89**

Appendix 9 Average concentrations (bold mean ± SD) of HDL (mmol/L) and LDL (mmol/L) in vervet monkeys after 12 weeks of exposure to diet supplemented with *T. sericea* root and an additional 4 weeks washout period

	Monkey No	Baseline	Week 4	Week 8	Week 12	Week 16
HDL						
Experimental						
	413	2.10	2.10	2.30	2.60	2.70
	254	1.90	2.00	2.10	2.30	2.10
	391	1.60	1.60	1.40	1.70	1.90
	394	2.40	1.70	1.70	2.20	3.10
	Mean	**2.00**	**1.85**	**1.88**	**2.20**	**2.45**
	SD	**0.34**	**0.24**	**0.40**	**0.37**	**0.55**
Control						
	390	2.80	2.90	2.80	2.70	2.80
	389	2.00	2.10	2.10	2.30	2.20
	244	2.50	2.30	2.30	2.20	2.20
	130	2.10	2.00	1.90	2.00	1.90
	Mean	**2.35**	**2.33**	**2.28**	**2.30**	**2.28**
	SD	**0.37**	**0.40**	**0.39**	**0.29**	**0.38**
LDL						
Experimental						
	413	1.40	1.70	1.80	1.60	1.40
	254	2.30	2.40	1.90	1.80	2.30
	391	2.50	3.20	3.20	2.40	2.60
	394	1.60	2.40	2.20	2.10	2.20
	Mean	**1.95**	**2.43**	**2.28**	**1.98**	**2.13**
	SD	**0.53**	**0.61**	**0.64**	**0.35**	**0.51**
Control						
	390	2.70	2.90	2.50	2.30	2.30
	389	3.10	3.50	3.20	2.60	2.50
	244	2.00	2.00	1.90	1.70	1.70
	130	1.80	1.80	1.90	1.80	1.30
	Mean	**2.40**	**2.55**	**2.38**	**2.10**	**1.95**
	SD	**0.61**	**0.79**	**0.62**	**0.42**	**0.55**

Appendix 10 Average concentrations (bold mean ± SD) of urea (mmol/L) and creatin kinase (U/L) in vervet monkeys after 12 weeks of exposure to diet supplemented with *T. sericea* root and an additional 4 weeks washout period

	Monkey No	Baseline	W4	W8	W12	W16
Urea						
Experimental						
	413	4.30	4.80	3.90	2.00	3.30
	254	3.20	1.50	4.30	3.60	3.30
	391	4.40	4.20	2.20	5.80	3.40
	394	5.50	3.40	2.80	3.70	5.40
	Mean	**4.35**	**3.48**	**3.30**	**3.78**	**3.85**
	SD	**0.94**	**1.44**	**0.97**	**1.56**	**1.03**
Control						
	390	5.20	3.70	4.30	4.30	4.10
	389	4.60	3.80	2.50	2.70	3.20
	244	5.70	3.70	3.50	3.50	3.60
	130	5.60	4.70	4.40	3.20	5.40
	Mean	**5.28**	**3.98**	**3.68**	**3.43**	**4.08**
	SD	**0.50**	**0.49**	**0.88**	**0.67**	**0.96**
Creatin kinase						
Experimental						
	413	624.00	727.00	8742.00	1187.00	307.00
	254	477.00	737.00	643.00	1219.00	1008.00
	391	727.00	1297.00	1448.00	1425.00	310.00
	394	290.00	446.00	301.00	514.00	335.00
	Mean	**529.50**	**801.75**	**2783.50**	**1086.25**	**490.00**
	SD	**189.78**	**356.66**	**4001.33**	**395.81**	**345.56**
Control						
	390	7886.00	842.00	2170.00	3508.00	2513.00
	389	980.00	581.00	381.00	602.00	619.00
	244	1819.00	559.00	1451.00	550.00	1926.00
	130	774.00	662.00	1715.00	978.00	516.00
	Mean	**2864.75**	**661.00**	**1429.25**	**1409.50**	**1393.50**
	SD	**3377.87**	**128.54**	**759.31**	**1411.94**	**984.33**

Appendix 11 Average concentrations (mean ± SE) of serum electrolytes in vervet monkeys after 12 weeks of exposure to diet supplemented with *T. sericea* root and an additional 4 weeks washout period

Parameters	Baseline	Week 4	Week 8	Week 12	Week 16
Na (mmol/L)					
Experimenta l	149.25±0.48	146±1.29	144.75±2.39	146.25±2.02	156.5±5.84*
Control	148.75±0.25	149.25±0.48	148.5±0.64	149.25±0.48	152.0±0.82
Mg (mmol/L)					
Experiment	0.57±0.01	0.73±0.03	0.81±0.03	0.73±0.02	0.76±0.04
Control	0.53±0.04	0.56±0.03	0.60±0.04	0.58±0.04	0.68±0.05
K (mmol/L)					
Experiment Control	3.68±0.14 3.53±0.09	2.60±0.18* 2.98±0.17#	2.30±0.06* 2.85±0.18#	2.70±0.15* 2.83±0.15#	2.58±0.08* 2.38±0.05#
Ca (mmol/L)					
Experimenta l	2.19±0.01	2.16±0.02	2.11±0.04	2.11±0.01	2.32±0.03*
Control	2.14±0.05	2.14±0.01	2.15±0.01	2.20±0.02	2.17±0.05
Phosphate (mmol/L)					
Experimenta l	0.81±0.12	1.13±0.12*	1.17±0.15*	1.15±0.11*	1.15±0.17*
Control	0.90±0.1	0.63±0.02	0.75±0.05	1.08±0.1	1.10±0.09
Chloride (mmol/L)					
Experimenta l	108.5±0.29	107±1.63	104.75±3.28	107±2.34	108.25±1.25
Control	108.5±0.5	108.5±0.87	107.5±0.29	108.5±0.87	107.5±0.65
Anion gap (mmol/L)					
Experimenta l Control	12.5±1.5 10±0.91	11.25±0.63 11.5±0.29	11.25±0.95 12.25±1.97	9.5±0.29 10.5±1.5	14±0.71 14±1.35#
CO_3^{2-} (mmol/L)					
Experiment	28.25±1.0	27.73±1.5	28.78±0.87	29.83±0.48	27.6±0.55
Control	29.95±1.32	29.3±0.85	28.88±2.03	30.22±1.26	30.68±1.41

Appendix 12 Average concentrations (mean ± SE) of haematological parameters in vervet monkeys after 12 weeks of exposure to diet supplemented with *T. sericea* root and an additional 4 weeks washout period

Parameters	Baseline	Week 4	Week 8	Week 12	Week 16
RDW (%)					
Experiment	14.43±0.23	13.65±0.13	13.83±0.48	14.80±0.63	16.75±0.28*
Control	13.95±0.58	14.45±0.37	13.88±0.34	13.90±0.34	15.43±0.47#
MCH (pg)					
Experiment	24.5±0.29	24±0.41	23.5±0.29	23.75±0.25	24±0.41
Control	25±0.41	24.75±0.63	24.25±0.48	25±0.41	25±0.41
MCHC (g/dL)					
Experiment	32.50±0.29	31.75±0.25*	31.75±0.25*	31.25±0.25*	31±0.00*
Control	32.75±0.25	31.25±0.25#	31.50±0.29#	31±0.00#	31.75±0.25#
MCV (fl)					
Experiment	75.75±1.03	75.25±1.31	74±1.29	75.75±1.8	78.75±1.70
Control	76.5±1.19	78.75±1.25	78±1.08	79.25±1.31	80.25±1.31
WBC ($x10^9$/L)					
Experiment	2.88±0.53	5.9±1.37	5.05±0.68	5.40±1.23	3.63±1.42
Control	6.95±1.46	4.48±0.90	7.23±1.52	8.03±1.65	4.45±0.27
Neutrophils ($x10^9$/L)					
Experiment	1.93±0.48	4.78±1.37	4.03±0.61	4.43±1.12	2.63±1.3
Control	6.15±1.46	4.03±0.67	6.38±1.51	6.92±1.53	3.56±0.27
Lymph. ($x10^9$/L)					
Experiment	0.83±0.09	0.83±0.06	0.80±0.18	0.70±0.09	0.89±0.16
Control	0.55±0.06	0.67±0.07	0.68±0.08	0.71±0.08	0.71±0.04
Monocytes ($x10^9$/L)					
Experiment	0.15±0.03	0.48±0.28	0.20±0.04	0.23±0.10	0.35±0.23
Control	0.28±0.05	0.25±0.06	0.33±0.09	0.39±0.13	0.20±0.04

***Significantly different ($p < 0.05$) from the experimental group baseline. # Significantly different from the control group baselin**

Appendix 13 Average physical and physiological (mean ± SE) parameters in vervet monkey after 12 weeks of exposure to diet supplemented with *T. sericea* root and an additional 4 weeks washout period

Parameters **B.T (°C)**	Baseline	Week 4	Week 8	Week 12	Week 16
Experimental	38.95±0.31	38.55±0.21	38.73±0.38	38.93±0.23	38.98±0.08
Control	38.63±0.31	39.25±0.18	39.23±0.24	39.05±0.25	39.30±0.57
R.R (breaths/min)					
Experimental	25.0±1.91	26.0±2	30.0±4.76	29.0±1	34.0±3.46*
Control	27.0±1	28.0±1.63	29.0±1	32.5±2.06	32.0±0
S.P (mmHg)					
Experimental	80.0±6.82	79.25±2.32	106.25±4.01	67.5±3.66	89.0±2.35
Control	92.75±14.67	108.25±8.06	73.25±9.87	105.25±13.87	109.0±5.76
D.P (mmHg)					
Experimental	39.50±5.42	41.5±6.33	34.75±6.14	34.75±2.25	36.5±4.48
Control	54.75±7.59	48.0±1.29	55.25±1.65	51.25±4.87	47.25±5.94
M.A.P (beats/min)					
Experimental	59.75±7.92	56.25±7.43	50.25±10.07	48.25±3.35	54.25±7.26
Control	70.25±9.83	71.50±2.5	82.25±2.5	76.25±7.61	72.50±7.23
Pulse (beats/min)					
Experimental	108.0±16.90	97.75±11.92	97.5±13.36	118.0±7.05	142.5±20.12
Control	107.75±9.46	125.5±13.65	138.50±11.72	125.25±14.54	133.5±0.46

***Significantly different from the experimental baseline**