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DEPARTMENT OF CHEMISTRY

Phytochemical studies of extracts of the *Kirkia wilmsii* tuber.

PhD Research Thesis

By

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20 May 2020

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Declaration

I Kayini Chigayo hereby declare that the whole of this study is my own work and has not been copied from any other source, where use has been made of the work of others, it has been duly acknowledged. This work was done at the University of Venda under the supervision of Dr S. S. Mnyakeni-Moleele and Dr. J. M. Misihairabgwi.

20 May 2020

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Signature Date

As the study supervisors, we approve of this work for submission and certify that we have read this work and, in our opinion, it meets the academic and professional standards required by the University as a thesis for the degree of Doctor of Philosophy in Chemistry.

29 June 2020..

Signature Date

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Signature Date

Publications

The thesis is based on work reported in the following manuscripts;

1. **Chigayo, K., Mojapelo, P.E.L., Bessong, P. and Gumbo, J.R. (2014):** The Preliminary Assessment of Anti-Microbial Activity Of HPLC Separated Components Of *Kirkia Wilmsii,* African Journal of Traditional, Complementary and Alternative Medicines. 11(3): 275-281
2. **Chigayo, K., Mojapelo, P.E.L., Mnyakeni-Moleeele, S. and Misihairabgwi, J. M. (2016):** Phytochemical and antioxidant properties of different solvent extracts of Kirkia Wilmsii tubers, Asian Pacific Journal of Tropical Biomedicine. 6(12): 1037-1043
3. **Kayini Chigayo, Simon Mnyakeni-Moleele, Jane Masiiwa Misihairabgwi, Percy Maruwa Chimwamurombe，Cindiria Gleophline Van Wyk** (2020): Antimicrobial Activity of different *Kirkia wilms*ii (Legaba) extracts (Submitted to the Journal of Ethnopharmacology in Feb 2020)
4. **Kayini Chigayo, Simon Mnyakeni-Moleele, Jane Masiiwa Misihairabgwi** Proximate analysis and heavy metal analysis of *Kirkia wilms*ii tuber (Submitted to Journal of Applied Research on Medicinal and Aromatic Plants in Feb 2020)

Abstract

Ethnobotanical surveys have shown that *K. wilmsii* is used traditionally for the treatment of hypertension, diabetes, fever, hypertension, malaria, toothache and diabetes mellitus. Despite all this interesting information, there is no known literature on the phytochemistry of the tubers is recorded. This research seeks to gain an in-depth understanding of the structure of the bioactive compounds that can be extracted from *K. wilmsii* tubers.

The major objective of this research was to do an extensive study on *K. wilmsii* plant by extracting, isolating and identifying phytochemical(s) that are bioactive from the plant. This was achieved through, the determination of suitable phytochemical extraction solvents, development and optimisation of analytical HPLC methods, biological activity studies, investigation of levels of metal contamination and structural elucidation of the bioactive compounds by nuclear magnetic resonance (NMR), fourier transform infrared spectroscopy (FTIR), ultraviolet-visible spectroscopy (UV-Vis) and gas chromatograph-mass spectrometry (GC-MS).

In this research, tubers were air dried and ground into a fine powder. Extraction was performed on the powder. In the extraction and phytochemical extraction tests, extraction under the ultrasonic method was done by adding 1g of powder to 25ml solvent and extracting for the specified time. For the extraction test studies, the tests were performed for 15, 13 and 60mins. Extraction for phytochemical tests were done according to adapted recent methods. Quantitative tests were also carried out according to recent methods with minor modifications.

Phytochemical screening confirmed the presence of phenolics, flavonoids, terpenoids, tannins, cardenolide deoxy sugars and reducing sugars. The extraction results showed that from the 12 solvent extracts used, six gave yields higher than 5%, while the other six gave yields less than 1%. The highest extract yield of 52.9%was obtained using 80%methanol while the lowest yield of 7.3% was obtained using ethanol at 60 min. The 80% methanol, methanol/chloroform/water (12:5:3) (MCW) and 60% methanol extracts were significantly higher than those of ethanol, methanol and water ($P < 0.05$). Therefore ethanol, methanol, methanol/chloroform/water, 80% methanol, 60% methanol and water can be used as suitable phytochemical extraction solvents for *K. wilmsii* tubers. Total phenolic content and total flavonoid content analysis proved the presence of high levels of phenolic compounds as well as flavonoids. The presence of phenols and flavonoid could be

responsible for the radical scavenging activities observed. Total phenolic content recorded extracts ranged from (45.32 ± 0.50) to (122.84 ± 0.31) mg gallic acid equivalent per gram.

A maximum total flavonoid content of (917.02 ± 0.10) mg quercetin equivalent per gram and a minimum of (206.26 ± 0.10) mg quercetin equivalent per gram were recorded for methanol and water, respectively. The flavonoid content for methanol was significantly higher than all the other extracts ($P < 0.05$).

The scavenging profiles of *K. wilmsii* extracts were significantly lower ($P < 0.05$) than that of ascorbic acid and IC_{50} values ranged from 129.94 mg/mL for methanol to 225.04 mg/mL for water. An IC_{50} value of 56.52 mg/mL was obtained with ascorbic acid.

Antimicrobial activity tests were performed using the disc diffusion (Kirby-Bauer) method using 9 micro-organisms. Sub-culturing of the microbes was performed before using them in the test. The minimum inhibitory concentrations were determined using the micro titre plate method.

In the antimicrobial tests the positive control, amoxycillin, inhibited growth of all the tested bacteria except for *K. pnuemoniae.* All the *K. wilmsii* extracts also tested negative for inhibition of *K. pneumoniae*. The most potent extracts were found to be extracts from 80% methanol and 50%acteone followed by MCW and 80% ethanol. As expected, the minimum inhibitory concentration (MIC) values for the positive control were very low (0.031-1mg/ml). Only the 80% acetone extract had one comparable MIC values (0.031 -1 mg/ml) against *S. aureus*. The 80% methanol extract also had 2 encouraging results with MIC value of 0.031mg/ml against *E. coli* and *S. typhi*. The absolute solvent extracts did not yield interesting results as their antimicrobial activities were not pronounced. The water extracts also tested negative for inhibition of all pathogens used in the current work. These water extract results are in contrast to a lot of other work done on natural products.

Proximate analysis tests were done are moisture content and ash content, which done according to recent methods. Elemental analysis was performed on the XRF. Proximate analysis studies revealed that the average moisture content of the dried *K. wilmsii* tuber was found to be 3.44±0.11% which is less than the recorded moisture content for most natural plants. Our moisture result is well below the maximum 10% suggested for herbal remedies (EDQM, 2007).

The average total ash, acid insoluble ash and water soluble content of the *K.wilmsii* roots was found to be 12.43±0.41, 1.22±0.3 ad 8.18 ±0.39% respectively. The higher the total ash value, the

higher the chances of contamination, especially by minerals, less than the allowed 14% maximum. The result is close to the upper limit, possibly due to inorganic matter getting embedded in the tubers as they grow in the soil. The acid insoluble ash was found to 1.22% lower than 2% maximum. The XRF results for Cr, Pb, As, Cd and Hg levels for the *K. wilmsii* tuber were 1.48, 3.55, 0.14, ND and ND ppm respectively (mg/kg) are lower than the maximum allowed concentration of 25, 0.5, 1.5, 0.5, and 3ppm (EDQM, 2007; ICH., 2014) respectively for the metals except for Pb whose limits are above specifications. In our samples, Cd and Hg were not even detected. These results agree with our sub-chronic toxicity studies that showed that all the monkeys that were fed with a *K. wilmsii* meal did not die and did not show signs of poisoning.

Toxicological tests were carried out on vervet monkeys at the South African Medical Research Council, Primate Unit and Delft Animal Centre in Cape Town. One set of monkeys was fed with *K. wilmsii* while the other set of monkeys was fed with the normal food. The biochemical, haematological and physical and physiological tests were done at the centre.

In the toxicology studies there were no signs or symptoms as tha[illegible]ere indicative of unwell-being or distress during the 120day study period. The LD_{50} value was not determined as no mortalities were recorded. In the tested group of monkeys, no significant difference ($p>0.05$) in body weight gain, food and water intake were recorded. Similarly, the haematological and biochemical parameters and organ weights did not record any significant alterations ($p>0.05$) in the test (*K. wilmsii*) group when compared to the control results. The biochemical and haematological tests did not indicate any damage of the kidneys and liver and consequently any cell damage. Although not significant, the results indicate possible anti-hypertension properties of *K. wilmsii*.

HPLC methods were developed from scratch using the researcher prior knowl[illegible]e. Column purification methods were performed and adapted from recent methods cited in publications.

The purification of extracts yielded several extracts with different proportions and only 3 were selected for the final structural elucidation tests. The 3 extracts had UV-VIS spectra that exhibited maxima at 247nm (KTC3), 290, 295 and 358nm (KTC4) and 287nm (KTC5).

KTC3, KTC4 and KTC5 chromatograms were recorded and the resolution was generally satisfactory and a few components need to be separated. The HPLC profiles were meant to provide a tool for the purification of the extracts.

FTIR, GC-MS and NMR were performed according either according to normal laboratory methods or were adapted from recent research.

The GC produced a chromatogram with a peak at 5.77minutes for KTC3, whose MS produced the base peak with a mass to charge ratio of 77 and the parent peak gave a mass to charge ratio of 110 implying that KTC3 compound is likely to have a molar mass of 110g/mol. KTC4's main peak was at 5.75 minutes exhibiting a mass spectrum with a base peak of 97 and parent peak of 110. The GC chromatograms for KTC5 had a retention time of 6.75minutes, a base peak of 100 and the parent molecule of molar mass 113.

The structure of the 3 compounds that is KTC3, KTC4 and KTC5 were proposed which are closed related.The structures of the three compounds show that the compounds contain ether groups, hydroxyl functional groups, cyclic heterocyclic unsaturated compounds (tetrahydropyrans) which fall into the deoxy sugars and tannins.

Dedication

This work is dedicated to God who strengthens me every day. He has blessed me with a wonderful family, relatives, wife Zahara, sons Tauya Stephen and Munashe Beason. Surrounded by such company, I am motivated to work even beyond my imagined capabilities.

I would like to offer my deepest and sincere gratitude to the dearly departed Mr P. E. L. Mojapelo, who identified and initiated this research work.

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I would like to thank the Holy Spirit which guides us every day in everything we do.

Secondly, I would like to express my sincere gratitude to my supervisors Dr S. S. Mnyakeni-Moleele and Dr J. M. Misihairabgwi. Their assistance went beyond the normal duties of supervisors. I hope our continued relationship will lead us to achieving great goals. We owe much respect and sincere gratitude to Mr P. E. L. Mojapelo who passed on in July 2016 and was not there to see the conclusion of this research.

A round of thanks goes to the all the people who supported me in one way or the other, especially my wife and the children.

Abbreviations

1D	One Dimensional
2D	Two Dimensional
Å	Angstrom
AAS	Atomic Absorption Spectrophotometry
AIDS	Acquired Immune Deficiency Syndrome
ALP	Akaline Phosphatise
ALT	Alanine AminoTransferase
ANOVA	Analysis of Variance
APCI	Atmospheric Pressure Chemical Ionization
AST	Aspartate AminoTransferase
ATCC	American Type Culture Collection
BSAC	British Society for Antimicrobial Chemotherapy
CI	Chemical iIonisation
CK	Creatine Kinase
CLSI	Clinical Laboratory and sStandards Institute
COSY	COrrelated SpectroscopY
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DNA	DeoxyriboNucleic Acid
ECD	Electron Capture Detector
EDTA	Ethylenediaminetetraacetic acid
EI	Electron Ionisation
EUCAST	European Committee for Antimicrobial Susceptibility Testing
EDQM	European Directorate for the Quality of Medicines & HealthCare
Hb	Haemoglobin
HCT	Haematocrit
FTIR	Fourier Transform Infrared Spectroscopy
GAE	Gallic Acid Equivalent
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrometry

GGT	Gamma-Glutamyl Transferase
GHz	Giga Hetz
GSK	Glaxo Smith Kline
HIV	Human Immuno Virus
HMBC	Heteronuclear Multiple Bond Correlation
HMDE	Hanging Mercury Drop Electrode
HPLC	High Performance Liquid Chromatography
IC	Ion Chromatography
IC_{50}	Concentration for an antioxidant to reach 50% of scavenging DPPH free radical activity
INT	Iodonitrotetrazolium chloride
IR	Infrared Spectroscopy
kHZ	KiloHetz
K. wilmsii	*Kirkia wilmsii*
kg	kilogrammes
LD_{50}	Dose killing 50% of the tested animals
LD_{90}	Dose killing 90% of the tested animals
LOD	limit of detection
LOQ	Limit of quantitation
λ	Wavelength
MAP	Mean Arterial Pressure
MBC	Minimum Bactericidal Concentration
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MCW	Methanol/Chloroform/Water (12:5:3)
mg	milligrames
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometer
NCE	New Chemical Entities

NIST	National Institute of Standards and Technology
nm	nano meters
NMR	Nuclear Magnetic Resonance
NOAEL	No Observed Adverse Effect Level
NOESY	Nuclear Overhauser Enhancement SpectroscopY
NUST	Namibia University of Science and Technology
OD	Optical Density
PDA	Photo Diode Array
PUDAC	Primate Unit and Delft Animal Centre
QE	Quercetin Equivalent
RDW	Red Blood Cell Distribution
RBC	Red Blood Cells
RF	RadioFrequency
R_f	Retention Factor
SD	Standard Deviation
SNR	Signal to Noise Ratio
T	Tesla
TB	Tuberculosis
TFA	TriFluoroAcetic Acid
TLC	Thin Layer Chromatography
TFA	Trifluoroacetic acid
TM	Traditional Medicines
TMS	TriMethylSilane
TPC	Total Phenolic Content
USO	Underground Storage Unit
UNAM	University of Namibia
UV/Vis	Ultraviolet/Visible Spectroscopy
WBC	White Blood Cells
WHO	World Health Organisation
XRF	X-Ray Fluorescence

CHAPTER ONE: INTRODUCTION

Bioprospecting has become important in the modern world as we are faced with new challenges, such as new diseases as well as drug resistance that need new solutions. One definition of bioprospecting is that it is the process by which new products that originate from biological sources are discovered and commercialised (Müller, Obermeier and Berg, 2016).

Natural products are substances produced by living organisms such as secondary metabolites which are typically small organic molecules whose molecular weight is typically below 2000amu. These are produced by plants, fungi, bacteria, sponges, snails and amphibia (Salim, Chin and Kinghorn, 2008). On the other hand, living organisms also produce primary metabolites (macromolecules) such as proteins, polysaccharides and lipids which are nutrients and compounds needed for their survival.

Plants naturally produce chemical compounds as defence mechanisms to survive in the harsh ecological environments where are growing (Wurtzel and Kutchan, 2016). The necessity to produce these compounds is due to but not limited to the following factors; dry conditions, nutrient limitations, defence mechanisms and idiophase metabolites. The plants have manufactured bitter chemicals (glucosinolates and pyrrolizidine alkaloids) to deter herbivores, brightly coloured flowers (carotenoids and anthocyanins) to attract insects for pollination purposes, to name a few examples. Some of these chemicals have found therapeutic uses which are beneficial to humans. However, it is estimated that only a few plants (less than 15%) have been fully explored for their medicinal potential (Cragg and Newman, 2013).

The World Health Organisation (WHO, 2013) defines traditional medicines (TM) as knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness. The WHO bemoans the underestimation of the use of these TM as an integral part of health services in many countries. The term TM is sometimes used interchangeably with the term complementary medicines.

Before the invention of modern pharmaceutical preparations and remedies, plants were the only sources of medicinal remedies available to humans for treatment of various ailments (Atanasov *et al.*, 2015). They were used for many years the world over, and this trend still continues, especially

in developing countries where primary health care facilities are limited, as remedies for many ailments, (Balunas and Kinghorn, 2005; Cragg and Newman, 2013). The custodians of the health of the world, the (WHO, 2013), estimates that about 80% of the population in the developing world still depends on traditional medicines, mainly plant extracts, for the treatment of diseases (Harvey, 2000; WHO, 2013).

The main two reasons for the use of TM are limited health care facilities and cultural reasons (WHO, 2013). In Africa and the poorer nations, the WHO recorded that health care facilities are limited and expensive and hence the communities would prefer to make use of the more readily available and cheaper TM. For instance, a study conducted in Africa by WHO revealed that the ratio of traditional healers compared to the rest of the population was 1: 500 whereas the ratio of medical doctors to the population was a staggering 1:40 000. The second major reason for the use of the TM is cultural and historical reasons. For instance, Singapore and South Korea have proper up and running health facilities and yet very high numbers amongst their populations, 76 and 86% respectively still prefer to use TMs (WHO, 2013).

This information concurs with information gathered by Qiao who found out that TMs are mainly used by poor communities in places such as, Africa, China, India, etc., whereas in the Western world these medicines are used mainly because of Greek and Roman cultures (Qiao *et al.*, 2012). Locally, in South Africa, it was reported that despite the fact that rural communities have been using TM for thousands of years, the practice continues up to today, where some individuals prefer TM to Western medicine (Masoko and Nemudzivhadi, 2015). Cragg and Newman reported that Arabs collected vast amounts of Greco-Roman TM knowledge and added their own medicinal expertise coupled TMs from the native Chinese and Indian traditional to create a pool of knowledge of the medicines (Cragg and Newman, 2013).

Farnsworth also states that Hong Kong has the largest herbal market in the world, with Japan and China also claiming large volumes of consumers passing through herbal markets (Farnsworth, 1988). A survey carried out in India illustrated that documented scientific TM research has been on-going since the 1930s (Salim, Chin and Kinghorn, 2008).

In the past, natural products were used with little or no understanding of the actual properties of the plants or herbs and the actual science behind the plant or its treatment pathway (Atanasov *et al.*, 2015). Scientists have developed sophisticated equipment and techniques to unravel the

mysteries of nature, and we are no exception as we also attempt to add to the knowledge base through this research. Modern science has made it possible to investigate and characterise the active components in medicinal plants.

Some of these plants are used with little or no processing, in the form of tinctures, teas, powders, poultices or other forms (Bajpai, Majumder and Park, 2016). Great strides into drug research were made after the isolation and purification of opium from morphine in the early 19th century (Hamilton and Baskett, 2000). This led to drug discovery research from medicinal plants, which then led to the isolation of drugs such as cocaine, codeine, quinine and others. Although drugs like cocaine and morphine have been abused, they still find use as analgesics and anaesthetics. Furthermore, medicinal remedies from plants such as cinchona, digitalis and opium were replaced in the 1950s by the purer isolated active forms (Phillipson, 2007). Thus, natural products have been used as lead material for drug development by pharmaceutical companies, as noted by (Borris, 1996; Turner, 1996), from their study of perspectives on the development of drugs from natural products by pharmaceutical companies Glaxo Smith Kline (GSK) and Merck. Pauli and co-workers reported that a large percentage of new drugs introduced into the markets in the last few decades in the Western world were derived from TMs (Pauli *et al.*, 2012).

The search for new lead drug molecules from natural sources has been maintained as scientists are battling to counter adverse effects found in modern medicines (Dev, 2010). An example in question was the discovery that the human body easily absorbs natural products as compared to synthesised drugs, as most of the natural products are small molecules (Harvey, 2008).

Although there are hundreds of thousands of plant species that are found in many places around the world, only a small fraction of these plants have undergone extensive research (Hostettmann, 1998). The obvious implication here is that there are various plants and herbs that can still be subjected to research activities to find out the possibility of using them. The research requires that efficient methods for rapid chemical and biological screening of the plants must be found. Scientifically, even semi-processed herbal remedies can be considered to be crude, as not much is known about them. In most societies, the knowledge of these medicinal plants is held by the old and illiterate generations, who are most unlikely going to hand down the information to the younger generation. This is exacerbated by the fact that the younger generation might regard the use of TMs as primitive and would also not want to be associated with TMs. This might mean that

the TM information might slowly but surely disappear, as the information is not being inherited by the younger generation. Even in the societies where the information is passed down, it is not documented, and the information is passed down orally from one generation to another, and hence may not be reliable.

The use of TM for the development of new drugs has been necessitated by the advent of new diseases such as Acquired Immune Deficiency Syndrome (AIDS) and related illnesses, chronic diseases such as cancer, and the need for cheaper medicines. Closely related to this is the emergence of drug resistant tuberculosis (TB) strains (Pauli *et al.*, 2005). A good example is the occurrence of opportunistic infections, which include candidiasis and cryptococcosis that are more pronounced in individuals with compromised immune systems, for which there are a limited number of effective antifungals (Hostettmann, 1998). Natural products can then be used as a solution for such problems. Thus, in the quest to find new drugs, natural products offer a good alternative.

Some of the synthesised drugs that are currently used for treatment of ailments are not always effective and may have side effects, which can be fatal. The Chagas disease, common in Central and South America, is a good example. The drugs used for the treatment of this disease are only effective in the acute stage (Tonin *et al.*, 2009). To exacerbate the situation, those drugs also have serious side effects such as cardiac toxicity. This has necessitated the need to search for plant drugs. However, the search for new plant drugs has followed a wide range of routes. The most effective has been found to be the testing of plants that are currently in use in some communities. In this way, the chances of success are higher subject to the availability of sufficient funding for the research (Farnsworth, 1988).

The use of TMs in Limpopo in particular, has been widely reported on. Some of the reports focus on documenting the use of the TMs, while others actually investigated the plants in the laboratories. One ethnobotanical study conducted in Venda to find out which TMs traditional healers use for the treatment of Candida revealed the widespread use of TM (Masevhe, McGaw and Eloff, 2015). In the study, the various traditional healers revealed that they use a wide variety of preparation, and some of their claims were supported by documented laboratory scientific research while others were not. The most common preparations were roots, bark and leaves or their combinations. Therefore more scientific research needs to be carried out to harmonise these

different opinions from the traditional healers. Steenkamp and co-workers actually performed antimicrobial activity tests for plants that were reported to be used by traditional healers (Steenkamp, Fernandes and van Rensburg, 2007). They reported mixed results, with some plants showing no antimicrobial activity at all while others were active.

The anti-inflammatory properties of TM used on the VhaVenda people for the treatment of venereal diseases by traditional healers was confirmed by the results obtained laboratory investigation performed on the same plants (Mulaudzi *et al.*, 2013). In another study, Muladzi and co-workers also investigated the plants used for the treatment of cough and fever (Mulaudzi *et al.*, 2012). They also reported that their results confirm that the traditional healers were using the correct medicinal plants for the treatment of these two ailments. Many other researchers have reported the widespread use of TM in Venda.

Use of TM has also been widely reported in Limpopo and South Africa at large. In 2015, Semenya and Potgieter reported that the Bapedi people of Polokwane use the *Kirkia wilmsii* plant for the treatment of hypertension (Semenya and Potgieter, 2015). An interesting study was carried out on the TMs used as skin care products (Lall and Kishore, 2014). They reported that although the plants are used as alternative skin care products, little scientific research has been conducted on the products. These are just a few examples of the publications on TM use in Venda, Limpopo and South Africa.

TMs use has also been published in Southern Africa, Africa and the world. Generally, the authors all come to the same conclusion that the traditional use of TMs needs to be backed up by laboratory scientific evidence. This has motivated this research which hopes to unravel new knowledge with respect to *Kirkia wilmsii* plant that has been reported to have traditional benefits that are not backed up by scientific research.

The WHO has encouraged the use of TM and research into TMS. One disadvantage of finding lead molecules from TMs is that the isolation of pure and biologically active components from plants is a long and tedious process. Furthermore, this process needs multi-disciplinary expertise, such as, chemical, biological, botanical, ethnobotanical and phytochemical skills. The other challenge is that most of these plants are found in developing countries, which are commonly not politically stable, hence the continued supply of the raw material is not guaranteed.

Finally, the process is very long and some documentation indicates that it may take up to 10 years or sometimes more, to successfully complete the whole new drug discovery process (Balunas and Kinghorn, 2005).

We hope that this research will assist in the development of new drug(s). High performance liquid chromatography (HPLC) methods will be developed and validated so that the methods can be documented and be available for use by analysts which might be incorporated in the new WHO herbal medicines pharmacopoeia. Bioactivity studies will be conducted to establish the possible pharmaceutical properties of importance. The research may lead to better and cheaper alternatives to the currently available medication.

1.1 Research Problem

For centuries, herbs and herbal preparations were used as medicines in the world as there were no other alternatives in the early years (Atanasov *et al.*, 2015). Furthermore, research in traditional medicines is important as the WHO estimates that 80% of the people living in the developing world rely on them for treatment of diseases (WHO, 2013). Thus research into traditional medicines is as important today as it was a long time ago.

The need is also exacerbated by the emergence of new diseases as well as drug resistant strains that have forced scientists to go out to seek new medicines and alternatives that can deal with drug resistance. HIV related diseases, cancer, drug resistant TB, invasive candidiasis and malaria are examples that have been cited regularly (Watkins and Bonomo, 2017).

South Africa is endemic to a lot of plants that are used as traditional medicines in the country and in one ethnobotanical survey, *K. wilmsii* was cited as a treatment for hypertension (Moeng and Potgieter, 2011). Another study involving ethnobotanical surveys, phytochemical and pharmacological studies revealed that *K. wilmsii* is being used for the treatment of diabetes, fever, hypertension, malaria and toothache (Maroyi, 2016). This information coincides with other investigations done by other researchers who came to the same conclusions regarding the use of the plant for hypertension (Moeng and Potgieter, 2011; Semenya and Maroyi, 2013; Semenya and Potgieter, 2015), diabetes mellitus (Semenya and Maroyi., 2013), malaria and fever (Suleiman *et al.*, 2010).

All the studies mentioned above involve mainly the ethnobotanical survey, which confirm the use of *K. wilmsii* as traditional medicines and yet there is no documented research on its chemistry. Although there is little documented research on *K. wilmsii,* there is more literature on the ethnobotanical surveys on *K wilmsii* than phytochemical studies. Other than the above cited few cases of research on *K. wilmsii*, little literature has been published on *K. wilmsii,* yet it has been widely used amongst the Northern Sotho (Bapedi) people of South Africa for the treatment of a wide range of diseases.

To fill up those gaps, this research seeks to gain an in-depth understanding of the structure of the bioactive compounds and maybe suggest synthetic pathways for the compounds. We hope that this research will assist in the development of new drug(s).

Further, there is a growing need for the provision of validated methods of analysis, especially as the WHO has now encouraged the use of TM as an alternative source of medication. In this research high performance liquid chromatography (HPLC) methods were developed and validated so that the methods can be documented and be available for use by analysts. This might also be incorporated in the new WHO herbal medicines pharmacopoeia. This would help in the regulatory branch of pharmaceutical industry research in the quest to regulate the use of traditional medicines through the production of regulated and validated compendial methods of analysis.

Bioactivity studies were conducted to establish the possible pharmaceutical properties of importance. The research may lead to better and cheaper alternatives to the currently available medication.

In conclusion, the fact that little research has been documented on the *K. wilmsii* tubers, coupled with the documented traditional use of *K. wilmsii* tubers for curative purposes by the Bapedi people, motivates this study.

1.2 Hypothesis

The *Kirkia wilmsii* can be used to cure many diseases, therefore it can be used as a precursor to manufacture wide spectrum medicines.

1.3 General Objective

The major objective of this research was to do an extensive study on *K. wilmsii* plant by extracting, isolating and identifying phytochemical(s) that are bioactive from the plant.

1.4 Research objectives

- To determine suitable phytochemical extraction solvents for *K. wilmsii*.
- To develop, optimise and validate analytical HPLC methods for separation and isolation of K. wilmsii extracts.
- To investigate the biological activity of crude and purified extracts of *K. wilmsii*
- To determine the levels of metal contamination in the plant tubers
- To determine the toxicological effect of the tubers on monkeys
- To determine the structure(s) of compound(s) extracted from *K. wilmsii* tubers

CHAPTER TWO: LITERATURE REVIEW

2.1 *Kirkia wilmsii*

The name *Kirkia wilmsii (K. wilmsii)* was derived from the names of two scientists. Dr John Kirk (1832-1922) was a plant collector and explorer who travelled with David Livingstone during their expeditions on the Zambezi escarpment. People used to refer to him as Kirkii, where the genus name *Kirkia* was later derived from. The species name *wilmsii* came from Friedrich Wilms (1848-1919), a German Pharmacist, plant collector and botanist (http://pza.sanbi.org/kirkia-wilmsii).

K. wilmsii is commonly known as wild pepper or Mountain Seringa (English) and the local name is Legaba or Modumela (Northern Sotho) (Semenya, Potgieter and Erasmus, 2012). According to work done by Bachelier and Endress, *K. wilmsii* belongs to the *Sapindales* family and they content that it was wrongly placed in the *Simaroubaceae* family for a long time (Bachelier and Endress, 2008).

It can grow to a height between 8 and 10 metres, in rocky soils and can survive short periods of drought as it can store water in the roots. It is has adapted to dry season conditions, by developing underground storage organs (USOs) (Landen and Wrangham, 2005), which enable plants to store their energy. These USOs include bulbs (formed from the stem and leaves), tubers (formed from the stem or roots), corms (formed from the stem) and rhizomes (formed from the stem), which are usually large enough to store a substantial amount of food (Dominy *et al.*, 2008). The USOs of the plant *K. wilmsii* is identified as a tuber (Semenya, Potgieter and Erasmus, 2012). Indigenous people chew the bulb root for both medicinal and thirst quenching purposes (Smith, 1991).

Like any other living organism, plants naturally adapt to fight against unfavourable conditions during their growth phases or seasonal changes. Therefore the plant storage system is affected by its location and the surrounding adverse conditions. This means that the nutritional value of the underground storage organs varies widely within species and between species, depending on the area, season and energy (Pillay, Maharaj and Smith, 2008). Water, toxins and the presence of other nutrients in the environment are also factors that affect the nutritional value.

In South Africa, the tree is mainly found in the Mpumalanga and Limpopo provinces. In fact, the *K. wilmsii* is the main vegetation that is found in the Leolo Mountain range, situated in Sekhukuneland, Limpopo Province (Venter *et al.*, 2007). Elsewhere on the continent, this family

(Kirkiaceae) includes a wide range of small to medium sized trees, mainly found in eastern tropical Africa and Madagascar.

The trees from which the tubers were obtained did not have leaves as shown in Figure 1 below:

Figure 1: Pictures of the *K. wilmsii* trees without leaves during the dry season.

However another picture taken in February after substantial amount of rain had fallen, showed that the tree had developed some leaves as show in Figure 2 below:

Figure 2: Pictures of the *K. wilmsii* trees with leaves during the rainy season.

One research on the trade in TM showed that of the 231 plants surveyed, roots were the most preferred and *K. wilmsii* was mainly used for the treatment of hypertension (Moeng and Potgieter, 2011). They were particularly concerned that most of the plants are not even protected by conservation laws, including *K. wilmsii*. A mixed study including ethnobotanical surveys,

phytochemical and pharmacological study revealed that *K. wilmsii* is being used for the treatment of diabetes, fever, hypertension, malaria and toothache (Maroyi, 2016). This information coincides with other investigations done by other researchers who came to the same conclusions regarding the use of the plant for hypertension (Moeng and Potgieter, 2011; Semenya and Maroyi, 2013; Semenya and Potgieter, 2015), diabetes mellitus (Semenya and Maroyi., 2013), malaria and fever (Suleiman *et al.*, 2010).

In our earlier studies, we reported antimicrobial activity against *Aeromonas hydrophilia, Candida albicans, Enterobacter aerogenes, Escherichia coli, Proteus mirabilis, Salmonella typhii, Shigella dysenteriae, Stapphylococcus aureas* and *Vibrio cholera* (Chigayo *et al.*, 2014). This proved the potency of the extracts from *K. wilmsii*.

Research performed on the leaves showed that the *K. wilmsii* dichloromethane/methanol and water extracts have anti-plasmodial properties (Clarkson *et al.*, 2004; Pillay, Maharaj and Smith, 2008). Leaf extracts exhibited biological activity against *Staphylococcus aureus* (acetone, methanol, hexane and dichloromethane extracts), *Enterococcus faecalis* (acetone, methanol and hexane extracts), *Escherichia coli* (acetone, hexane and dichloromethane extracts), *Pseudomonas aeruginosa* (acetone, hexane and dicholoromethane extracts), *Sporothrix schenckii* (methanol and hexane extracts), *Microsporum canis* (acetone extract), *Cryptococcus neoformans* (acetone, hexane and dichloromethane extracts), *Candida albicans* (acetone and hexane extracts) and biological activity against *Aspergillus fumigatus,* a fungus that causes breathing problems in poultry (Suleiman *et al.*, 2010). Hexane, dichloromethane and methanol extracts of the dried and powdered bark of the *K. wilmsii* were investigated and found to contain secondary metabolites (Mulholland, Cheplogoi and Crouch, 2003). These were identified as lignans, iso-coumarins, flavonoids and nor-carotenoids.

This tree has generally been found to be free from pests (Grant and Thomas, 2006) . In other research activities for the future, the insect repellent properties of the tree must be investigated to rationalise this information.

Of the *Kirkia* genus, it has been noted that *Kirkia acuminata* is the most common species in the family naturally occurring in the Sub-Saharan African region (Maroyi, 2017). Maroyi reported that ethnobotanical surveys showed that *Kirkia acuminata* has been used for the treatment of abdominal

pains, backache, cholera, constipation, cough, diarrhoea, dysentery, snake bites, toothache and wounds.

Phytochemical studies on *Kirkia acuminata* showed the presence of tannins, which are naturally occurring polyphenolic compounds that form complexes with proteins (Aganga and Mosase, 2001).

Although there is little documented research on *K. wilmsii,* there is more literature on the ethnobotanical surveys on *K wilmsii* than phytochemical studies. Other than the above cited few cases of research on *K. wilmsii*, little literature has been published on *K. wilmsii,* yet it has been widely used amongst the Northern Sotho (Bapedi) people of South Africa for the treatment of a wide range of diseases.

We hope that this research will assist in the development of new drugs. Analytical chemistry is a tool that can be used as part of the regulatory branch pharmaceutical industry research in the quest to regulate the use of traditional medicines through the production of regulated and validated compendial methods of analysis. The traditional use of TMs involves the use of unknown doses, unknown chemical composition and consequently unknown quality of the product (WHO, 2013). Furthermore, the natural resources from which traditional medicines are derived are continuously being depleted without replacement, although efforts are being made to resuscitate these natural resources (Balandrin, Kinghorn and Farnsworth, 1993). Most of the plants have not been categorised as protected species under the law and thus harvesting is continuing uncontrolled.

A global effort has to be made to find solutions before the resources are completely depleted. Therefore, it has become imperative to find a solution to this problem. This research, which seeks to gain an in-depth understanding of the structure of the bioactive compounds and maybe suggest synthetic pathways for the compounds, will go a long way in trying to solve the problem.

2.2 Extraction of phytochemicals from *K. wilmsii*

2.2.1 Sample preparation

The first step after identification of a plant of interest is the extraction of the secondary metabolites from the plant. Extraction involves the identification of possible solvents to be used and extraction procedures which affects the extracted bioactive compounds. The selection of the plant of choice is followed by the choice of the part of the plant to be used, that is, whether to use the leaves, bark,

fruits, flowers or roots. This choice depends on the ethnobotanical studies, similar plants of the same genus showing activity, interests and selection criteria of the individual researchers. For instance, we chose to use the roots as they were not studied at all and yet they are widely used by the local communities. Further, a choice has to be made whether to use fresh or dried samples. It is often very difficult to maintain fresh samples and thus many samples are dried before use and some researchers reckon that plants lose their freshness after 3 hours (Sulaiman *et al.*, 2011). Therefore, a lot of researchers prefer to dry samples before extraction can be performed.

Drying can be carried out in different ways, such as, drying in air, freeze drying, drying in microwave and normal oven drying. Air drying is performed under ambient conditions and thus can sometimes take very long depending on the properties of the sample. However, this can be good for samples that might be heat sensitive, unlike normal oven drying which can affect heat sensitive samples. Even though oven drying is the simplest method to use, it is quite difficult to select the best temperature. Drying plants in the microwave has very complicated implications, one of them being the high temperatures generated by the electrical effect of the microwave and the magnetic effect can change the dipole moments of the chemical constituents of the samples dried (Kaufmann and Christen, 2002). Therefore, both oven and microwave drying can lead to sample degradation.

For heat sensitive samples or samples whose chemical composition is completely unknown, freeze drying is always recommended as the drying process actually occurs at very low temperatures. Temperatures as low as -80°C are used. This process involves sublimation of the solvent and the consequent removal of the gas leads to drying. The major drawback of this method is that it can take very long to accomplish complete drying.

The extraction process follows a series of simple steps such as grinding (size reduction) which is meant to break the cell walls so as ensure maximum solvent exposure as the plant material surface area is increased.

2.2.2 Extraction methods

Extraction involves the separation of the soluble secondary plant metabolites from the insoluble ones with the help of different solvents (Banu and Cathrine, 2015). A lot of factors such as time, solvents, temperature, purpose of extraction and prior knowledge have to be considered in extraction (Blicharski and Oniszczuk, 2017).

Different solvents are used for the extraction of different phytochemicals. Polar solvents are used to extract polar compounds and likewise non-polar solvents extract non-polar compounds. Organic solvents are mainly used, even though many traditional healers use water for herbal preparations. The polarity of the solvents increases in the following order: hexane, cyclohexane, benzene, dichloromethane, chloroform, acetone, ethanol, methanol and water. Some work done on different vegetables proved that the various solvents extracted different compounds with varying contents of phenolics, antioxidants and free radical scavenging activities (Sulaiman *et al.*, 2011).

There are many different extraction methods to be chosen from ranging from, the traditional methods such as maceration and soxhlet extraction to the newer methods like supercritical fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction and surfactant mediated techniques (Azmir *et al.*, 2013; Chua, 2013). The old classical traditional methods take advantage of the extraction power of the solvents, temperature and agitation to achieve maximum extraction.

Maceration is one of the oldest extraction techniques applied to plant extraction. According to Handa and co-workers, this procedure was "borrowed' and improved from wine production techniques (Pauli, Pro and Friesen, 2008). In this technique, the plant material is soaked into the solvent of choice and left standing for a pre-determined length of time. In some procedures occasional agitation of the container is advised whereas some leave it to stand for the entire extraction period. The period ranges from a few days to many days, typically 3 to 7 days, after which filtration retrieves the pregnant extract solution (Azwanida, 2015). Infusion, percolation and decoction are similar methods except that they use hot samples which are more suitable for heat resistant samples. Maceration is the simplest and easiest to perform but the length of time taken as well as the huge amounts of organic solvents used are major drawbacks.

Decoction is an extraction method where hot solvents especially water are used to extract active compounds from plants. The procedure is similar to maceration except the temperature of the water, and is used to extract hard materials, such as roots and bark (Azwanida, 2015). Further, these materials must be heat stable. Heat is applied until the initial solvent volume is reduced to about 25%.

Soxhlet extraction is one of the oldest methods that has continued to be used despite recent advances in extraction technologies. According to research, the soxhlet extraction method is one

of the most quoted methods with regards to extraction of medicinal plants (Luque-Garcia and Luque De Castro, 2004). The sample is placed inside a thimble and the extraction sample is heated below. The solvent evaporates and cooled by the condenser and extracts the TM from the finely ground sample and it is collected back into the bottom flask through the side arm, inside which there is a siphon tube. The condenser cools down the vapours and it also returns to the thimble. No solvent escapes as the process continues for a determined length of time, thus considerable amounts of extracts can be obtained using a small amount of solvent. The process is stopped when no more solvent is released by the siphon tube. This normally takes about 12 hours to complete (Stalikas, 2007).

There are several modern extraction methods that have been developed to improve the efficiency of the extraction process. Out of the new methods such as accelerated solvent extraction, supercritical fluid extraction, negative pressure cavitation extraction, the ultrasonic assisted extraction method, used in this research uses ultrasound of frequencies between 20 and 2000kHz to break down the cell walls thereby facilitating the contact between the phytochemicals and the solvent (Banu and Cathrine, 2015). This method is a direct improvement of the maceration method. One disadvantage is that this method is thought to produce radicals, which means that the phytochemicals chemical structure and configuration might be altered. The temperature and length of time can also be controlled on the ultrasonic bath to improve extraction.

Microwave assisted extraction method is another modern method which uses electromagnetic energy of between 0.3 to 300GHz which consists of electric and magnetic fields (Kaufmann and Christen, 2002). This makes the temperature to rise which is a disadvantage for thermo-labile samples. Other modern methods include supercritical fluid extraction which uses compressed gasses such as carbon dioxide to effect the extraction process. The gases can be converted to liquid and back to gas by changing the pressure and temperature in the vessel.

In a literature survey done by Chuang and co-workers, it was generally shown that ultrasonic assisted extraction gave higher extraction yields than any other method (Chuang *et al.*, 2015). This also made our reasons for choosing the ultrasonic extraction stronger.

2.3 Drug Discovery from Plants

For centuries, herbs and herbal preparations were used as medicines in the world as there were no other alternatives in the early years (Atanasov *et al.*, 2015). Before the advent of modern medicinal

science, herbs and their preparations were the only therapeutic agents that humankind would use to cure diseases and sickness (Ganesan, 2008). During that time, the people used these medicines with little or no in-depth knowledge of the chemistry of these natural products (Atanasov *et al.*, 2015). Later on and in recent times, advanced techniques and instrumentation were developed to help in the study of the phytochemicals which are used to study and eventually positively identify the bioactive components of the plants.

In terms of the number of small molecules, nature by far produces more molecules than humans have tried to synthesise and thus remain the best bet in search of new drugs and drug leads (Newman and Cragg, 2012). TM continue to play an important role in our lives today and a more recent case is the award of the 2015 Nobel Prize for Physiology or Medicine to William C. Campbell, Youyou Tu and Satoshi Omura for the discovery of avermectins class of compounds which cure river blindness and elephantiasis as well as artemisinin which cures malaria (Shen, 2015). These natural products were both extracted from plants, which still give impetus and hope to natural product research.

The discovery of new medicines from plant materials has developed from ancient times. One of the first known drugs derived from plants was morphine, which was isolated from opium. Opium preparations are used as anaesthetics was well documented for surgery and post-surgery relief of pain (Hamilton and Baskett, 2000). A junior scientist, Friedrich Serturner, isolated morphine from opium around 1800. This is one of the first recorded successful isolation of phytochemicals from plants, although it was not used until about 50 years later when the hypodermic needle and syringe were developed. This significantly changed medicine as the first post-surgery pain killer had been developed. This led to an avalanche of research into TM.

The advent of new diseases as well as drug resistant strains has also forced scientists to go out to seek new medicines and alternatives that can deal with drug resistance. HIV related diseases, cancer, drug resistant TB, invasive candidiasis and malaria are examples that have been cited regularly (Watkins and Bonomo, 2017). The drug quinine which cures malaria was developed from the bark of a plant, Cinchona officinalis, which was being used for the treatment of fever by the Amazon people (Cragg and Newman, 2013). The drug was later derivatised to form the more effective chloroquine. In the 1960s, some chloroquine resistant strains of malaria were reported and new plant derived drugs were discovered from plants, especially, artemisinin (Shen, 2015).

Th need for research into natural products was supported by Chen and co-workers, who also noted that good leads can be identified from natural sources but often they have to be modified to cover up for their shortcomings (Chen *et al.*, 2015).

Other problems associated with drug discovery from natural sources are the length of time from bush to the pharmaceutical plant production, expenses involved as opposed to the costs for synthesis and the low rates of success (Cragg and Newman, 2013).

TM research has also been necessitated not only by drug resistance, but also by the surfacing of difficult to treat diseases such as cancer and incurable diseases such as AIDS. Chemotherapy, surgery or radiation treatments are often used for the management of cancer cases. Chemotherapy is the use of drugs to cure or reduce the effects of diseases on the body. This term is often only used with reference to cancer. Cancer drugs have high success rates if the cancer is detected early. However in cases where the tumours are detected late, curing the disease using drugs becomes almost impossible and drugs are prescribed to manage and minimise the tumours. In this respect, natural product research has been launched to try and find solutions to this problem.

A few examples of active agents against cancer that were developed from plants are, Paclitaxel (whose trade name is taxol) from yew plants and bark of brevifolia, vincristine and vinblastine from Catharanthus roseus and camptothecin from Camptotheca acuminata Decne (Cragg, 1998; Atanasov *et al.*, 2015). The complex structures of these compounds are shown in Figure 3. Taxol is by far the most publicised anticancer drug derived from plant and has been approved for treatment against ovarian and breast cancer. Taxol signifies the popularity of some natural product derived drugs with annual sales of above US$1billion (Cragg and Newman, 2013).

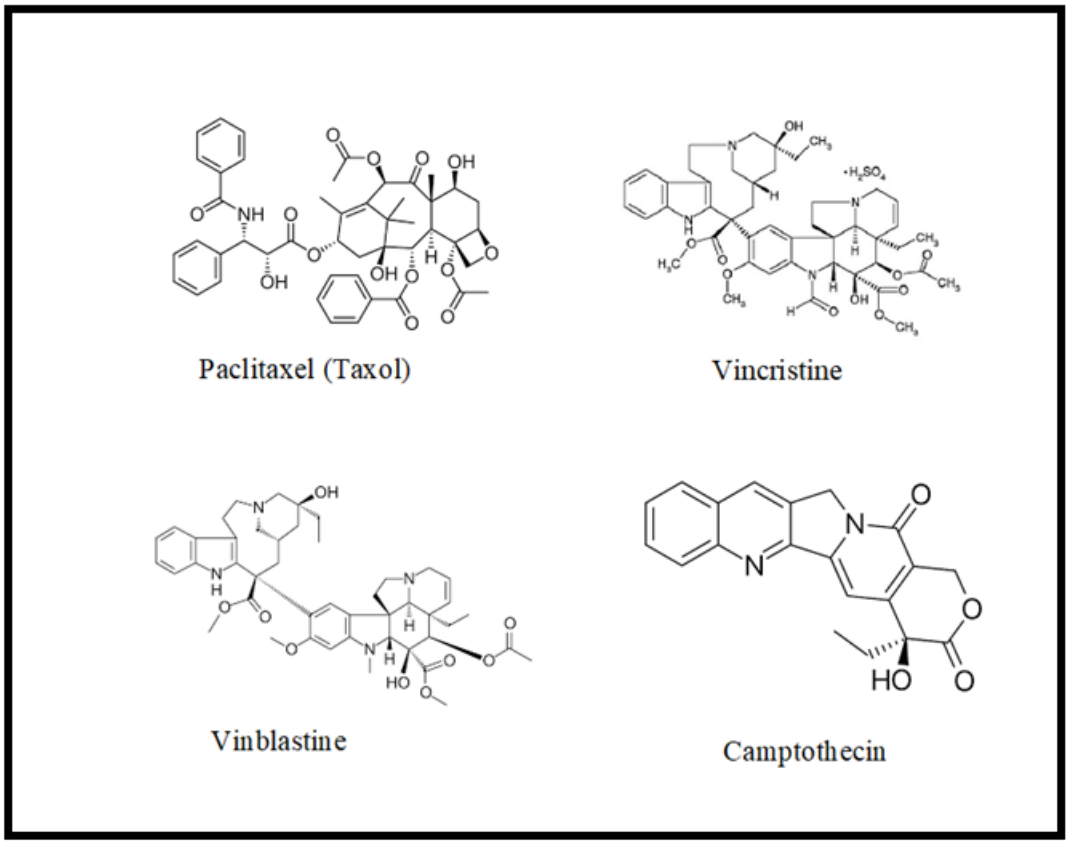

Figure 3:Structures of some anticancer medicines developed from natural products

The HIV/AIDS problem has challenged the best brains in science and continues to be a major health problem which has claimed a lot of lives. The cure or a vaccine has eluded scientists up to date, as the virus evolves to keep ahead of possible cure. A lot of research into natural cures has taken place but tangible results are yet to be produced. A case study was done in China by Jin and co-workers, who reviewed data on the treatment of HIV/AIDS related diseases in China where they compared government/WHO sponsored patients against those receiving natural traditional Chinese Medicine (Jin *et al.*, 2014). Their study over a 6-year period showed that the mortality rate was lower for the patients taking traditional medicines as opposed to those taking medicines. Their study suggests that traditional Chinese medicines may be used to improve the lives of people living with HIV.

Despite the successes of traditional medicines, some drawbacks have been recorded. The depletion of natural resources has become an obvious consequence of over reliance on TM in some societies.

The ‘taxol supply crisis” is one example, when the drug could only be derived from the brevifolia plant, the plant became a target when people realised that it could “treat” cancer (Kusari, Singh and Jayabaskaran, 2014). This problem is prevalent and governments are moving in to protect the vital natural resources. Furthermore, countries where the natural products are found expected financial windfalls from the drug manufacturing companies, which was not to be as the American National Cancer Institute reports that more than one hundred thousand extracts were tested and only taxol and camptothecin were developed into pharmaceutical drugs (Atanasov *et al.*, 2015).

Other challenges faced during the process of drug discovery from natural products is that the natural products are not readily soluble and may also not be stable (Chen *et al.*, 2015). Instability of the lead compounds is a major challenge as it makes experimental work very difficult. The conditions which make the molecule stable must be investigated, such as temperature and isomerism. Modification of these compounds may be necessary.

Research into TM generally starts with identification of the plant(s) of interest, collection and subsequent positive identification of the herbal plants of interest by a botanist, ethnobotanist, ethno-pharmacologist or plant ecologist. One disadvantage is that different common names are used for the same plant and confusion ensues regarding the naming of the plants (Elkington *et al.*, 2014).

A biological screening, using the relevant biological activity assays is carried out after extraction. Active compounds are then isolated, purified and characterised in the laboratory using modern analytical techniques such as chromatography and nuclear magnetic resonance. Computational chemistry can then be employed to see if the identified compounds would show any biological activity towards several organisms using theoretical simulations.

Despite various drawbacks, natural products have been used as lead material for drug development by multinational pharmaceutical companies (Borris, 1996; Turner, 1996). Both authors studied the perspectives of pharmaceutical companies such as GlaxoSmithKline and Merck. They pointed out that, although significant, research from natural products only plays a small part in drug development.

In summary, research in TM is important as estimates state that more than half of the drugs in clinical use today were derived from natural sources (Cheuka *et al.*, 2017). Noted examples are drugs such as morphine, quinine and penicillin among many others.

2.4 Isolation and purification of extracts

Nature has proven to be an excellent and complex "laboratory" for the synthesis of mixtures of complex phytochemicals. This attribute is still unchallenged by human beings. As part of the drug discovery process it is necessary to separate these compounds to understand better the bioactivity of natural plants. Therefore isolation and purification of our plant extracts is a very important step in our research. Improved technology is available for this purpose.

A chilling example of the dangers of using medicines which are not properly purified was recorded in Pakistan where 125 poor people died after taking pyrimethamine which was contaminated by isosorbide 5, which caused severe bone marrow suppression (Nishtar, 2012). Consequently the WHO issued a drug safety alert for this incident. This negligent loss of lives could have been avoided if the authorities had taken action on an earlier WHO report which indicated that up to 50% drugs used in Pakistan were either of low quality or counterfeit (WHO, 2005). To exacerbate the situation, the use of TM was not regularised and the parliament was sitting on a proposal on the use of TM for 10 years. We can only wonder how many more unreported victims/cases worldwide go unreported because of un-purified products.

In the process of purifying natural products, an interesting concept of typical pathways of impurities detected is always evident, referred to as residual complexity (Chen *et al.*, 2009). Residual complexity has an impact on the accuracy of microbiological activity tests, which might reduce the accuracy of the tests. There are two types of residual complexity, that is, static and dynamic residual complexity. Static has to do with natural purity presence of minor impurity and dynamic complexity with chemical instability and reactivity. While static complexity can lead to imperfect biological activity, dynamic may lead to imperfect results over time.

2.5 Methods used for analysis of natural products

In the purification of natural products, first, the actual purification is performed and then the actual assessment of the purity follows (Pauli *et al.*, 2012). Chromatography is the most commonly used method as it allows the separation of complex mixtures of compounds.

2.5.1 Chromatographic methods

Chromatography describes a group of separation methods used for qualitative and quantitative analysis of components of a given analyte. This technique is a powerful analytical tool that can even separate components of complex mixtures. The separation techniques are based on the differences in interactions of the components of the analyte between the stationary phase (static) and the mobile phase (moving). Depending on the nature of the mobile phase, when a liquid stationary phase is employed, it is named liquid chromatography whereas gas chromatography makes use of gaseous mobile phase.

Chromatography was developed around 1906 by the Russian Botanist, Mikhail Tswett, which he coined from the two Greek words, chroma (colour) and graphein (to write). The words were combined to literally mean "colour writing". The technique has evolved from the early years where analysis times where typically very long to modern times where rapid analysis can be performed.

When performing chromatographic separations, the analyte is carried through the column by a mobile phase or eluent. The stationary phase is fixed in a column. The sample components distribute themselves between the mobile and stationary phase to varying degrees. The components that are strongly retained by the stationary phase move slowly with the flow of the mobile phase and components that are weakly held by the stationary phase travel rapidly.

Consequently, because of these different migrations rates, sample components separate into discrete bands that can be analysed qualitatively (identification) and quantitatively (concentration). Chromatography as a separation technique is relevant to plant extracts as they are made up of various phytochemicals/bioactive compounds which sometimes may be of different polarities but can be separated by chromatography (Boligon and Athayde, 2014).

2.5.1.1 Thin layer chromatography (TLC)

In TLC studies the stationary phase is attached to an inert material such as plastic, glass or metal. The stationary phase also referred to as the sorbent allows for the separation of different analytes applied on the TLC plate. The mobile phase is placed in a TLC tank and after saturation, the plate is carefully placed inside and the mobile phase is allowed to travel up the plate by capillary action. This action carries the sample up the plate on the stationary plate and separation of the components takes place as the mobile phase rises, which can be described either as adsorption or partition

chromatography according to the nature of separation. TLC is applicable to a wide range of compounds. The most common detection method is examination under the UV lamp.

2.5.1.2 Column chromatography

When chromatography was discovered experiments were conducted through column chromatography, where the stationary phase was packed in a column and elution was performed through the continuous addition of the mobile phase through the top of the column. This method is still applicable to the modern-day research as it is used for the separation and purification of compounds. The setup is made up of a glass or plastic column packed with silica or alumina and a solvent is poured from the top. The mobile phase can be run though gravity or at a low pressure powered by a medium pressure pump. Early work was performed under gravity and the major setbacks for this method include the manual operation which involves long hours of attention, large volumes of solvents are wasted, and the run times are typically very long (Marston, 2007; Stalikas, 2007). Furthermore, another procedure, especially TLC, is needed to process the results. The advantage of the technique is that no expensive equipment is required and large samples can be loaded onto the column at a time. As in TLC, separation can be liquid/solid (adsorption) or liquid/liquid (partition).

As the solvent migrates down the column, separation of components takes place using the properties of the sample, solvent and stationary phase elutes the sample through the column, allowing the components to separate. Non-polar mobile and polar stationary phases are normally employed to effect separation although the polarity of the mobile phases can be greatly varied. Most often separations are not completed in one run. The column chromatography/TLC cycle has to be repeated several times until satisfactory results are obtained.

Solid phase extraction is a method modified from column chromatography. In this method, cartridges are used, normally containing specific stationary phases, such as, C18 and a vacuum is used to control the flow of the eluent through the cartridge.

2.5.1.3 High Performance Liquid Chromatography (HPLC)

HPLC was developed as an improvement to the classical column chromatography. The improvements led to high column efficiency, achieved through the reduction of the particle size of the stationary phase. This consequently led to high pressures in the system as the smaller particles

and liquids needed more pressure to pass through the system (Marston, 2007). Glass could not withstand the pressure, generated by the pump and metal columns were developed. This new method was then named HPLC. Initially the P stood for pressure and was replaced by performance. The mobile phase, and when one eluent (one pump) is used, the method is called isocratic and when the eluent is changed (through multiple pumps) with time changing during analysis, then the term gradient elution is used. Dissolved gasses must be removed as they can lead to irreproducible flow rates, cause band spreading or interfere with the performance of the detector. Dust and small particles must be removed from the mobile phase by filtration. Filtration through a millipore filter under vacuum can be done before introduction of the mobile phase into the system. A pressure of up to 6000 psi can be reached.

Stainless steel columns are usually used, with supports prepared from silica, or silica based compositions such as hydrolysed silica with chemically reactive silanol groups. Heavy walled glass tubing and polymer tubing can also be used to withstand the high pressures generated by the pump. Most columns range from 5 to 25 cm long with an internal diameter of 3 to 5 mm.

In HPLC, control of column temperature may be needed, and more reproducible chromatograms are usually obtained by maintaining the temperature.

Methods tend to be complex in liquid chromatography because the sample components interact with both the stationary phase and the mobile phase. Separation of the components of natural products has been done by column chromatography, which is tedious and takes a very long time to complete. Further complete separation has to be confirmed by other methods such as thin layer chromatography (TLC). Most often, separations are not completed in one run. The column chromatography/TLC cycle has to be repeated several times until satisfactory results are obtained. Recent advances in HPLC have seen the development of fraction collectors which can be attached to an HPLC system that can collect the separated components which may or may not possess the same polarity.

The use of HPLC in the analysis of TM is made more attractive by the fact that naturally the plant extract occurs as a combination of many compounds and chromatographic techniques were designed to tackle such problems.

HPLC is divided into normal and reversed-phase chromatography (Table 1). Normal phase refers to chromatography where the stationary phase is polar and the mobile phase in non-polar. This

was the basis of the early work done by Tswett, although it is much less widely used than reverse phase. In reverse phase, the stationary phase is non-polar and the mobile is polar. It is estimated that most HPLC work (about 75%), is based on reversed phase (Boligon and Athayde, 2014; Banu and Cathrine, 2015).

Table 1 : Comparison between normal and reverse phase chromatography

	Normal Phase	**Reversed Phase**
Stationary phase	Polar stationary phase e.g. alumina, silica	Non polar e.g. C8, C18
Eluent	Non polar	Polar
Samples	Non-polar elute fast	Polar elute fast
Mode of separation	Adsorption	Partition

The HPLC method is much faster and the resultant chromatogram can be seen, clearly showing whether the components are separated or not. When the components are separated, then fraction collection can commence. HPLC has generated a lot of interest from researchers as a method of separation and purification of herbal medicines, as it can achieve analyse much faster than most method (Boligon and Athayde, 2014).

Detectors used in HPLC analysis include photodiode array, UV-Vis, refractive index, fluorescence, evaporative light scattering and conductivity detectors. The choice of detector is influenced by the type of analysis and nature of the analyte. Out of these detectors, the UV-Vis detector is the most commonly used (Sasidharan *et al.*, 2011). Besides it being sensitive, the majority of the compounds isolated from plants have some UV-Vis properties and therefore analysed using the UV-Vis detector (Cannell, 1998; Pauli *et al.*, 2012).

Recent advances in HPLC have seen the development of fraction collectors which can be attached to an HPLC system that can collect the separated components. Many HPLC methods have been developed for the analysis of natural products. There are various methods documented for the HPLC analysis of herbals ranging from simple to complex analysis. Some work done in India in the *Millingtonia hortensis* involved among other purification tests the isocratic HPLC with photo diode array detector (PDA) for purification of the methanolic extracts (Kumar, Talluri and Rajagopal, 2015). The HPLC analysis was simple as only one component was isolated. In another

one component study of herbal medicines, analysis and validation of HPLC methods for the analysis of cucurbitacin E in herbs was performed in India (Chanda *et al.*, 2019). The researchers reported that Cucurbitacin E possesses numerous biological effects including anti-inflammatory, antioxidant, cytostatic and anti-angieogenesis.

Hyphenated techniques, such as HPLC-MS have also been employed in some analytical procedures. These procedures offer an upper hand over conventional methods as they give more information, at a cost none the less. Stavrianidi and co-workers explored the saponins that are found in ginseng (Stavrianidi *et al.*, 2017). In their work they proved that for the saponins, the results obtained from the UV-Vis detection could be slightly misleading as it produced exaggerated values. Furthermore, the MS can be used as a qualitative analysis tool, thereby hitting the "double target" to achieve both quantitative and qualitative results.

HPLC was also used to investigate potential herbal anti-cancer agents that have even reached the clinical trial stage(Wu *et al.*, 2018). The Honokiol is reported to be have strong anti-tumour agent properties against lung, gastric and breast cancer. Other than developing and validating the method, impurities and degradation products were also successfully determined.

Several other documented HPLC research work used of natural products has been reported in literature(Zhang *et al.*, 2018; Xie *et al.*, 2019; Zhao *et al.*, 2019)

HPLC is one of the strongest chromatographic procedures used for the separation of natural products in a complex matrix, and it is accurate, precise not limited by sample preparation and reproducible (Aubin and Cleary, 2010).

TLC can be used as a preliminary technique before HPLC is employed for instance the polarity of each component can be checked by TLC when designing a gradient elution programme (Li *et al.*, 2011). TLC is more widely used in qualitative analysis; whereas HPLC is a better tool if quantitative analysis is needed. Furthermore TLC is more likely to miss co-products than HPLC (Tian Ye *et al.*, 2011)

One of the most useful techniques for the analysis of natural products is achieved when HPLC is coupled to a MS. This technique, known as HPLC-MS has the advantage of combining the "good" qualities of HPLC with those of MS. The HPLC is used to separate components of a sample and then the library of the MS is used to identify the separated components. Atmospheric pressure chemical ionization (APCI) has been used for the analysis of triacylglycerols, instead of normal

chemical ionisation (CI) or electron ionisation (EI) (Holčapek *et al.*, 2005), which has the advantage of being very simple.

In other studies isoflavones were rapidly separated on HPLC where in retention times have been reduced to as low as 5 minutes (Klejdus *et al.*, 2007). The method was also adapted for the analysis of pesticides and similar results were achieved. The reduction in retention times were achieved through the use of extremely high pressures.

2.5.1.4 Gas chromatography-Mass Spectrometry (GC-MS)

In this technique a Gas Chromatograph (GC) is coupled to a Mass spectrometer (MS) to give the hyphenated GC-MS. As in all chromatographic methods the GC separates components in a sample and the MS acts as the detector and at the same time helps to identify the separated components using the mass to charge ratio.

The mobile phase is a gas which is also called the carrier gas and must be chemically inert. Helium, argon, nitrogen and hydrogen are used as the carrier gas. The sample is introduced into a heated sample port located at the head of the column. The sample port is ordinarily set at about 50°C above the boiling point of the least volatile component of the component of the sample. Thus the sample is immediately volatized. Very low volumes of the samples are injected into the GC such as 1μℓ.

Two types of columns are used in GC; packed columns and open tubular columns (also called capillary). In the earlier work, GC analysis used packed columns only. Recently, packed columns have been replaced by the more efficient open tubular columns. Column temperature is very important and must be closely controlled for precise work. Thus the column is housed in the temperature controlled oven. The temperature depends on the boiling points of the sample and the degree of separation required. For samples with components that greatly vary in boiling point, temperature programming is employed. Here, column temperature is increased either continuously or in steps as the separation proceeds.

After separation, the sample is transferred to the MS where atomisation occurs after which conversion of the atoms to ions (usually singly charge positive ions) is done. The MS also separates the formed ions on the basis of mass-to-charge ratio, counting the number of ions of each type or measuring the ion current produced when the ion strikes the transducer. Since most of the ions are singly charged, m/z is usually the mass of the ion (m/z is the mass to charge ratio).

One advantage of this technique over conventional GC is that the MS scans the masses repetitively during a chromatographic experiment. Consequently, incompletely separated compounds can be analysed by MS.

2.6 Spectroscopic techniques

Spectroscopy refers to a group of techniques where electromagnetic radiation is applied on a sample and either the incident absorbed energy or the emitted radiation is measured. Depending on the type of interactions, there are several different types of spectroscopic methods available but only 3 will be discussed here. When light interacts with matter, excitation from the ground state to the excited state occurs. The absorbed light is measured and sometimes the emitted energy also gives useful information as relaxation occurs.

2.6.1 Infrared Spectroscopy (IR)

In IR, the molecules in samples are irradiated with infrared light and the absorbed light is measured. The results give information about the functional groups that are found in the molecules. The absorbed radiation is characteristic of the functional groups present in the molecules under assessment. Therefore, IR is typically used for qualitative analysis.

2.6.2 Ultraviolet -visible Spectroscopy (UV-Vis)

In UV-Vis Spectroscopy only radiation that is used is in the UV-Vis range of the electromagnetic spectrum, that is from about 200-900nm. This process results in the excitation from the ground to the excited energy levels. The UV-Vis can be used for both qualitative and quantitative analysis. In the research it was used for qualitative analysis only. The characteristic absorbed maximum wavelength (λ_{max}) is used for identification purposes whereas the amount of energy absorbed (Absorbance) is used for quantitative analysis.

2.6.3 Nuclear Magnetic Resonance Spectroscopy (NMR)

Atomic nuclei that are positively charged (nuclei with an odd number of protons), spin on an axis thereby generating some electromagnetic field. In this way they behave like small magnetic bars. Some of the nuclei that behave like this are ^{1}H and ^{13}C which makes them good targets for NMR tests. In the absence of an external magnetic field the H and C nuclei, like magnets can take any orientation (random) or direction of spin. However, if an external field is applied, the nuclei will

be aligned either against the direction of the external field or along the same direction with the applied filed. If the applied filed has a proper frequency, the nuclei will absorb the energy and be able to resonate and align against the applied field. The nuclei are then said to be in resonance with the field.

Practically, superconducting magnets are used to generate the field of up to 21T and lower fields of 4-7T can also be used depending on the need. NMR is a strong tool for the structure elucidation of organic samples and many different experiments are carried out to come up with the final structure.

NMR is one tool that has been widely used in the study of natural products (Halabalaki *et al.*, 2014). The identification and structure elucidation have been helped by using a combination of NMR experiments such as, 1D (^{1}H, ^{13}C and ^{15}N) and 2D (COSY, TOCSY, NOESY, HSQC, HMBC and HNMBC).

2.6.3.1 One dimensional Proton NMR (1D NMR)

Classical 1D NMR experiments involve the exposure of the sample under a magnetic field, to radiofrequency (RF) pulses and the outgoing response is recorded as a 1D spectrum with chemical shift values. The RF pulses interact with protons in the molecule and produce a frequency signal (chemical shift) and the area under the graph shows the number of protons present. The chemical and electronic environment data is deduced from the chemical shifts and the splitting pattern gives information about the neighbouring protons.

2.6.3.2 One dimensional Carbon NMR (^{13}C-NMR)

^{13}C-NMR experiments are similar to 1D NMR where signals from different carbons are obtained and analysed according to their chemical shifts. Unlike in the proton results, the peaks normally appear as singlets as the protons are decoupled. However the different carbons can be identified such that primary, secondary, tertiary and quaternary carbons can be distinguished.

2.6.3.3 Two dimensional NMR (2D)

2D experiments are more complicated and more time consuming than 1D experiments. In 2D experiments, there are changes in pulses, length of time, angles and mixing times. The pulses are delayed after the previous pulse.

2.6.3.3.1 2D ^{1}H-^{1}H COrrelated SpectroscopY (COSY)

This technique helps to identify which protons are coupled to each other through showing relationships between neighbouring protons through the coupling constants. Furthermore, this experiment gives an indication of how the atoms are connected within the molecule. This can be achieved by drawing a straight line that passes through each axis from any spot and reveals which protons are connected to each other and to which carbons.

2.6.3.3.2 2D Nuclear Overhauser Enhancement SpectroscopY (NOESY)

The technique is used for the determination of spatial proximity as it gives a correlation of nuclei through space in distance typically of less than 5Å. The homonuclear correlations are directed through dipolar couplings, which might be caused by chemical exchange interactions or the nuclear Overhauser effect. In this technique, the protons that are close to each other can again be identified by drawing the straight line from the dark spots to the axis.

2.6.3.3.3 Heteronuclear Multiple Quantum Correlation (HMQC)

This technique is similar to the COSY experiment but unlike in COSY, the HMQC experiment provides correlation between protons and their attached hetero-nuclei through the heteronuclear scalar coupling. COSY only deals with the relationships between protons whereas HMQC seeks to elaborate the relationship between protons and carbons. Proton signals that are not coupled to and carbon atoms or any other hetero-nuclei are removed from the output signal.

2.6.3.3.4 Heteronuclear Multiple Bond Correlation (HMBC)

The HMBC experiment is similar to the HMQC experiment however, it has the advantage of being able to detect long range coupling between protons and carbons which are two or three bonds away with great sensitivity.

It is essential to determine the structure of chemical compounds isolated from natural products as the structure directly affects the biological activity of the actives (Pauli, Jaki and Lankin, 2007; Pauli *et al.*, 2014). The chemical shift and coupling constants values need to be accurate to a high degree in order for the proper elucidation information to be deciphered. The spectra are normalised using internal standards such as, tetramethylsilane or trimethylsilyl propionic acid (TMS).

2.7 Antimicrobial Activity

Antimicrobial activity testing is an important stage in natural product research. Biological activity or pharmacological activity refers to the ability to effect a change, constructive or detrimental, which any material of interest has on living things (Jackson *et al.*, 2007). In this research, we are particularly interested in the detrimental effects of *K. wilmsii* on pathogenic bacteria. The activity is strongly related to the amount of material used.

Bacteria, fungi and viruses have been identified as the most common causes of most diseases in humans and animals. Scientists have undertaken different bioassays which include but are not limited to antimicrobial, antiviral and enzyme inhibition tests, to assess the effectiveness of plant extracts (Mulaudzi *et al.*, 2012). If the antimicrobial activity assays show high inhibition potential, the hypothesis that plants are a potential source of new forms of medicinal products holds.

Natural products have been shown to exhibit microbiological activity against several microorganisms (Mann and Markham, 1998). In the Limpopo Province of South Africa , several herbal/traditional medicines tested showed remarkable antimicrobial activity towards *E. coli*, *E. faecalis* and *P. aeuginosa* (Matotoka and Masoko, 2018). The same researchers also recorded some mild anti-diarrhoeal activity from the plants. In another study conducted with plants from Nelspruit, Newtonia (Fabaceae), acetone and the dicholoromethane/methanol extracts showed the highest activities against *B.cereus*, and *P. aeuroginosa* (Motlhatlego *et al.*, 2018).

The antimicrobial properties of plants used by VhaVenda people for the preparation of concoctions for the traditional treatment of venereal diseases were investigated and susceptibility was recorded against *C. albicans*, *E. coli*, and *S. aureus* (Mulaudzi *et al.*, 2011).

Results of many other studies that were conducted in the rest of the world, show that a variety of plant extracts exhibit different biological activity. Plant extracts have been reported to have anti-diabetic (Chadwick *et al.*, 2007), antibacterial (Kumar, Talluri and Rajagopal, 2015) properties. The importance of plant extracts on the activity against several microorganisms is also highlighted by several authors such as effectiveness against erythromycin resistant bacterial strains (Kreander, Vuorela and Tammela, 2005), anti-cyanobacterial (Lürling and Beekman, 2010; Yang, Kwon and Kim, 2015), or antioxidant (Fernandes, Amador and Prudêncio, 2013).

2.7.1 Minimum Inhibitory Concentration (MIC)

In biological activity studies, the effectiveness of a molecule is given in terms of the minimum inhibitory concentration (MIC), which is the lowest concentration of that particular molecule capable of stopping the growth of a particular organism (Mann and Markham, 1998). This procedure is performed through serial dilutions until the lowest effective concentration is found. This measurement is very important as it is loosely considered to be a standard for the determination of susceptibility of organisms to antimicrobials. The MIC is further used to confirm border line cases of microbial activity, as the lowest concentration that can stop the growth of microorganisms is determined. The determination of MIC is performed through two major methods, diffusion or the dilution methods. In the diffusion method, agar is prepared and the surface of the microbe to be used in the test is spread uniformly over the agar. The substance under test is inoculated onto a hydrophilic round disk placed onto the surface of agar and it diffuses radially across the microbe infested agar. The resultant visibility on non-appearance of zones of inhibition inform scientists about the potential antimicrobial activity of the substance under investigation.

Dilution methods use the agar disks or the micro-titre plates where the different concentrations of the substance under investigation are added to the broth or agar where different microorganisms have been placed. The test is performed in a similar way to the agar diffusion method where all the plates and tubes are inoculated with microorganisms before-hand. The detection can be done by visual methods or using turbidity.

Both the broth and agar dilution methods have been employed for the determination of MICs. These methods have been adapted by several researchers (Jacobus Nicolaas Eloff, 1998; European Committee for Antimicrobial Susceptibility Testing (EUCAST), 2003; Giti, Mehdi and Nasser, 2005; Chorianopoulos *et al.*, 2006; Wiegand, Hilpert and Hancock, 2008).

MIC determinations are performed taking into consideration several standards such as the USA's Clinical Laboratory and standards Institute (CLSI), British Society for Antimicrobial Chemotherapy (BSAC) and EUCAST.

The MIC is closely related to the minimum bactericidal concentration (MBC), which is the minimum concentration that causes bacterial death. For the most desirable results the MIC and the MBC should be very close to each other (Owuama, 2017). These two quantities are important in

the application of administering of antibiotics to humans and animals as it helps to determine the smallest effective antibiotic concentration (Cira *et al.*, 2012; Owuama, 2017). This is particularly true for immunity compromised patients who can easily develop drug resistance due to the intake of ineffective drugs. Consequently, the MIC values can be used to make decisions on treatment of diseases (European Committee for Antimicrobial Susceptibility Testing (EUCAST), 2003).

By definition antibiotics are medicinal preparations that are used to slow down or completely destroy the growth of bacteria, hence the name anti-bacterials (Kocsis and Szabó, 2013). Antibiotics are classified into two categories according to this criterion; bactericidal (kill bacteria) and bacteriostatic (prevent multiplication), which are directly related to MBC and MIC respectively. This bacteriostatic/bactericidal quality sometimes depends on the concentrations of the antibiotics with some substances exhibiting both properties at specific concentrations.

2.8 Heavy Metal and Proximate Analysis

2.8.1 Proximate analysis

Proximate analysis is also referred to as the Weende System of plant materials and was developed by Henneberg and Stohmann for the testing of some parameters such as, dry matter, crude fibre, crude fat, crude protein and ash for stock feeds (Horrocks and Vallentine, 1999). The Weende analysis has now been adapted for the analysis of nutritional plants for human consumption, including plants materials consumed by human beings for medicinal purposes (Abdu *et al.*, 2015). The significance of this test is that it gives the first indication of the quality and purity of the plant under consideration (Rao and Xiang, 2009). Furthermore, the *K. wilmsii* roots can be added to food as a supplement, as we proved that it has antioxidant properties. Research has shown that the consumption of foodstuffs rich in antioxidants can decrease the risks associated with heart diseases (López-Cervantes *et al.*, 2018)

The WHO has set out testing parameters and specifications for the quality control testing of herbal preparations, including but not limited to, moisture content, acid insoluble ash, total ash and phytochemicals (WHO, 1998). Save for the test for phytochemicals, the other tests fall under proximate analysis.

The importance of the proximate tests is described shortly. According to the WHO, the moisture content test, although very simple has a huge influence on the quality of herbal preparations

because a high moisture content enhances microbial growth, attract insects and fungal growth and general loss of quality (WHO, 1998).

Total ash is the amount of ash left over after roasting the sample at high temperature for some time until a constant weight is obtained. The resultant ash is divided into two categories, physiological (which comes from the plant tissue itself) and non-physiological (which originates from external environmental sources, such as soil, sand or rocks) ash (Rao and Xiang, 2009; Abdu *et al.*, 2015). They reported that this test is one of the tests first cited in the Chinese Pharmacopoeia for herbal medicines in 2005 as well at the WHO's Quality Control Methods for Herbal Materials 1998.

The metal analysis coupled with the proximate analysis, are therefore used as a guide to the quality and safety of the herbal medicines.

2.8.2 Heavy metals

There are many different types of pollutants that have been dumped in the environment mainly due to human activities, industrial activities being the biggest contributor (Guveni and Akinci, 2011). These activities include mining, waste disposal, release of wastewater and burning of fuels. However, natural activities, such as rock weathering, leaching and volcanic activities can also lead to environmental pollution. The subsequent pollutants produced may find their way into plants and ultimately humans. Inorganic materials do not degrade unlike some organics and therefore they might bioaccumulate in the environment. Thus, they might end up being taken up by plants like the tuberous roots of *K. wilmsii*, posing potential danger to life.

The presence of metals in plant materials, although not always desirable, is sometimes necessary for carrying out metabolic processes in plants. For instance, the presence of metals such as copper and zinc, in the correct concentration have been reported to assist in the proper functioning of a variety of processes in plants (Gardea-Torresdey *et al.*, 1996). However, the same researchers reported that the presence of the same metals at elevated concentrations can disturb the normal functioning of metabolic processes and may cause stunted growth of plants.

Once heavy metals are introduced into the water and soil, plants take them up and they eventually accumulate in plants. The extent of the uptake depends on the available metals as well as the concentrations. Through the food chain, the metals that accumulate in the plants end up accumulating in humans and other living organisms that consume the contaminated plants causing possible health complications later on in life (Bhargava, Saluja and Dholwani, 2013). To

exacerbate the situation, once they are introduced into the environment, the metals are not easily degradable and thus persist for prolonged periods, causing harm to life (Yabanli, Yozukmaz and Sel, 2014).

The roots of the *K. wilmsii* are eaten by the Bapedi people for medicinal purposes. The toxicity levels of these roots have not been determined before. Therefore, the metal content of the roots would be an important parameter in assessing the preliminary safety of the roots. Acute toxicity tests were also performed to corroborate the metal analysis tests.

To safeguard the health of plants, animals and human beings, it is important to monitor the movement and concentration of the metals in the environment. A few examples of the adverse effects on life include, the stunted growth of plants (Gardea-Torresdey *et al.*, 1996), damage of cells and disturbance of the ionic balance (Yadav, 2010) and limited photosynthesis DNA and enzymes (Eutrópio *et al.*, 2015).

At high concentrations, some heavy metals have been reported to have high toxicity whereas trace elements, such as Fe, Zn, Cd, which are essential for supporting life, have been reported to cause neurotoxicity at high concentrations (Olowu *et al.*, 2015). These researchers reported numerous other adverse effects of elevated metal concentrations in plants, such as, reducing the probability of seed germination, stunted plant growth, reduction of plant populations. The metals can be distributed to the other parts of the plants from the roots. This is of major interest in our study as the roots have been reported to harbour the highest concentrations of metals as compared to the rest of the plant parts. Therefore, there is a need to address the concerns over the assimilation and bioaccumulation on the metals in the roots of the *K. wilmsii* tubers.

2.9 Toxicology Studies

Toxicology studies are carried out to determine the adverse effects of substances consumed by humans or other animals so that the safest way to use the chemicals may be adopted (Worth, 2018). Although the best test target for humans would be humans, as the extrapolation from other animals might not be necessarily accurate due to the differences in biochemistry, anatomy and physiology, other animals are tested first (Erhirhie, Ihekwereme and Ilodigwe, 2018).

The testing on animals commenced in the early years of the twentieth century, proposed by John William Trevan for the calculation of the minimum lethal dose (Gaddum, 1957). In their work,

Trevan and co-workers set up experiments where they fed different doses to animals to determine the lethal doses. Trevan explained his results in terms of LD_{50} and LD_{90}, which represent the dose killing 50% and 90% of the tested animals. Consequently, the use of LD_{50} was agreed as the standard (Gaddum, 1957; Erhirhie, Ihekwereme and Ilodigwe, 2018). The LD_{50} test is normally the first test to be conducted, used to estimate/predict the effects of the substance under investigation (Erhirhie, Ihekwereme and Ilodigwe, 2018).

Since then toxicology tests are performed by pharmaceutical companies to determine possible adverse effects of new drugs, we decided to perform the tests on *K. wilmsii* as a potential precursor (Robinson *et al.*, 2008). The determination of the baseline level, the no observed adverse effect level (NOAEL) dose, is an important parameter even in clinical trials to determine if a new drug is safe and effective (Parasuraman, 2011).

Toxicological studies have evolved since early years of Paracelsus in the early 1500s, who believed that what determines whether a substance is a poison or not is the dose (Borzelleca, 2000). He believed that above a certain dose any chemical can be a poison. This test has evolved and has been improved over the years, from the classical LD_{50} test where 100 animals where used. Due to the lobbying and protests animal rights groups, the target number of test animals has gradually gone down to outright zero in some tests (Wilson, Ahearne and Hopkinson, 2015). For extrapolation to humans, rodents, rabbits, dogs and primates have been used, which has now been expanded to worms, birds, bees and fish (Worth, 2018).

The test has gone through the phases; classical LD_{50} in the 1920s, the Karbal method in the 1930s to the Miller and Tainer method in the 1940s (Worth, 2018). In place of the traditional *in vivo* tests, new technologies such as *in vitro* and *in silico* tests have emerged (Costin, 2017; Balls, Combes and Worth, 2019). The 3Rs concept, to reduce, refine and replace was embraced by the environmentalists. Reduce, is the reduction of the number of tested animals without reducing the validity of the test results. Refine, refers to the provision of a much comfort to the test animal, such as reducing pain, distress and providing a good environment for the animals. The use of other methods such as *in vitro* and *in silico* are covered under replace. The use of animals is avoided totally.

The importance of such studies was emphasized by an unfortunate event in the early 1960s where over 10 000 babies were born with defects due to thalidomide (Wild, 2012). This was blamed on

the drug Contergan (Thalidomide), manufactured by the German pharmaceutical company Chemie Grunenthal. The drug, which was sold without the need for clinical trials, which were not a requirement at the time, was prescribed to pregnant women. Doctors started noticing a disturbing trend of birth deformities in children born to mothers who had taken the drug. Its distribution was subsequently stopped in 1961.

Toxicity experiments are divided into three different categories, acute toxicity, subacute and chronic toxicity studies(Parasuraman, 2011). Acute toxicity studies are meant to observe any adverse effects after a short period of exposure either after multiple doses or single dose with observation time of up to two months. Subacute studies involve the observation of the adverse effects after repeated daily doses are administered for a period not more than 10% of the life expectancy of the animal under investigation. Chronic tests are the long-term tests, typically, more than 3 months. Other authors categorised the toxicity tests into acute, allergenic, repeat dose toxicity, genotoxicity and mutagenicity (Worth, 2018).

Some of the reported toxicity test work include the tests on extracts of *Alpinia conchigera Griff* on rats where the LD_{50} was higher than 6.6mg/kg and the NOAEL was 2.22mg/kg with no significant adverse effects (Abdalla *et al.*, 2018). Porwal and co-workers also proved that the ethanolic extracts of *Marsdenia tenacissima* leaves were also not toxic to rats (Porwal, Khan and Maheshwari, 2017). These results may suggest that the phytochemicals under investigation may be safe for human use. Similar results were also obtained with the ethanolic leaf extracts of Punica granatum (Bhandary *et al.*, 2013). However, the ethanolic leaf extracts of the *Combretum Molle,* showed moderate toxicity on rats (Yeo *et al.*, 2012).

CHAPTER THREE: METHODOLOGY

3.1 Reagents and consumables

The ultrapure water was obtained from the Milli-Q Gradient Millipore deioniser that produced ultra-pure water with an electrical conductivity of 0.054µS.

For the chromatographic analysis, the solvents were Omni Solv HPLC grade and were purchased from Sigma-Aldrich. All other chemicals were analytical grade.

3.2 Sample Preparation

Kirkia wilmsii's common names are wild pepper or Mountain Seringa (English) and Legaba or Modumela (Northern Sotho). The tubers of the *Kirkia wilmsii* plant were collected from the Lebowakgomo region in Polokwane district, which is situated in the Limpopo province of South Africa. The tubers were collected between November 2013 and August 2019. The plant name was identified by the Department of Botany at the University of Venda and the name was further confirmed by the National Herbarium (Voucher number MPT00112) in Pretoria, South Africa. The plant name has been checked with www.theplantlist.org and has been reported as an accepted name (record 29400130), website accessed 28 November 2015.

In this study samples were collected from the *K. wilmsii* trees located in the Lebowakgomo mountains, located in the south east side of Polokwane. Most of the samples were dug from the tuberous roots of the trees around the coordinates; -24.234285, 29.501212.

A collection of some of the tubers is shown in the picture in Figure 4 below;

Figure 4: Picture of fresh *K. wilmsii* tubers

The bark of the tubers was removed, and the soft tubers were cut into small pieces and air dried in the laboratory. In this research, we decided to use air drying under room conditions as we felt the

other methods like oven and microwave drying might damage the unknown secondary metabolites in *K. wilmsii*. Freeze drying was chosen as the method of choice for *K. wilmsii.*

3.3 Preparation of extracts for phytochemical screening

The tubers were cut into small pieces and air dried in the laboratory, after which a Retsch Mill was used to grind the samples into a fine powder.

50g of raw extract was added to a conical flask and the prescribed extraction solvent was added to make a total volume of 100ml. The contents were covered with a parafilm to prevent solvent evaporation. The contents of the flask were placed in an ultrasonic bath and the bath was run for one hour. After one hour, the extract was filtered, the residue was returned to the conical flask and more solvent added to effect further extraction

3.3.1 Phytochemical Screening Tests

Phytochemical screening tests were performed according to the methods outlined by several authors.

Ferric chloride test(Khanam, Wen and Bhat, 2015)

50mg of each extract was dissolved in 5ml deionized water and 3 drops of 5% ferric chloride solution were added. The presence of a bluish black colour indicates the presence of phenolic compounds.

Alkaline reagent test (Khanam, Wen and Bhat, 2015)

Each extract was dissolved in water and a few drops of sodium hydroxide (0.1M) which gave an intense yellow colour. When 0.1M hydrochloric acid was added, the disappearance of the yellow colour showed the presence of flavonoids.

Lead acetate test (Bhandary *et al.*, 2012)

To a solution of each extract, a few drops of 10% lead acetate solution resulting in the formation of a yellow solution signifies the presence of flavonoids.

Salkowski's test (Khanam, Wen and Bhat, 2015)

About 50mg of each extract was dissolved in chloroform followed by concentrated sulphuric acid. A reddish brown colour at the interface indicates the presence of terpenoids.

Froth test (Khanam, Wen and Bhat, 2015)

Each extract was dissolved in 20ml water. The solution was shaken for 15 minutes in a graduated measuring cylinder. The development of 2cm layer of foam indicates the presents of saponins

Keller-Kiliani's test (Khanam, Wen and Bhat, 2015)

2ml glacial acetic acid was added to 50mg of each extract and one drop of 5% ferric chloride followed by 1ml of concentrated sulphuric acid. A brown ring at the interfaces is characteristic of cardenolide deoxy sugar. A violet ring below the brown ring and greenish ring in acetic acid layer indicates the presence of cardiac glycosides.

Tannins (John *et al.*, 2015)

50mg extracts were dissolved in 2ml water. 2 drops of 5% ferric chloride solution were added and the appearance of blue black colour shows the presence of tannins.

Phlobatannins (John *et al.*, 2015)

50mg was dissolved in 1ml water, 1ml of 1% hydrochloric acid was added. The mixture was boiled and production of a red precipitate indicates the presence of phlobatannins.

Anthraquinone glycoside (Borntrager'ss test) (Iqbal, Salim and Lim, 2015)

To 1ml of each extract, 1ml of 5% sulphuric acid was added. The mixture was boiled on a water bath and filtered. The filtrate was shaken with an equal volume of chloroform and allowed to stand for at least 5 minutes. The chloroform layer was shaken with half of its volume of dilute ammonia solution. Anthraquinone glycosides are present if a rose pink to red colour appears in the ammonia layer.

Liebermann-Burchardt test for steroids and terpenoids (Kumar *et al.*, 2007)

To 1ml of methanolic, 1ml chloroform was added and 3ml acetic anhydride followed by 2ml of concentrated sulphuric acid. Formation of a dark green colour indicates the presence of steroids whereas a dark pink or red colour indicates the presence of terpenoids.

Reducing sugars (Singh and Bag, 2013)

To 50mg of the extract, 2 ml of water was added followed by 2ml each of Fehling's solution A and Fehling's solution B. The mixture was boiled on a water bath. A brick red precipitate indicates the presence of reducing sugars.

3.4 Solvent Extraction

The bark of the tuber was removed, the tuber was cut into small pieces and air dried. The dry tuber was then ground into a fine powder using the mill. The powdered samples were then extracted

using different solvents with the help of an ultrasonic bath. The solvents were chosen from the results of the phytochemical screening tests, where methanol, acetone and water yielded the bigger number group of compounds, that is, phenolics, flavonoids, cardenolides, tannins and reducing sugars (Chigayo *et al.*, 2016). Water was used because it the most commonly used solvent in traditional medicine preparations. The solvents used were methanol, ethanol, dichloromethane, chloroform, acetone, MCW, 80% methanol, 60% methanol, water, hexane, diethyl ether and ethyl acetate.

1 g of the powdered extracts was weighed into different volumetric flasks. 25 ml of the solvent was added into each flask for extraction, and for each solvent, 3 different time intervals were employed; 15miuntes, 30 minutes and 60 minutes using the ultrasonic assisted extraction technique. This extraction method was adapted from the method used in similar work where the extraction of 1:10 was used (Olivier *et al.*, 2017). In this work, the ratio was increased to increase the extraction capacity as there is no documented work on *K. wilmsii*. Extracts were prepared in triplicate by ultrasonic-assisted extraction in the following solvents: petroleum ether, chloroform, ethyl acetate, methanol and water. The extracts were freeze dried and stored in a freezer at -18°C.

3.4.1 Successive Solvent Extractions

For the six solvents that gave the highest yields, successive extractions were performed for ethanol, methanol, MCW, 80% methanol, 60% methanol and water. About 2.5g of powder was weighed into the six different 50ml volumetric flasks. 25ml of the different extractants were added into each flask and sonicated for 30minutes. After 30 minutes, the extracts were filtered and the residue was returned to the respective volumetric flasks, 25ml of each solvent added and sonication was performed for a further 30 minutes. The procedure was repeated one more time, such that each powder portion was subjected to three successive extractions.

3.5 Quantitative tests

3.5.1 Antioxidant Activity - DPPH test

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the different plant extracts were compared by adapting the procedure reported by two researchers (Sahu, Kar and Routtray,

2013; Iqbal, Salim and Lim, 2015). Each extract was dissolved in an appropriate volume of methanol to give final concentrations ranging from 10.00 - 250µg/ml. Standard ascorbic acid solutions were also prepared in methanol to give the same concentration range. 2ml of each solution was transferred to a test tube and mixed with 4ml of 0.3mM DPPH. A control solution was prepared by adding methanol (2ml) to 0.3mM DPPH (4ml). The experiment was run in triplicate. The solutions were mixed well and left in the dark for 30 minutes. After 30 minutes the solutions were analysed on a Thermo Scientific Genesys 10S UV-Vis spectrophotometer at 517nm. The percentage of antioxidant potential was calculated using the formula:

$$\% \text{ inhibition} = \frac{(A_c - A_s)}{A_c} \times 100$$

A_c = Absorbance of the control solution

A_s = Absorbance of the sample or standard

3.5.2 Total Phenolic Content

A modified method for the determination of total phenolic content was carried out using modification of the method cited by (Sahu and Saxena, 2013).

Gallic acid standard solutions were prepared in methanol to give the following final concentrations; 20, 40, 60, 80, and 100µg/ml. Each plant extract was dissolved in methanol to give a final concentration of 1mg/ml and 0.5ml of each sample and standards were introduced into different test tubes and mixed with 2.5ml of a 10-fold dilute Folin-Ciocalteu reagent and 2ml of 7.5% sodium carbonate were added. The test tubes were covered with parafilm and allowed to stand for 30 min at room temperature before the absorbance was read at 760nm. The results of the total phenolics were expressed as gallic acid equivalents (GAE), (mg of gallic acid/g of sample) and calculated by the formula (Singh and Bag, 2013) :

$$TPC = (C \times V) / M$$

TPC = total phenolic content (mg/g plant extract in GAE),

C = concentration of gallic acid established from the calibration curve (µg/ml),

V= volume of the extract (ml),

M= mass of the extract of the plant (g).

3.5.3 Total Flavonoid Content

Total flavonoid content was estimated using standard methods with minor modifications (Kapewangolo, Hussein and Meyer, 2013; Sahu and Saxena, 2013) using quercetin as a standard. The standard solutions with the following final concentrations were prepared; 50, 100, 150, 200 and 250µg/ml. 1ml of each standard solution and extract solutions was taken into 10ml volumetric flask, containing 4ml of deionised water. 0.3ml 10% $AlCl_3$ was added to the mixture. At the 6^{th} minute, 2ml NaOH (1M) was added and volume made up to 10ml with distilled water. The absorbance was read at 510nm using Beckman Coulter DU 650i UV-Vis spectrophotometer. The results of the total flavonoids were expressed as quercetin equivalents (QE).

3.6 Antimicrobial Activity

The extracts were tested for biological activity before and after the separation of their components. The extracts that gave positive results in the biological activity tests were further used in the study. The extracts that tested positive in the biological activity test were purified by column chromatography coupled with HPLC and further taken for structural elucidation tests, that is IR, GC-MS and NMR tests.

Antimicrobial activity tests were carried on the raw extract, different solvent extracts and finally the separated/purified components of the extracts.

The following part of the research was carried out in the Microbiology Laboratory, at the Namibia University of Science and Technology (NUST), in Windhoek, Namibia.

After extraction, all plant extracts were tested for their antimicrobial activity, using the agar disc diffusion method (Kirby Bauer method). All the active extracts were further tested for their MICs.

3.6.1 Agar disc diffusion method

The standard agar disc diffusion (Kirby – Bauer) method (Sasidharan *et al.*, 2012) was adopted for this research. The following microorganisms were obtained from the Microbiology Department at the Namibia University of Science and Technology (NUST) and the School of Medicine, University of Namibia (UNAM);

1. *Escherichia coli* (*E. coli*), ATCC number 25922
2. *Staphylococcus aureus* (*S. Aureus*), ATCC number 25923
3. *Staphylococcus epidermidis* (*S. epidermidis*), ATCC number 14990

4. *Streptococcus pyogenes* (*S. pyogenes*), ATCC number 21853
5. *Staphylococcus saprophyticus* (*S. saprophyticus*), ATCC number 15305
6. *Klebisiella pneumoniae* (*K. pneumoniae*), ATCC number 13882
7. *Streptococcus agalactiae* (*S. agalactiae*), ATCC number 12386
8. *Salmonella typhimurium* (*S. typhimurium*), ATCC number 13311
9. *Candida albicans* (*C. albicans*), ATCC number 10231

The microorganisms were sub-cultured on nutrient agar, lot number 104068, sourced from Scharlau Chemicals. The agar was prepared by dissolving 28g of the agar powder in 1L de-ionised water, with the aid of heating on a hot plate. The heat helped with the dissolution process. When the powder was completed dissolved, the agar was sterilised in a Steridium micro-digital autoclave at 121°C and a pressure of 115kPa for 30 minutes, after which the autoclave door was opened, and the agar was allowed to cool down to about 45°C. The agar was poured into petri dishes and stored in a fridge for later use.

3.6.1.1 Sub-culturing and antimicrobial testing

The microorganisms were cultured on the agar plates by the streaking method and incubated at 37°C and 24 hours in an MRC LOM 150 orbital shaker incubator.

13g of nutrient broth, lot number 105031, sourced from Scharlau Chemicals was dissolved in water and diluted to 1000ml with ultrapure deionised water. The broth was autoclaved for 30 minutes at 121°C and when the sterilisation cycle was completed, the broth was left to cool down.

The broth (10 ml) was poured into different test tubes with screw caps. One colony from each organism was added to each test tube and incubated for 24 hours at 37°C. The cloudiness of each test tube indicated the growth of the organisms.

They were further diluted to an optical density (OD) equivalent to that of 0.5 McFarland. 5ul was applied to each petri dish containing nutrient agar and the organism spread evenly all around the plate using a spreader.

All extracts and amoxycillin were dissolved in water to give a final concentration of 1mg/ml. Amoxycillin was chosen as is a broad-spectrum antibiotic, arising from the group of penicillins, aminopenicillins which has enhanced activities against gram-negative bacteria (Watkins and Bonomo, 2017).

The dishes were divided into 4 quadrants. 20µlof each test solution was added to a paper disk and allowed to dry before insertion, one into each respective quadrant. The petri dishes were incubated at 37°C for 24 hours. The zones of inhibition were measured using a Vernier Calliper for each test extract. The results were recorded.

The extracts that gave positive results were used for further tests.

3.6.2 Determination of minimum inhibitory concentrations

The minimum inhibitory concentrations (MIC) were determined using the method detailed below which was adapted from Mwuama, 2017). The concentration of the extracts used was 1mg/ml.

Nutrient broth (180µl) was added to row A while 100µl of broth was added to the rest of the wells in the microplate using a multichannel micropipette. Each extract (20µl) was added to row A of the microplate (that is, column 1 for extract 1, column 2 extract 2, etc), until column 9. In column 10, 20µl of deionised water was added, 20µl of amoxycillin was added to column 11(positive control); and 20µl of the organism under test was added to column 12 (negative control).

The contents of row A were thoroughly mixed using a multichannel pipette. Eight serial dilutions were performed by withdrawing 100µl from row A and depositing them in all the wells of row B. This process was repeated up to row H.

Microorganisms (100µl) were added to all the microplate. One microorganism was tested in one plate. Different plates were prepared for testing each microorganism.

The microplates were covered with para-film and then incubated at 37°C for 24 hours in the shaking incubator.

After 24 hours, 40µl of p-iodonitrotetrazolium chloride (INT), 0.02mg/ml, was added to all the wells in all the microplates.

The results were observed visually. The wells which exhibited a purple pink colour showed that there was microbiological activity going on. The wells were the microorganism activity was inhibited, became colourless or showed pale colour.

3.7 Proximate and Metal Analysis

The proximate analysis experiments of *K. wilmsii* root powder were conducted according to the methods employed in recent research (WHO, 1998; Bhargava, Saluja and Dholwani, 2013; Zou *et al.*, 2017; Kassegn, 2018) which were adapted from the Official Methods of Analysis of the Association of Official Analytical Chemists.

3.7.1 Moisture Content/Loss on drying

The moisture content was determined by taking a quantity of the dried ground sample and drying it at 60°C until constant weight in a Scientific Oven Series 2000 (López-Cervantes *et al.*, 2018).

3.7.2 Total Ash

Empty crucibles were ignited at 600°C for 30 minutes, cooled in a desiccator and weighed. Accurately weighed dried root samples were subjected to a temperature programme of gradually increasing to 350°C, 500°C and finally a temperature plateau of 600°C for one hour in a muffle furnace. The ashes were cooled down in a desiccator and weighed (Abdu *et al.*, 2015).

3.7.3 Acid-insoluble Ash

10 ml of 2M HCl was added to the ash obtained in the "Total Ash' step and boiled for 5 minutes. The resultant mixture was filtered on an ashless filter paper. The collected residue was ignited in the crucible at 450°C for one hour, cooled and re-weighed (Bhargava, Saluja and Dholwani, 2013).

3.7.4 Water-soluble Ash

25 ml of de-ionised water was added to the ash obtained in the "Total Ash' step and boiled for 5 minutes. The resultant mixture was filtered on an ashless filter paper. The collected residue was ignited in the crucible at 450°C for one hour, cooled and re-weighed (Bhargava, Saluja and Dholwani, 2013)

3.7.5 Metal/Elemental content

The ground powder was prepared for analysis on the Rigaku NEX CG1384 XRF elemental analyser. About 400 mg sample was compressed for 1 minute at 400 Pa pressure, after which the sample was run on the XRF instrument for elemental analysis at the Namibia University of Science and Technology.

3.8 Toxicology Studies

In this research the toxicology studies were carried out at the South African Medical Research Council at the Primate Unit and DELFT Animal Centre in Cape Town where the monkeys were housed and maintained. The care and maintenance of the animals was done according to the South African National Standard for the Care and Use of Animals for Scientific purposes (SANS 10386:2008). The study was approved by the Ethics Committee for Research on Animals of the South African Medical Research Council (Project number 09/13).

3.8.1 Nonhuman primates, environment and housing

Sixteen male captive-bred Vervet monkeys were selected for this study and were identified with numbers in ink tattoo. A maintenance diet consisting of 100g stiff maize porridge with micro- and macronutrient supplementation was fed throughout the study, and water was available *ad lib*. The individuals were maintained under identical housing conditions in single cages and had also regular access to exercise cages and environmental enrichment. Additionally, 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 12h.

3.8.2 Plant material

The plant materials were milled into powder form. The milled powder was sterilized by irradiation at 18kGY for not more than 24 hours by the company HEPRO, (Montague Gardens, Cape Town). The powders were tested before and after irradiation for bacterial and fungal contamination by Swift Silliker (Claremont, Cape Town) (Appendix H). The same batch of plant material was used for the entire study.

3.8.3 Formulations and administration of compounds

One experimental sample of *K. wilmsii* was administered as a powder mixed in a ball of 100g of food (pre-cooked maize meal). The animals received the food bolus once per day (7:00am). The food bolus was consumed voluntarily by animals and food intake was recorded daily. This is the standard method used at PUDAC.

3.8.4 Animal selection

Adult Vervet males (n=8) were randomly assigned into two groups of four individuals each. The first experimental group received the study test material at a dose that is 25 times higher than the recommended daily dose, once a day (Table 2). The second group served as a negative control and received maintenance diet throughout the study.

Table 2: Group allocation and dosages

Groups	Monkey numbers	Baseline weight	Feeding schedule
2.14 g/kg 25 x daily dose	374 292 397 387	4.55 5.90 5.56 5.57	Once/day
4: Control Maintenance diet	390 389 244 130	5.52 5.45 5.42 5.46	N/A

3.8.5 Duration of animal intervention

The overall duration of the study was 120 days (90 days treatment and 30 days washout) and includes five time points: Baseline, week 4, week 8, week 12 and week 16 (washout).

3.8.6 Blood collection

All animals were fasted overnight for clinical evaluation and blood sampling. Toxicity assessment included the standard biochemical, haemato-chemical, clinical and behavioural parameters. Blood samples (6 ml) were drawn from each monkey (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 all the groups. Blood was obtained via femoral venipuncture after Ketamine anaesthesia at 10mg/kg bodyweight. The blood volume

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.

3.8.7 Clinical biochemistry analysis

The clinical tests were performed at the accredited SAMRC laboratories in Cape Town using the Architect ci 4100 and a c702 high throughput clinical chemistry module.

Blood (2 ml) was collected every four weeks into SST, Sodium Fluoride/Potassium Oxalate and EDTA tubes to perform clinical biochemistry and haematology tests. The clinical chemistry included test for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatise (ALP), gamma-glutamyl transferase (GGT), total protein, bilirubin (total and direct), albumin, globulin, cholesterol (total, LDL-C, HDL-C), urea, creatinine, triglyceride, calcium (Ca), sodium (Na), magnesium (Mg), chloride (Chl), anion gap, phosphate (P), glucose, potassium (K), total bicarbonate (CO_2) and creatine kinase (CK). Complete blood count examination included 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), neutrophils, eosinophils, basophils, lymphocytes, monocytes and platelets count. Additional test included physiological variables such as weight, body temperature, respiratory, systolic and diastolic pressure, pulse and mean arterial pressure (MAP).

3.8.8 Observations

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

3.9 Purification of Extracts

50g of raw extract was added to a conical flask and the prescribed extraction solvent was added to make a total volume of 100ml. The contents were covered with parafilm to prevent solvent evaporation. The contents of the flask were placed in an ultrasonic bath and the bath was run for one hour. After one hour, the extract was filtered, the residue was returned to the conical flask and more solvent added to effect further extraction. This process was repeated one more time, the extracts were mixed and dried on the freezer drier. After extraction, all the extracts were tested for their antimicrobial activity, using the agar disc diffusion method (Kirby Bauer method). All the active extracts were further tested for their MICs.

3.9.1 Purification/ Column chromatography

The extracts that exhibited antimicrobial activity were then chosen for the purification process. The extracts were first dissolved in water, extraction was done with chloroform and finally with hexane. This constituted the first step in purification. After that a column was set up to separate the components of the extracts, and solvents of different polarities were added to the column to aid the separation process.

The solvent system was continuously changed from n-hexane, chloroform, ethyl acetate, methanol and water (Table 3). These solvents were added as combinations with the next solvent, for example moving from 100% n-hexane to 75% n-hexane and 25% chloroform, then 50% n-hexane and 50% chloroform. This sequence was continued until a 100% water solvent system was reached. The extracts were collected into different test tubes as shown in Figure 5.

Table 3: Preparation of the composition of different eluents used for purification.

	Solvent mixture (ml)				
	n-hexane	Chloroform	Ethyl acetate	Methanol	Water
1	100	0	0	0	0
2	75	25	0	0	0
3	50	50	0	0	0
4	25	75	0	0	0

5	0	100	0	0	0
6	0	75	25	0	0
7	0	50	50	0	0
8	0	25	75	0	0
9	0	0	100	0	0
10	0	0	75	25	0
11	0	0	50	50	0
12	0	0	25	75	0
13	0	0	0	100	0
14	0	0	0	75	25
15	0	0	0	50	50
16	0	0	0	25	75
17	0	0	0	0	100

3.9.2 50% Acetone Extracts

A sample of the 50% acetone extract (4.782g) was dissolved in water and chloroform was added for extraction. To the aqueous layer methanol was added to 80% methanol and extraction with hexane to give three final extracts, aqueous (3.94g), chloroform (0.031g) and hexane (0.016g).The aqueous extract was diluted to 60% methanol and further separation was performed using successive 25ml of the mobile phases with changing polarity of mobile phases as shown in the Table 3.

3.9.3 80% Methanol Extracts

A sample of the 80% methanol extract (3.97g) was dissolved in water and chloroform was added for extraction. To the aqueous layer methanol was added to 80% methanol and extraction with hexane to give three final extracts, aqueous (3.02g), chloroform (0.057g) and hexane (0.028g).The aqueous extract was diluted to 60% methanol and further separation was performed using successive 25ml of the mobile phases with changing polarity of mobile phases as shown in the Table 3.

3.9.4 Methanol Extracts

A sample of the 80% methanol extract (6.735g) was dissolved in water and chloroform was added for extraction. To the aqueous layer methanol was added to 80% methanol and extraction with hexane to give three final extracts, aqueous (6.332g), chloroform (0.122g) and hexane (0.098g).The aqueous extract was diluted to 60% methanol and further separation was performed using successive 25ml of the mobile phases with changing polarity of mobile phases as shown in the Table 3.

3.9.5 MCW Extracts

A sample of the 80% methanol extract (4.02g) was dissolved in water and chloroform was added for extraction. To the aqueous layer methanol was added to 80% methanol and extraction with hexane to give three final extracts, aqueous (3.56g), chloroform (0.038g) and hexane (0.128g).The aqueous extract was diluted to 60% methanol and further separation was performed using successive 25ml of the mobile phases with changing polarity of mobile phases as shown in the Table 3.

Thin layer chromatography (TLC) was run on all the contents of the test tubes and extracts that have similar retention factor (R_f) were mixed into separate containers, depending on their R_f values. Further, UV-Vis spectra of the purified extracts were run on a Thermo Scientific Genesys 10S UV-Vis spectrophotometer.

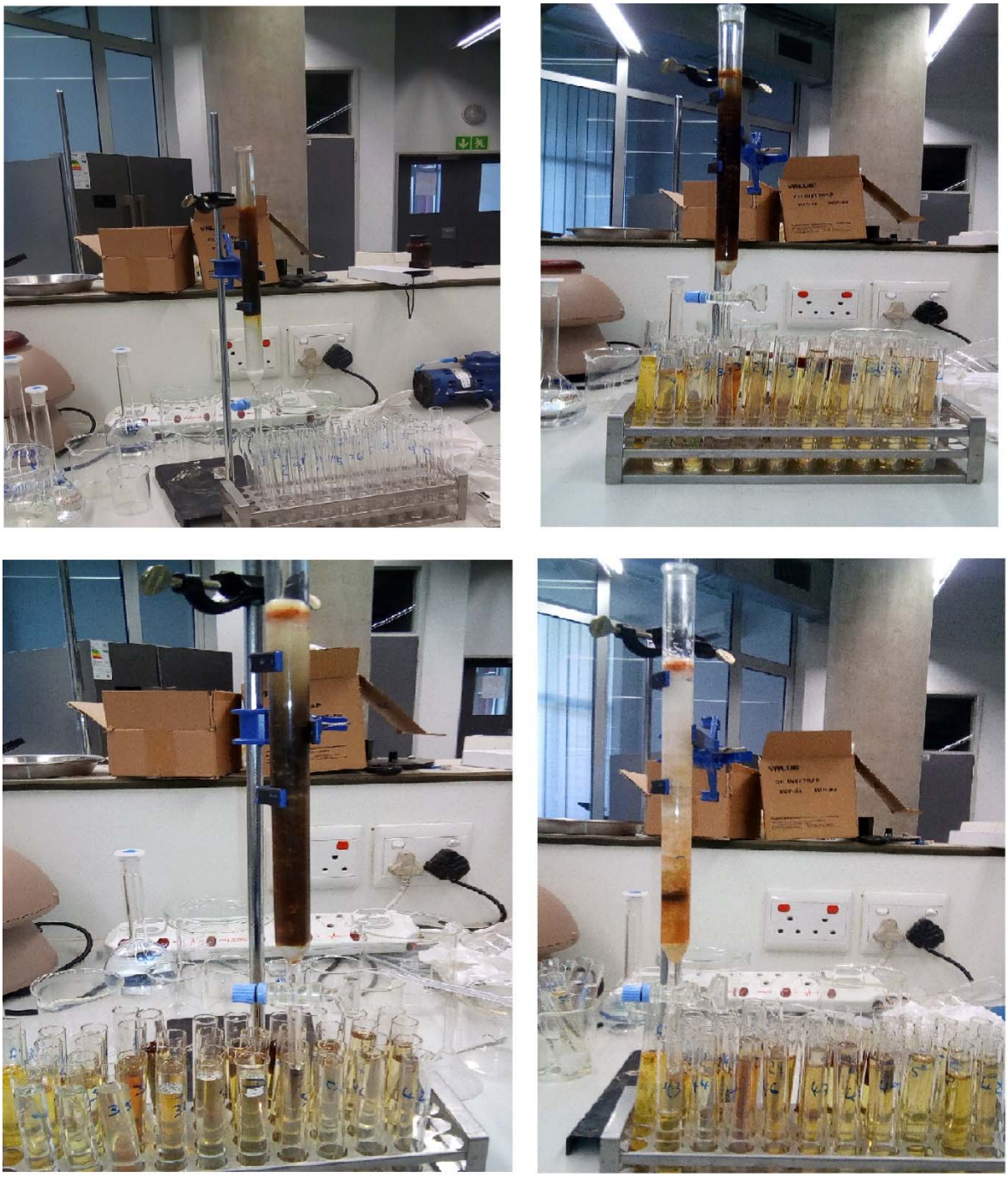

Figure 5: Column chromatography purification of extracts sequence

3.10 HPLC Method Development

A Waters HPLC 2535 with a quaternary Gradient Elution Module, Water 2707 Autosampler, Waters Fraction Collector III, Photo Diode Array (PDA) Detector and a Waters 2707 Autosampler was used for the separation of the components of the extracts.

The picture of the HPLC is shown in Figure 6:

Figure 6: Picture of the Waters HPLC used in this project

A procedure was developed in the analytical mode of the HPLC. In this mode normal analysis takes place. After optimisation, the procedure was then adapted to the HPLC preparative method which was then used to collect the different fractions of the plant extract.

The best method was found from following the optimisation of various methods that were developed. A gradient elution system was used. The method consists of two mobile phases: Mobile phase A; 0.5%v/v trifluoroacetic acid (TFA) in water whereas mobile phase B was 0.25%v/v TFA in acetonitrile.

The PDA was set to run from 200 to 800nm and the total run time was 60 minutes.

The first trial injection was done with an isocratic system with the eluent made up of 95% mobile phase A and 5% mobile phase B and run for 60 minutes at 0.5ml/min. The results were not good and gradient elution programmes were tried and the best of which was the one shown on in Table 4 below.

Table 4: The initial gradient programme used on the HPLC

Time(min)	Flow(ml/min)	PumpA (%)	Pump B (%)
Initial	2.0	95	5
20.00	2.0	5	95
28.00	2.0	5	95
28.10	2.0	95	5
40.00	2.0	95	5
55.00	2.0	95	5
60.00	2.0	95	5

The analytical column used for this stage was Waters BEH300, C18, 5µm, 4.6 x 2500mm.

The resolution was not so good thus the flow rate was reduced to 0.5ml/minute.

The gradient elution programme was then adapted from the analytical mode to the preparative mode using the prep calculator. The syringes, columns and sample vials are different in the analytical and preparative mode of the HPLC. In the preparative mode, all these accessories are much larger and hence take more volumes of samples and eluents. A prep calculator gives the optimum conditions that must be used when moving from the analytical to the preparative mode and vice versa.

The conditions obtained from the prep calculator were then used for the analysis. The following prep method was used and the gradient elution is shown in Table 5.

The mobile phases were not changed, that is, mobile phase A: 0.5%v/v TFA in water whereas mobile phase B was 0.25%v/v TFA in acetonitrile.

The chromatographic system consisted of Waters BEH300 19 x 150mm 5µm Prep C18 column, PDA detector set to scan from 200 to 400nm and the run time was 36 minutes;

Table 5: Improved gradient elution programme

Time(min)	Flow(ml/min)	PumpA (%)	Pump B (%)
Initial	8.53	95	5
12.00	8.53	5	95
16.80	8.53	5	95
16.86	8.53	95	5
24.00	8.53	95	5
26.40	8.53	95	5
36.00	8,53	95	5

The chromatograms that were obtained from this adapted method were not satisfactory as the resolution was poor and the method optimisation was undertaken again.

The method was then further optimised by reducing the flow rate and maintain the gradient programme the same which was reduced from 8.53 to 4ml/min. The chromatograms obtained showed a great improvement.

After minor modifications good chromatograms were finally obtained from the following gradient elution parameters and the subsequent fraction collection parameters. The final gradient elution systems are shown in Tables 6 and 7.

Table 6: Continuously improved gradient elution method

Time(min)	Flow(ml/min)	PumpA (%)	Pump B (%)
Initial	2	95	5
30.00	2	95	5
31.00	2	40	60
48.00	2	40	60
50.00	2	40	60
51.00	8	95	5
52.00	4	95	5
64.00	4	95	5
65.00	0.2	95	5

Mobile phases A and B remained the same, the PDA scanned from 200 to 400nm and the total run time was increased to 65 minutes.

There were some unresolved peaks at the end and the chromatograms showed that the run time had to be increased and hence the method was also further developed to the most satisfactory chromatograms that could be obtained. First, it was clear that the run time had to be increased as there were some peaks that would appear to be coming from the previous injection which were overlapping with next injection. Secondly, although the chromatogram was generally much improved, some of the peaks were not completely resolved. Using the conditions shown in Table 7 below the residual overlap was eliminated and the peak resolution was greatly improved as well. The method development was now finalised.

Table 7: Final optimised gradient elution method

Time(min)	Flow(ml/min)	PumpA (%)	Pump B (%)	Pump C (%)	Pump D (%)
Initial	2.0	95	5	0	0
48.00	2.0	70	30	0	0
50.00	2.0	5	95	0	0
54.00	2.0	5	95	0	0
55.00	2.0	95	5	0	0
70.00	2.0	95	5	0	0
71.00	2.0	5	95	0	0
75.00	4.0	5	95	0	0
88.00	4.0	5	95	0	0
89.00	2.0	5	95	0	0
90.00	4.0	0	0	50	50
110.00	4.0	0	0	50	50
110.01	2.0	95	5	0	0
129.00	2.0	95	5	0	0
130.00	0.2	95	5	0	0

3.11 Fourier – Transform Infrared Spectroscopy

FTIR tests were run on a Bruker Alpha FITR with no further sample preparation as the samples were run as the dried and ground powder. The background was run in air.

3.12 Gas chromatography – Mass Spectrometry (GC-MS)

The GC-MS method procedure was performed on a Perkin Elmer Clarus 680 GC (operating on the Turbomass software) coupled to a Clarus SQ 8 T MS shown in Figure 7 below. The GC column used was Perkin Elmer ELITE-5MS which has the dimensions, 30 m length, 0.25mm internal diameter and a temperature range of -60°C to 350°C.

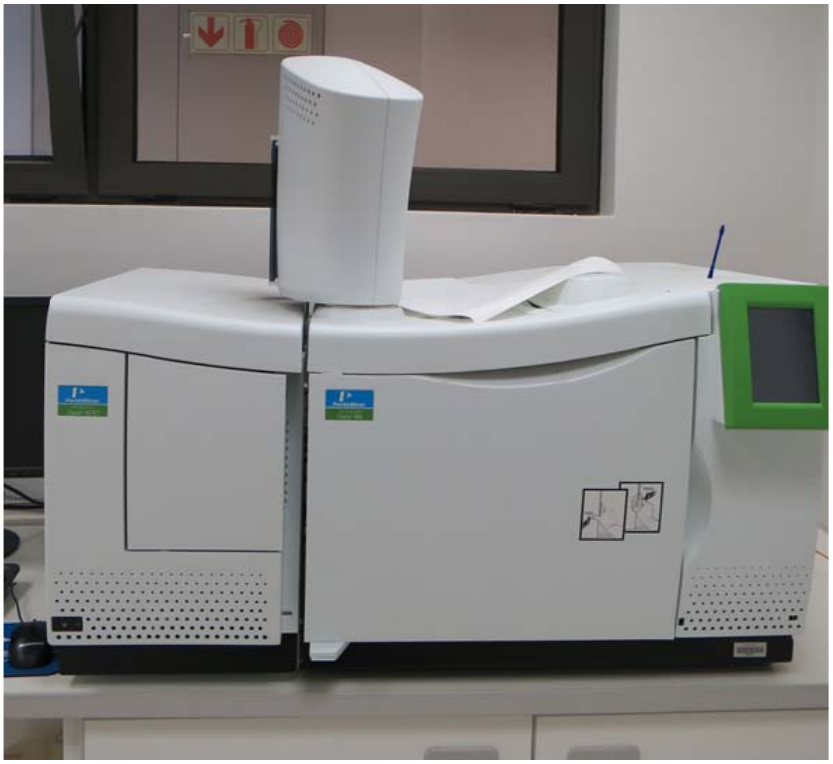

Figure 7: Perkin Elmer Clarus SQ8 GC-MS

The GC method was run according to table 8 below.

The samples were dissolved in methanol to give a concentration of 1mg/ml and the injection volume was 10µl and the eluent was helium gas.

Table 8: GC running conditions

Oven ramp	Rate(°/min)	Temperature(°C)	Hold(min)
Initial	10	110	4
1	10	150	4
2	10	200	4
3	10	300	10

The MS parameters used are given below:

Solvent delay: 3 minutes

Scan time: 32 minutes

Scan range: 30 to 450 Da

Run time (min): 36 mins

3.13 Nuclear Magnetic Resonance

^{1}H and ^{13}C NMR analysis was performed at 100MHz and 400Hz with a Bruker 400 Avance Spectrometer using deuterated methanol and TMS as an internal standard. All the samples were dissolved in dueterated methanol. The samples were all filtered through cotton and transferred into NMR tubes. The following experiments were carried out: 1D (1H, 13C,) and 2D (HMBC, HSQC, COSY) using the TopSpin 3.2 software and IconNmr automation arm.

3.14 Statistical Analysis

Means of triplicate analysis were calculated and data was expressed as mean ± std dev (standard deviation) where applicable. Post hoc ANOVA statistical analysis was performed using SPSS 24 software for comparison between two or more treatments. A difference was considered to be statistically significant when $p<0.05$.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Phytochemical Screening Results

The phytochemical screening tests of the tuber revealed the presence of phenolics, flavonoids, terpenoids, tannins, cardenolide deoxy sugars and reducing sugars. A summary of the results obtained from the phytochemical screening tests is shown in Table 9 below.

Table 9: Phytochemical test results

Test	**Extract**					
	Water	Methanol	Acetone	Ether	Ethyl acetate	Chloroform
Phenolics	+	+	+	-	+	-
Flavonoids (Alkaline test)	+	+	+	-	+	-
Flavonoids (Lead Acetate)	+	+	+	-	+	-
Terpenoids	-	-	-	-	-	+
Saponins	-	-	-	-	-	-
Cardiac glycosides	-	-	-	-	-	-
Cardenolide deoxy sugar	-	+	+	-	-	-
Tannins	+	+	+	-	-	-
Phlobatannins	-	-	-	-	-	-
Anthraquinone glycosides	-	-	-	-	-	-
Reducing sugars	+	+	+	-	-	-

The ether extracts were not active for all the tests carried out whereas the chloroform extracts were only active for the test for terpenoids. Generally, the polar solvents were found to be more active for a wider range of compounds. The methanol and acetone extracts show that they contain the highest number of compounds of interest, that is phenolics, flavonoids, cardenolide deoxy sugars, tannins and reducing sugars. The water extracts also were highly active save for terpenoids, saponins, cardiac glycosides, phlobatannins, anthraquinone glycosides and cardenolide deoxy sugars. These groups of compounds have been found to be important as they have been reported

as exhibiting healing properties, which include anti-inflammatory, antioxidant activity and anti-allergic properties (Pandey and Tripathi, 2014; Khanam, Wen and Bhat, 2015).

These results are similar to the results reported by other researchers, where methanol extracts tested positive for the highest number of different classes of phytochemicals in *Bauhinia variegata L.* bark (Parekh, Karathia and Chanda, 2006) as well as the tests on the extracts from *Anamirta cocculus* seeds (Qadir, Paul and Ganesh, 2015). In another study, the root and bark of *Eurycoma longifolia* also showed that the methanol, ethyl acetate and chloroform extracts were good sources of different classes of compounds (Khanam, Wen and Bhat, 2015).

Some researchers reported different results with regards to the methanol extracts. In one study, the methanol and ethanol extracts of *Strychnos minor dennst* leaves yielded low levels of flavonoid content (John *et al.*, 2015). However some extracts gave contrasting results with methanol, ethyl acetate and chloroform extracts testing positive for phenolics, flavonoids, terpenoids, alkaloids, proteins and cardiac glycosides in the study done on root and stem extracts of wild *Eurycoma longifolia Jack* (Khanam, Wen and Bhat, 2015). The phytochemical screening tests for the stem methanol extracts gave more positive results than the roots.

In our study ethyl acetate and chloroform extracts tested negative for most groups of compounds. The ether extracts proved negative for all the tests conducted. We therefore conclude that water, methanol and acetone or their combinations would give good extraction yields that can be used for the phytochemical screening of *K. wilmsii*.

4.2 Solvent Extraction

The results of the solvent extraction are given in Figure 8 below. Extractions were performed at 15, 30 and 60 minutes.

In this section, only the solvents which extracted high yields were used for the triplicate analysis. The highest extract yield (52.9%) was obtained from extraction with 80% methanol (Figure 9). This result was not significantly different from 48.2% to 46.9% extract yields for MCW and 60% methanol, respectively ($P > 0.05$). The results for ethanol (7.3%), water (20.9%) and methanol (36.6%) were significantly lower than the above-mentioned results. An increase in the extraction time also showed a significant increase in the % yield obtained ($P < 0.05$).

We conclude that 80% methanol is the best solvent to use for extraction as the extracts are more soluble in polar solvents and 80% methanol has more polar organic properties.

Since biologically active compounds occur naturally in very small concentrations, the choice of an extraction method and the corresponding suitable solvent is an important step in the drug discovery process.

Solvents with a wide range of polarity were used for the extraction. The results suggest that polar solvents gave better extraction yields (Ahmed *et al.*, 2014), which is true in our study. Therefore, the *K. wilmsii* tuber secondary metabolites can be extracted with polar protic solvents with high yields ranging from 7.3% to 52.9%. Non-polar solvents, such as hexane and ether produced very small amounts of extracts, less than 1%. This observation is also supported by results obtained from the extraction from *Paramignya trimera* root where the polar protic solvents, methanol and water gave the best extraction yields (Nguyen *et al.*, 2015).

Extraction with a MCW combined solvent also produced a significantly high yield (48.2%), consistent with other research work using the same solvent (J. N. Eloff, 1998). However, we reported a slightly higher extract yield of 48.2% as compared to 45% and 35% extraction yields for *Anthocleista grandiflora* and *Combretum erythrophyllum* leaves reported by Eloff (J. N. Eloff, 1998).

However, the MCW combination tended to separate into different phases as chloroform and water are immiscible. Therefore, addition of water to methanol proved that the extraction efficiency can be increased significantly as the extraction yields obtained with 80% methanol (52.9% yield) and 60% methanol (46.9% yield) were much higher than the yield obtained from the use of pure

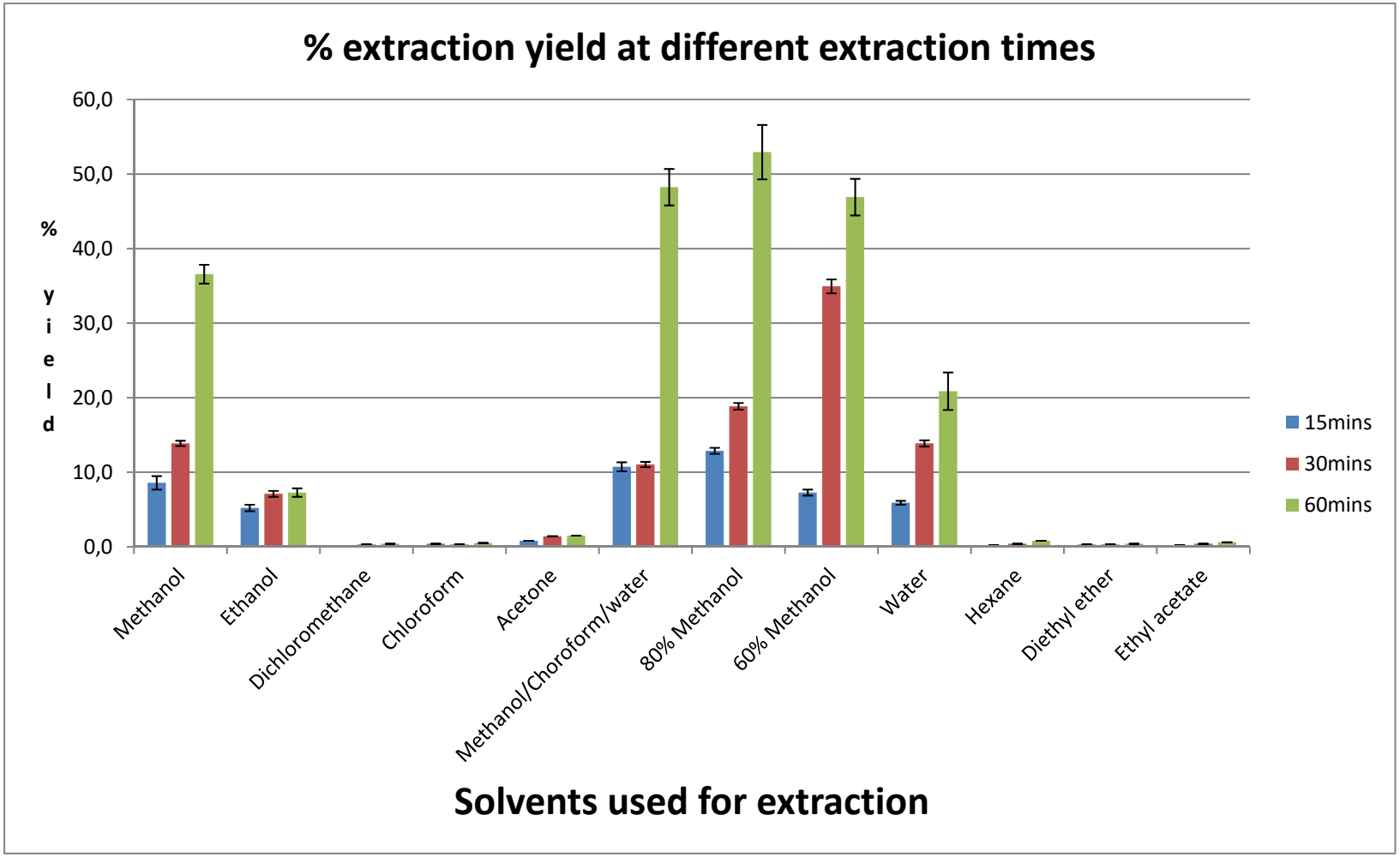

Figure 8: Graphical depiction of the solvent extraction results

methanol (36.6% yield). This observation is consistent with other reported results (Sultana, Anwar and Ashraf, 2009; Xie *et al.*, 2015), as the water tends to increase the polarity of the extractant.

The results show that the % yield significantly increased as a result of successive 30-minute extractions rather than one 60-minute extraction (Table 10).

Table 10: Successive solvent extraction yield

Solvent	% yield (1 x60min)	% yield (3 x30min)
Methanol	36.6	44.0
Ethanol	7.3	21.5
MCW	48.2	56.9
80% Methanol	52.9	75.8
60% Methanol	46.9	61.6
Water	20.9	66.1

4.3 Quantitative tests

4.3.1 Antioxidant Activity - DPPH test

The scavenging profiles of the extracts and ascorbic acid are shown in Figure 9, from which it can be observed that all the extracts possess radical scavenging potential. Methanol extracts proved to be the most active of the extracts while the water extracts were the least.

In our follow up study, where we investigated phytochemical screening, phenolic content and antioxidant activity, the plant proved the importance as a wide range of possible groups of interest were identified (Chigayo *et al.*, 2016).

The IC_{50} (Table 10) values of all our extracts are significantly different from each other ($P < 0.05$) except for MCW and 60% methanol extracts which are not significantly different (Table 11). Therefore, the IC_{50} value for the methanol extracts is significantly lower than all the other values. The best activity was recorded by the methanol extracts with an IC_{50} value of 129.94 mg/mL while the water extracts gave the lowest IC_{50} around 225.04 mg/ml.

Antioxidant agents with high scavenging activity should have a low IC_{50} value (Zhang *et al.*, 2015). This is supported by the lowest value being exhibited by ascorbic acid, a well-known antioxidant. The IC_{50} results obtained are significantly higher than that of ascorbic acid (56 mg/mL). This is to

be expected as crude extracts were used before purification. Results for purified extracts are expected to be much more closely related to those of ascorbic acid.

The methanol extracts IC_{50} of 130 mg/mL result is comparable to that obtained from 90% ethanol *Cyclocarya paliurus* extracts (146 mg/mL) (Zhang *et al.*, 2015). Furthermore, this observation closely relates to results obtained by Alkhawalisy and Hossain (Alkhawalidy and Hossain, 2015), where they reported that they recorded the highest antioxidant activity with methanol extracts. However, the concentration of the DDPH concentration used in our study is higher (0.3 mmol/L) as compared to 0.1 mmol/L used by Xie et al. (Xie *et al.*, 2015).

Other IC_{50} results from *Pistacia atlantica subsp. mutica* extracts yielded IC_{50} values ranging from 0.6 to 1105.3 mg/mL (Bhandary *et al.*, 2012), with protic polar solvents giving higher activity, which is consistent with our work. The major differences observed can be attributed to the very low concentration of DPPH used by Rezaie et al (Rezaie *et al.*, 2015).

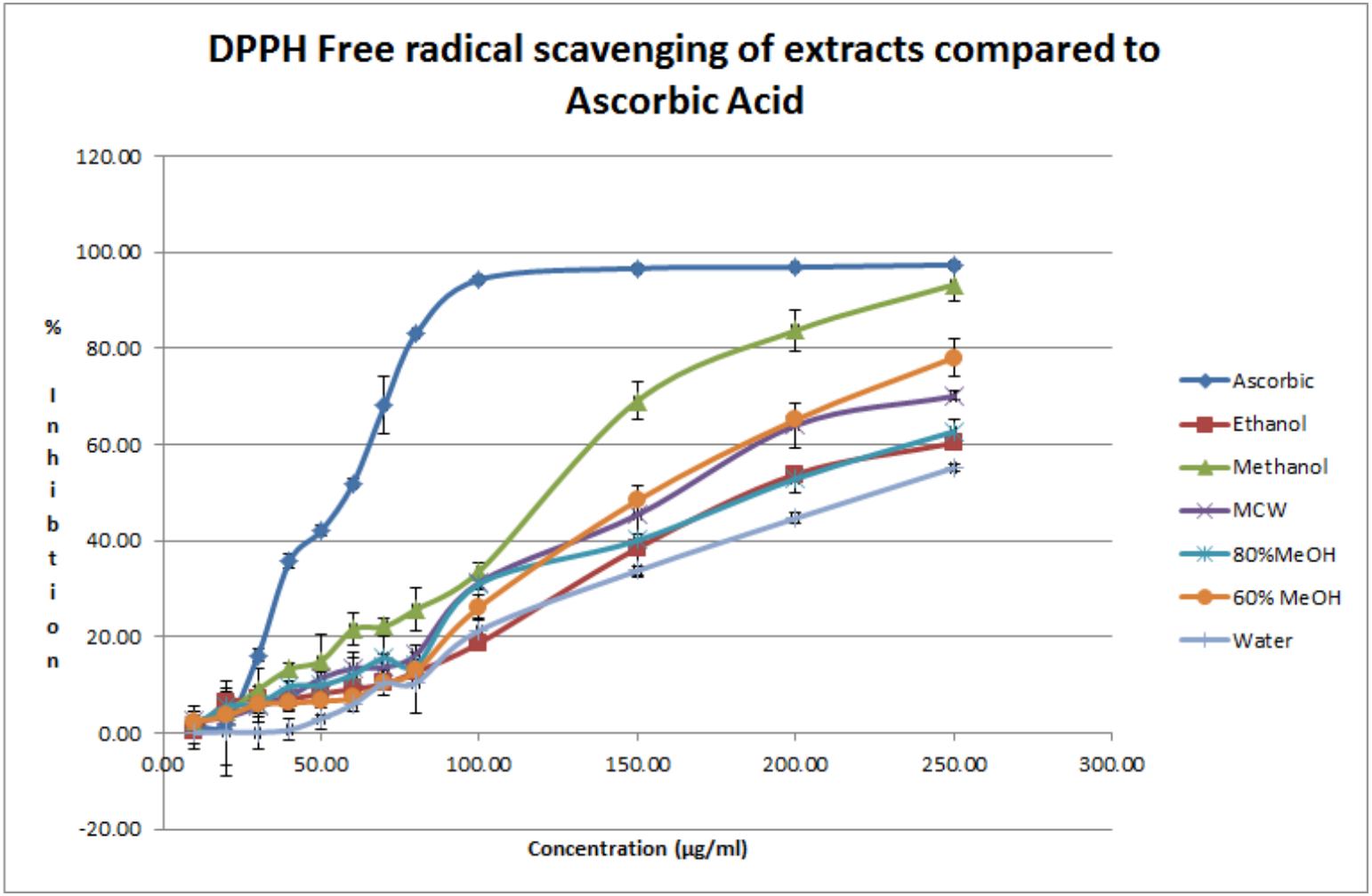

Figure 9: DPPH free radical scavenging of extracts compared to Ascorbic Acid

Table 11: IC_{50} values for the extracts as well as ascorbic acid.

Extract	IC_{50} (µg/g)
Ethanol	204.78 ± 1.51^{a}
MeOH	129.94 ± 0.20^{b}
MCW	172.44 ± 0.62^{c}
80% MeOH	194.62 ± 1.00^{d}
60% MeOH	167.27 ± 0.57^{c}
Water	225.04 ± 0.72^{e}
Ascorbic Acid	56.52 ± 0.07^{f}

All extracts with different superscripts are statistically different ($P < 0.05$) and extracts with the same superscript not statistically different. The results indicate that only the IC_{50} of the MCW and 60% methanol extracts are significantly the same while the rest of the values are significantly different form each other.

Our results are also comparable to IC_{50} values of 155 and 204 mg/mL from the leaves and bark respectively of the extracts of *G. velutinus* (Iqbal, Salim and Lim, 2015). An extract is considered to be active against free radicals if $IC_{50} < 5$ mg/mL is obtained (Abdillah *et al.*, 2015). All our extracts have IC_{50} values less than 5 mg/mL, therefore all the extracts for the solvents used are a possible good source of antioxidants. There is a positive correlation between the IC_{50}, total phenolic content and total flavonoid content ($r = 0.853$ for IC_{50} and total flavonoid content and $r = 0.899$ for IC_{50} and total phenolic content). Furthermore, there is also a very high correlation between total flavonoid content and total phenolic content ($r = 0.98$).

The phytochemical screening and solvent extraction analysis give a good guide of the phytochemicals present in the extracts as well as suitable extraction solvents. And 80% methanol is the best solvent to use for extraction as the extracts are more soluble in polar solvents and 80% methanol has more polar organic properties. Successive extractions proved that yields can be considerably increased by performing short repetitive extractions.

Total phenolic content and total flavonoid content analysis proved the presence of high levels of phenolic compounds as well as flavonoids. In comparison to other ethno-medicines studied, the flavonoid content was particularly high for *K. wilmsii*.

The results are consistent with the antioxidant activity produced by the extracts. Positive correlations between IC_{50}, total phenolic content and total flavonoid contents show that the antioxidant activity is caused by the presence of phenolic compounds and flavonoids.

Further studies would involve antimicrobial and anti-inflammatory activity tests, chemical properties tests, separation and identification of individual components.

4.3.2 Total Phenolic Content

The total phenolic content (TPC) was determined through the gallic acid calibration which is shown in Figure 10 below. The TPC of *K. wilmsii* extracts ranged from 45.32 to 122.84 mg GAE/g from water and methanol extracts, respectively (Table 12). Pure methanol produced extracts with the highest levels of total phenolics. The content is significantly higher than the phenolic contents of all the other solvents used ($P < 0.05$). No significant differences were recorded in the total phenolic contents of ethanol, 80% methanol, MCW, 60% methanol and water ($P > 0.05$).

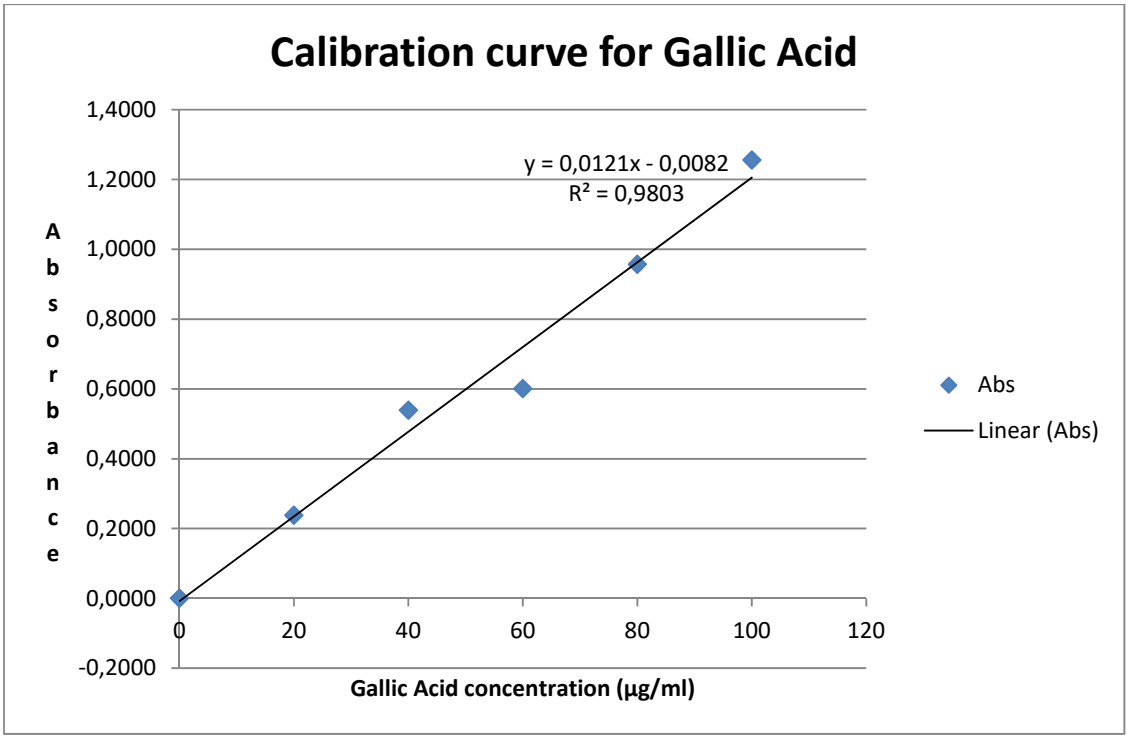

Figure 10:Gallic Acid calibration graph

Table 12:Total phenolic content of different extracts of K. wilmsii

Extract	TPC (mg/g)
Ethanol	58.98 ± 0.45[a]
Methanol	122.84 ± 0.31[b]
MCW	69.34 ± 0.91[a]
80% MeOH	57.16 ± 0.47[a]

60% MeOH	62.05 ± 0.30[a]
Water	45.32 ±0.50[a]

Results with different superscripts are statistically significantly different from each other ($P < 0.05$). The results indicate that all the TPC results are statistically the same except for the methanol extract results which is significantly higher than the rest of the results.

The total phenolic content results obtained in our study are higher than the results reported by Dhanani et al. (Dhanani *et al.*, 2017), who reported a maximum of 30 mg GAE/g for *Withania somnifera* roots extracted with ethanol, 10% ethanol and water (Dhanani *et al.*, 2017). Some other researchers reported total phenolic content values between 100 and 150 mg GAE/g, with the methanol and acetone extracts of the leaves, stem and flowers of *Thermopsis turcica*. These values are generally higher than our results and only our methanol extract matches these values. Our methanol extract compares well with these phenolic contents.

The other solvents produced extracts whose total phenolic contents is similar to those reported in literature, from the extracts of *Goniothalamus velutinus* (G. velutinus) whose bark extract gave 68 GAE mg/g and the leaves 78 GAE mg/g (Iqbal, Salim and Lim, 2015). *G. velutinus* is reported to have antitumor and anticancer properties. The water extracts of *Hedychium spicatum*, *Hedychium coronarium* and *Hedychium rubrum* were found to be 30, 35 and 67 GAE mg/g respectively (Iqbal, Salim and Lim, 2015). However, our results are much lower than total phenolic contents recorded for the resurrection plant *Myrothamnus flabellifolius* water, ethanol and methanol extracts (all 400 mg GAE/g) (Cheikhyoussef, Summers and Kahaka, 2015). Stanojević et al. (Stanojević *et al.*, 2009) also reported much higher total phenolic contents of 250 mg GAE/g when *Hieracium pilosella* water, ethanol and methanol extracts were tested.

Phenolic contents ranging 90–260 mg GAE/g were recorded with three species of Curcuma methanol extracts (Sahu and Saxena, 2013). All these results are also higher than our reported results except for our methanol extract. Curcuma species are used for the treatment of asthma tumours and also as antifungal.

Total phenolic content is an important factor in the consideration of antioxidant activity. Therefore, the higher the value of phenolic content, the more beneficial the extract is to human health as they can quench reactive free radicals or primary oxidants.

4.3.3 Total Flavonoid Content

The results of the total flavonoids phenolics were expressed as quercetin equivalents. Further, the IC_{50} values were calculated using linear regression.

The calibration graph for quercetin are shown in Figure 11 below.

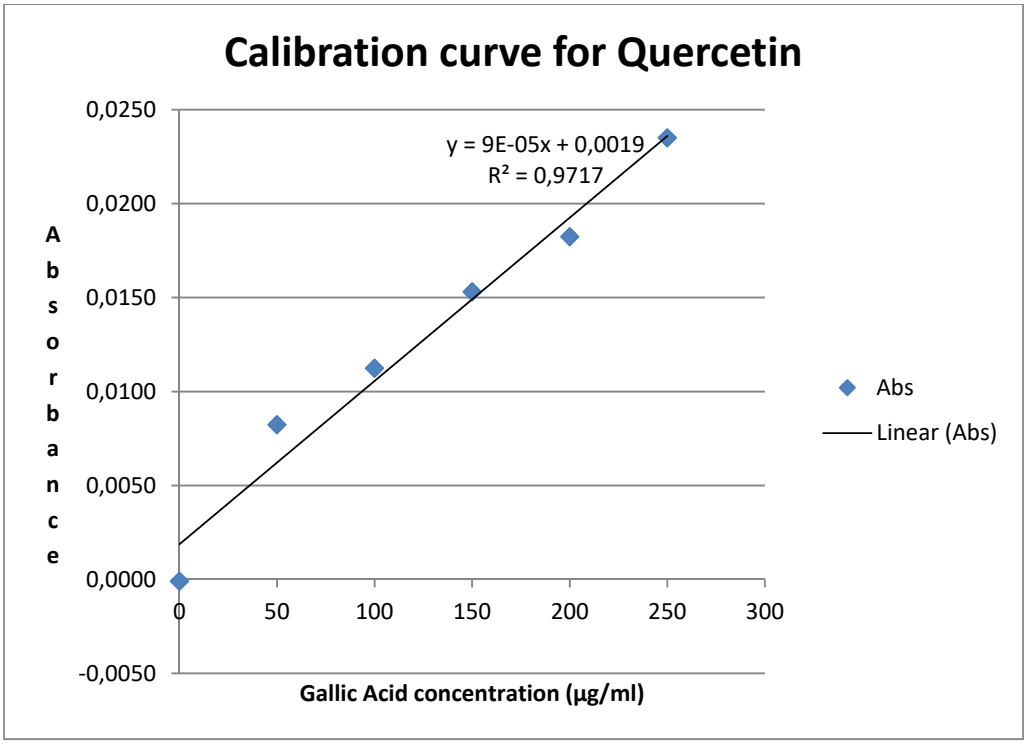

Figure 11: Quercetin calibration graph

The total flavonoid contents of *K. wilmsii* were recorded ranging from 206.26 mg QE/g to 917.02 mg QE/g from the water extract and the methanol extract respectively. The methanol extract exhibited a total flavonoid content that is significantly higher than the rest of the extractants ($P < 0.05$). The flavonoid contents of the rest of the solvents are also significantly different ($P < 0.05$) from each other except for ethanol, 60% methanol and water extracts whose flavonoid contents are not significantly different (Table 13).

The total flavonoid content for the methanol extract was very high (917 mg/g) and the other extracts exhibited results ranging from 200 to 450 mg/g.

Table 13: Total flavonoid content

Extract	Total flavonoid Content (mg/g)
Ethanol	256.95 ± 0.45[a]
Methanol	917.02 ± 0.10[b]
MCW	437.64 ± 0.17[c]
80% MeOH	351.37 ± 0.06[d]
60% MeOH	262.49 ± 0.25[a]
Water	206.26 ± 0.10[a]

The statistical significance of the results is indicated by the superscript letters in Table 13. The ethanol, 60% methanol and water extracts' total flavonoid contents are not significantly different. However, they are significantly different from the methanol, MCW and 80% methanol extracts.

The methanol extract's flavonoid content matches the very high flavonoid contents values obtained with wheat methanol extracts ranging from 791.3 to 987.7 mg QE/g (Sumczynski *et al.*, 2015). All our other results are comparable to the results reported with the hexane, chloroform, ethyl acetate, butanol, methanol and water extracts of *Azadirachta indica* with results ranging from 63 mg QE/g to 529.5 mg QE/g (Al-Jadidi and Hossain, 2015). In their work, all the other results are above 350 mg QE/g except for the butanol extract.

Research has proven that flavonoids are important in the fight against diseases and can also act as antioxidants depending on their structure (Iqbal, Salim and Lim, 2015). Coupled with phenols, flavonoids have been reported to have high antioxidant activity (John *et al.*, 2015). Therefore, these high total flavonoid content results could mean the abundance of possible compounds of pharmaceutical importance. Other published results are very low, pigeon pea extracts gave flavonoid contents ranging from 0.16 to 1.58 mg QE/g (Al-Saeedi and Hossain, 2015), and from 20 to 80 mg QE/g content for Curcuma extracts (Sahu and Saxena, 2013), 79.13–82.18 mg QE/g for *Hieracium* pilosella (Stanojević *et al.*, 2009). Further work is necessary to determine the flavonoid types, biological, anti-inflammatory activity, antimicrobial and anticancer activities of *K. wilmsii*.

4.4 Antimicrobial Activity

The sub-culturing was carried out using the T method and yielded colonies as depicted in Figure 12 below where one colony was taken for sub-culturing on the agar plates and incubated at 37°C for 24 hours . One colony was inoculated into the nutrient broth, prepared in the following manner.

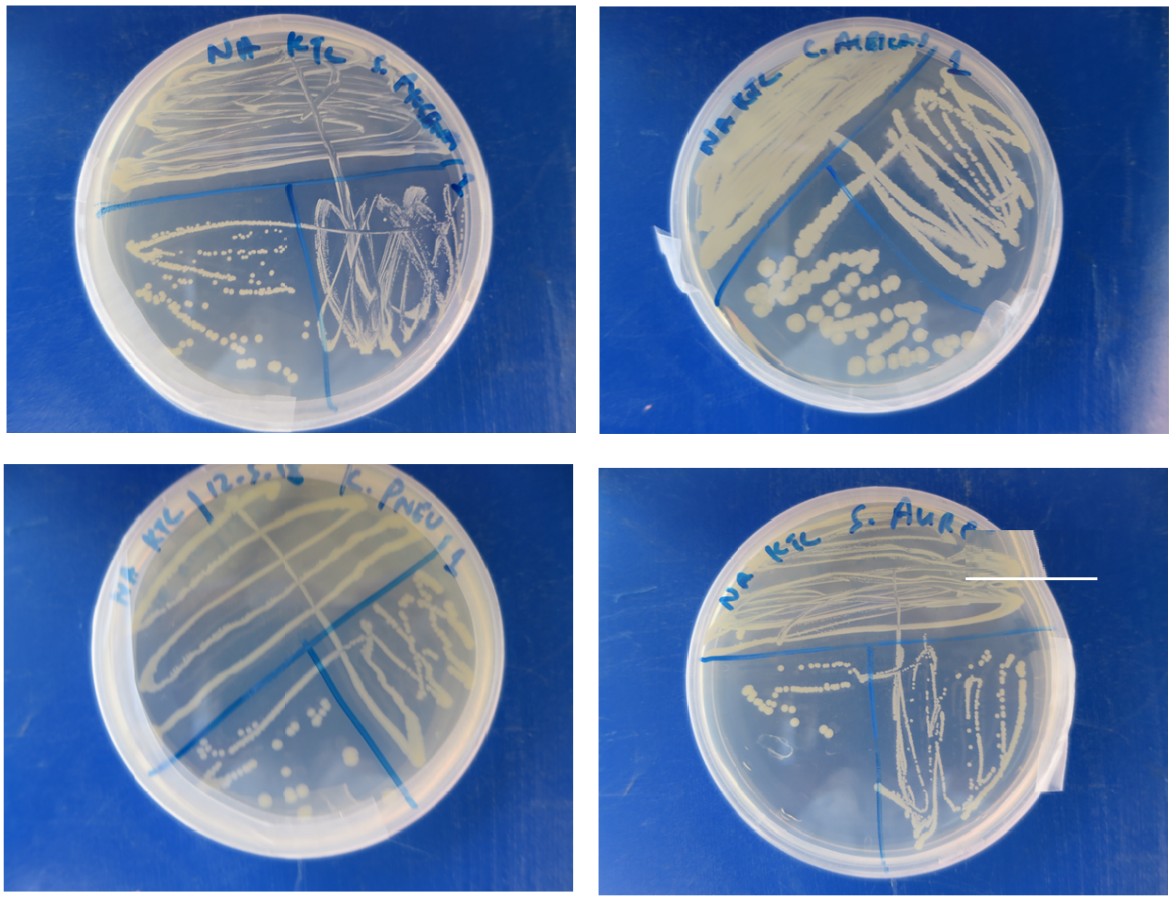

Figure 12: The results of T method of sub-culturing of a few selected organisms

Some of the plates showing the disc diffusion results are shown in Figures 13 show results of screening of the plant extracts for antimicrobial activity. The pure water extracts showed no antimicrobial activity against all pathogens tested. The clear zones indicate the area where the growth of bacteria was inhibited. Diluted organic solvent extracts showed more pronounced

antimicrobial activity as compared to absolute organic solvent extracts.

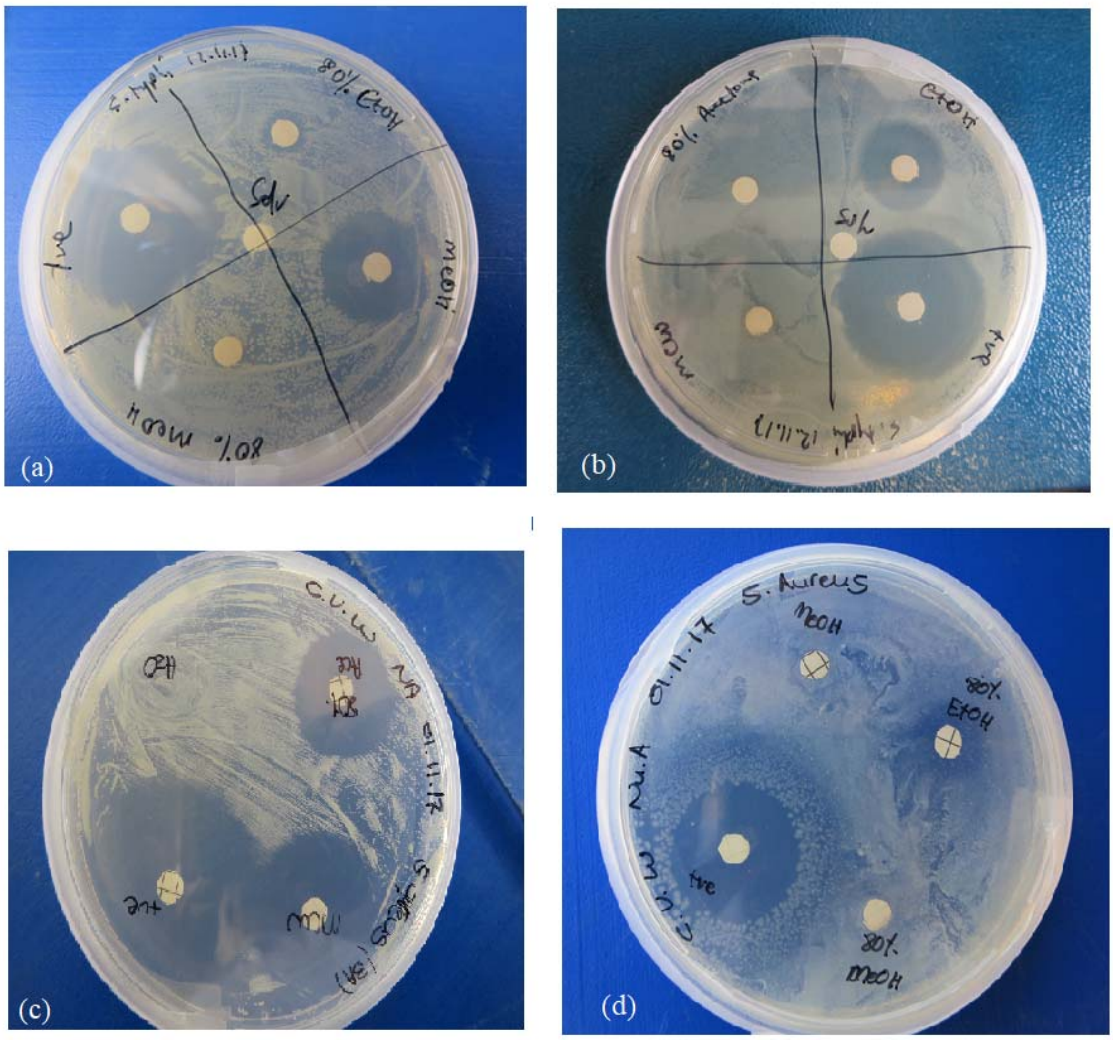

Figure 13: Some agar disc diffusion results showing antimicrobial activity of some extracts, positive control and solvent against some of the bacteria: (a) solvent (water) in the middle, amoxycillin (positive control, 80% ethanol, methanol and 80% methanol against *S. typhimurium*, (b) solvent (water) in the middle and MCW, amoxycillin (positive control, ethanol and 80% acetone against *S. typhimurium*, (c) solvent (water), amoxycillin (positive control, MCW and 80% acetone against *S. aureus* and (d) methanol, amoxycillin (positive control, 80% methanol and 80%ethanol *S. aureus*

The antimicrobial tests were tested on gram positive (S. *aureus, S. saprophycticus, S. epidermidis and S. pyogenes*), gram negative (*E. coli, K. pnuemoniae and S. typhimurium*) and yeast bacteria (*C. albicans*).

The negative control was pure deionised water, which was used to dissolve the extracts, which did not inhibit any bacteria to prove that the solvent did not interfere with results. A summary of the positive or negative results are shown in Table 14. The positive control, amoxycillin,

inhibited all the microorganisms except for *K. pnuemoniae*. The negative result for this widely used broad spectrum antibiotic is supported by research done on mice (Lin *et al.*, 2013). In their work, they proved that treating mice with ampicillin and amoxycillin killed the mice infected with *K. pnuemoniae* at a faster rate than mice that were fed on water only. This is further supported by literature where it has been well documented that β-lactams such as amoxycillin have been reported to have met with resistance against *K. pnuemoniae* (Fernandes, Amador and Prudêncio, 2013; Kocsis and Szabó, 2013). The resistance is caused by the hydrolysis of the antibiotic, thereby rendering it ineffective.

Similarly, the plant extracts tested negative for *K. pnuemoniae.* This led us to a conclusion that there might be a possibility that the mode of action of amoxycillin and the mode of action of our plant extracts might be the same. Amoxycillin activity actually occurs through stopping cell wall synthesis of the microorganisms (Kocsis and Szabó, 2013).

If the activity of our extracts is comparable to that of amoxycillin, it gives weight to our hypothesis as well as the claim by the local Bapedi people who claim that the tuber "cleans blood". We have interpreted the "cleaning of blood", to mean that it can be generally used for the general well-being of the human body by healing many sicknesses.

The most potent extracts were found to be 80% methanol and 50%acteone followed by MCW and 80% ethanol, which is depicted in the graph showing the antimicrobial activity results is shown in Figure 14. The 80% methanol and 50% acetone extracts were the most potent against 7 organisms, although their zones of inhibition were smaller in comparison with those of amoxycillin.

There is no recorded similar literature on *K. wilmsii* that we could use to compare with the results that were obtained in our research. However, a lot of antimicrobial activity research has been carried out on many other medicinal plants.

MIC results were computed and are shown in Table 15. Plant extracts whose antimicrobial activity has MIC values that are less than 1.00mg/ml are considered to be potentially potent against pathogens (Mabona *et al.*, 2013). A further definition is also described in literature according to the range of MIC, that is, high if MIC < 0.1mg/ml, moderate if 0.1mg/ml < MIC < 0.625mg/ml and low when MIC > 0.1mg/ml (Kuete *et al.*, 2014; Tankeo *et al.*, 2015) This is true in our case as all the results for amoxcyllin are way below those MIC values for most of the microbes we tested against. The lowest result is 0.016mg/ml for *C. albicans* and *S. saprophycticus*. Only the 80% acetone extract has one comparable result against *S. aureus*. The 80% methanol extract also has 2 results with 0.031mg/ml against *E. coli* and *S. typhi*. This is

in contrast to some work where most of the extracts were found to be more active than the positive control, ciprofloxacin (Tankeo *et al.*, 2015).

In work done by other researchers, the methanol extracts of *P. gingivalis* were found to be the most potent in contrast to our work were the diluted aqueous extracts were found to be most potent as compared to 100% solvents and nonpolar solvents (Mohieldin, Muddathir and Mitsunaga, 2017).

In other research work where pure solvents such as, ethanol were used for extraction, extracts of *juglandaaceae* were also very effective against tuberculosis organisms with MIC values around 0.1mg/ml (Bottari *et al.*, 2017). These results are in direct contrast with our findings where the aqueous/polar solvents exhibited higher activity.

According to literature, gram positive microorganisms are more susceptible than gram negative (Tuchilus *et al.*, 2017)

Table 14: Summary showing the positive/negative results for different extracts

Extract	Micro-organism inhibition zones (mm)							
	E. coli	*S. aureus*	*S. saprophycticus*	*C. albicans*	*S. epidermidis*	*S. pyogenes*	*K. pneumoniae*	*S. typhinurium*
Amoxycillin	+	+	+	+	+	+	-	+
80% Acetone	-	+	+	-	+	-	-	+
MCW	+	+	+	-	+	+	-	+
Water	-	-	-	-	-	-	-	-
80% Methanol	+	+	+	+	+	+	-	+
60% Methanol	-	-	+	+	-	+	-	-
Methanol	-	-	+	+	-	-	-	+
50% Acetone	+	+	+	+	+	+	-	+
80% Ethanol	+	+	-	+	+	+	-	+
Ethanol	-	-	+	-	-	+	-	+

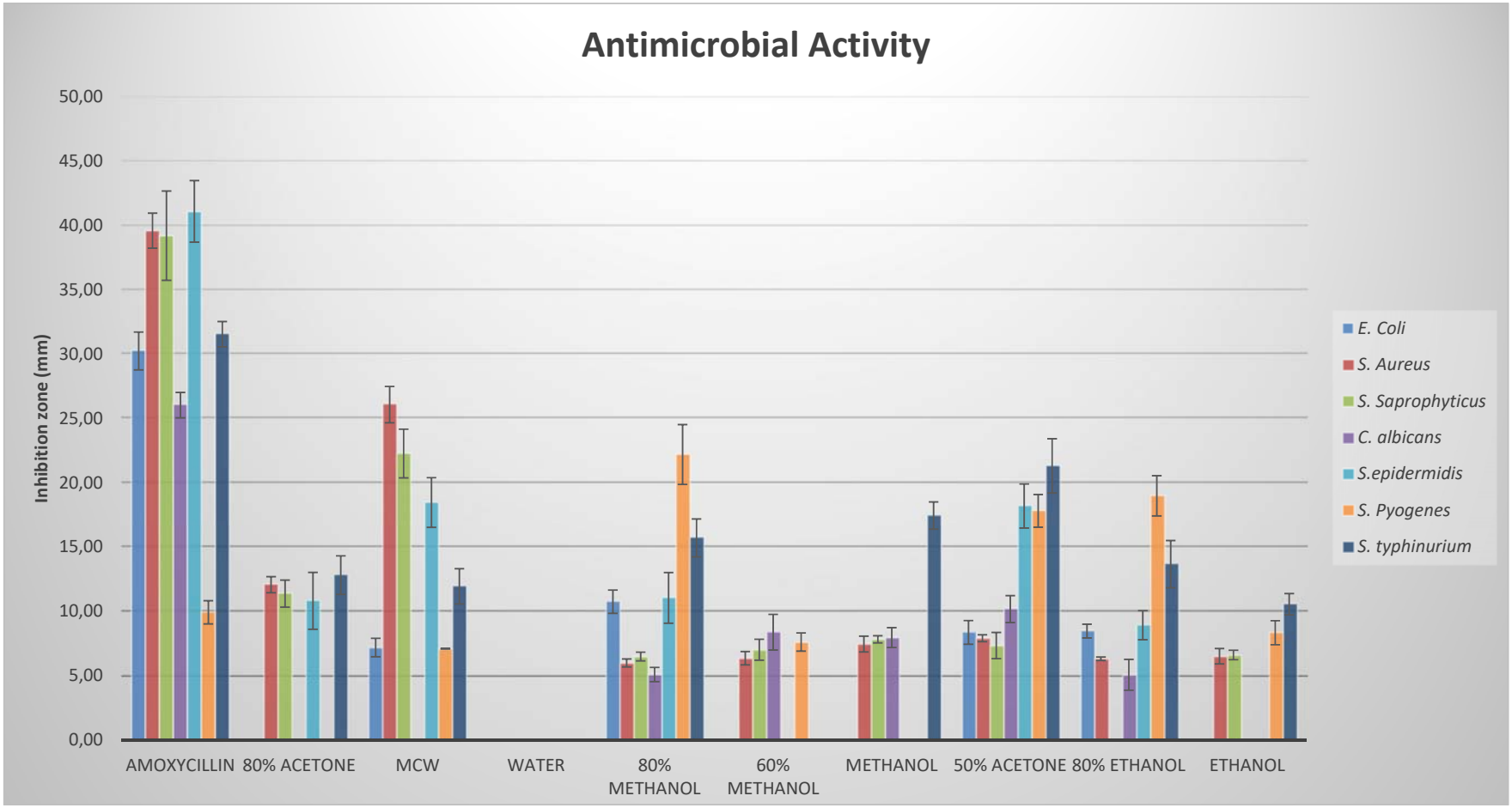

Figure 14: Summary of anti-microbial activity test results

Table 15: Summary of MIC Results

Extract	*E. Coli*	*S. Aureus*	*S. saprophyticus*	*C. albicans*	*S.epidermidis*	*S. pyogenes*	*K. pneumoniae*	*S. typhinurium*
Amoxycillin	1.000	1.000	0.016	0.016	0.063	0.031	-	1.000
80% Acetone	-	0.016	1.000	-	0.125	-	-	0.500
MCW	-	0.500	1.000	-	0.063	0.125	-	1.000
Water	-	-	-	-	-	-	-	-
80% Methanol	0.031	1.000	1.000	0.500	0.125	0.125	-	0.031
60% Methanol	-	-	1.000	0.250	-	0.031	-	-
Methanol	-	-	0.250	0.125	-	-	-	1.000
50% Acetone	0.125	1.000	1.000	0.125	0.125	0.125	-	0.063
80% Ethanol	-	1.000	-	1.000	0.063	1.000	-	0.500
Ethanol	-	-	1.000	-	-	0.500	-	0.250

Our water extract tested negative for all tests. This is in contrast to some work done by some other workers, who found that their water extracts gave the biggest antimicrobial activity results (shaikh, Rub and Sasikumar, 2016).

After a careful review of literature, to the best of our knowledge, the antimicrobial activities of the extracts of the roots of *K. wilmsii* are being reported here for the first time. The reported antimicrobial data in this work, proves that there is potential in the use of this plant and perhaps justifies its traditional use by the Bapedi people (Semenya and Maroyi, 2013; Semenya and Potgieter, 2015; Tankeo *et al.*, 2015)

For the purposes of our current study, the four extracts which proved to be more potent that the other extracts were taken further for structural elucidation.

4.5 Proximate and Metal Analysis

4.5.1 Moisture Content/Loss on drying

Moisture content results are shown in Table 16 below. The average moisture content of the dried *K. wilmsii* tuber was found to be 3.44±0.11%. This average moisture content is less than the recorded moisture content for most plat tubers. For instance the roots of *Anogeissus latifolia* recorded 7.8% (Bhargava, Saluja and Dholwani, 2013) and 5.66 to 7.55% for oil flaxseeds

(Zou *et al.*, 2017). This shows that the moisture content of our plant is low which might mean storage longevity and hence a good raw material.

If the amount of water found in medicinal plant materials is high, microbial growth is enhanced as well as the presence of fungi or insects (WHO, 1998). Consequently moulds and yeasts may become visible on the herbs. This is exacerbated by deterioration of the plant material caused by hydrolysis. Moisture is one of the factors mostly attributed to the deterioration of drugs and formulations (Bhargava, Saluja and Dholwani, 2013). However, the WHO contends that the specifications for this test should be set for each plant as some plants are hydro stable while others are hygroscopic and others deteriorate quickly if water/moisture is present. Our moisture result is well below the 14% suggested for vegetable drugs (Abdu *et al.*, 2015), as well as the maximum 10% suggested for herbal remedies (EDQM, 2007)

Table 16: Moisture/loss on drying

	Sample 1	Sample 2	Sample 3
Empty (g)	35.92	31.42	35.67
Container + sample (g)	37.78	32.76	37.53
Amount of sample (g)	1.86	1.34	1.86
mass after drying (g)	37.711	32.71	37.47
mass loss (g)	0.07	0.04	0.06
% mass loss	**3.56**	**3.35**	**3.40**

4.5.2 Total Ash, Acid-insoluble Ash and Water-soluble Ash

Ashing results were calculated with reference to the dried *K. wilmsii* powder. The total ash, acid insoluble and water soluble ash are shown in Table 17 below. The average total ash, acid insoluble and water soluble content of the *K.wilmsii* roots was found to be 12.43±0.41, 1.22±0.3 and 8.18 ±0.39% respectively. The total ash content value translates to a loss on ignition value of 87.57%. The higher the total ash value, the higher the chances of contamination, especially by minerals (Abdu *et al.*, 2015).

The European Directorate for the Quality of Medicines & HealthCare (EDQM) has set the maximum total ash at 14% (EDQM, 2007). Although our result is within the specifications, it is close to the upper limit. This could be because of inorganic matter getting embedded in the tubers as they grow in the soil. However the result shows that the ash value can be tolerated for drug use.

Table 17: Total, acid washable and water washable ash results

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Empty (g)	275.52	280.78	274.82	278.18	281.36	276.89
Container + sample (g)	277.74	283.46	277.21	279.86	283.36	278.94
Amount of sample (g)	**2.22**	**2.68**	**2.39**	**1.68**	**2.00**	**2.05**
mass after Ashing	275.81	281.11	275.11	278.38	281.61	277.15
mass of ash	**0.29**	**0.33**	**0.29**	**0.20**	**0.25**	**0.26**
Total ash (%)	13.06	12.31	12.13	11.90	12.50	12.68
mass after wash	275.54	280.82	274.85	278.25	281.44	276.98
amount remaining (g)	0.02	0.04	0.03	0.07	0.08	0.09
% acid insoluble ash / water soluble ash	0.90	1.49	1.26	7.74	8.50	8.29
	Acid (0.1M HCl) wash			Water wash		

The acid insoluble ash was found to be 1.22% while the water-soluble ash was 8.18%. The EDQM set the maximum of 2% for acid insoluble in ash, which indicates that our product complies with the specifications. The test evaluates the extent of the presence of unwanted minerals in samples and the results should be reasonably low. The results are complemented by the water-soluble ash results which show that most of the ash is actually not made up of metals.

In comparison with other plants our results compare favourably with 11.75%, 7.45%, 7.85% for total ash, acid insoluble ash and water soluble ash for respectively *A. latifolia* roots (Bhargava, Saluja and Dholwani, 2013), 10.85%, 6.35%, 3.65% wood, 12.1%, 4.5%, 7.35% bark (Khan *et al.*, 2009); 10.41% and 2.13% for *Prunellae spica* herbals (Rao and Xiang, 2009). Very high results were reported for different local herbal medicines ranging from 3.9 to 43.92% for total ash, 0.19 to 30.75% for water soluble ash and 0.69 to 86.47% for acid insoluble ash (Abdu *et al.*, 2015). This indicates that some of the plants are clearly not suitable for use as drugs.

4.5.3 Metal/Elemental content

The results from the XRF are shown in Table 18 below. The highest element recorded was potassium (K) with a reading of 43299ppm which translates to about 4.3% K in the analysed sample. This figure is followed by Mg and Cl with values of 2.6 and 1.4% respectively. We would assume that the tubers have accumulated a lot of salt from the surroundings as the highest concentrations were recorded with Mg, K and Cl elements.

Studies have shown that heavy metals accumulate in the roots of plants as compared to other parts such as the stem, bark and leaves (Yadav, 2010; Banu and Cathrine, 2015). Roots were put under study for this reason to prove/dispel any chances of dangers to human life. The heavy metals that have mainly been deemed to pose dangers to life and the environment include Cr, Pb, As, Cd and Hg. Conventionally the conversion from ppm to mg/kg is a 1:1 relationship such that 1ppm = 1mg/kg, we conducted a simple comparison with previous work done on plants as well as the accepted international pharmacopoeia methods.

The Cr, Pb, As, Cd and Hg levels for the *K. wilmsii* tuber were found to be 1.48, 3.55, 0.14, ND and ND ppm (mg/kg) respectively. This is lower than 9.07, 15.30, 0.36, 1.18 and 17.04 ppm obtained with the roots of *Myriophyllum spicatum L. (Haloragaceae)* (Yabanli, Yozukmaz and Sel, 2014). Metal analysis performed on contaminated areas produced much higher metal concentrations, for instance, Cd concentration of 30ppm and Pb concentration of 650ppm were recorded (Gardea-Torresdey *et al.*, 1996).

Table 18: XRF Elemental analysis of the powder

	Mg	Al	Si	P	S	Cl	K
Average	26211 ± 4.65	5243 ± 3.78	2230 ± 3.62	766 ± 1.15	2812 ± 0.16	14140 ± 0.13	43299 ± 0.03

	Ca	Ti	V	Cr	Mn	Fe	Ni
Average	8732 ± 0.60	21.7 ± 8.43	3.45 ± 30.72	1.48 ± 21.82	81 ± 5.4	234 ± 1.52	5.07 ± 1.84

	Cu	Zn	As	Br	Rb	Sr	Sn
Average	13.8 ± 4.87	27.8 ± 1.68	0.14 ± 17.61	14.4 ± 0.98	17.9 ± 1.67	19. ± 4.00	16.9± 7.61

	Au	Pb	Ta
Average	2.09 ± 9.75	3.55 ± 9.94	2.88 ± 41.11

Other K. wimlsii results include Pb 3.55ppm, As 0.14ppm and Cd was not detected. The results indicate that *K. wilmsii* could be safe for human consumption as the metal concentrations are very low compared to other herbal medicines. According to the EDQM, the maximum allowed concentration for Cd, is 0.5ppm, Pb 5.0ppm and Hg 0.1ppm (EDQM, 2007). In our samples, Cd and Hg was not even detected.

In this study the roots were of interest as the local people consume them for the cure of many diseases and we wanted to be sure of the safety of their health.

4.6 Toxicology Studies

The importance of toxicology study in the development of pharmaceutical products has been widely reported and is an accepted procedure although some activists are campaigning against it (Robinson *et al.*, 2008). The sustainable use (using he replacement, reduction and refinement models) of these primates for research is being encouraged as they are the best models for such studies because of their genetic similarity to humans. After toxicological tests are done, inferences on how K. wilmsii affects the organ systems, metabolic functions and pathophysiological responses on human being would be suggested.

4.6.1 Food intake and general observation

The selected animals consumed the treated 100 g portion of their food (Table 19 showing that the monkeys consumed all the extract and the control except week 4 where 97.7% of the food given was consumed. The consumption was considered to be satisfactory and was comparable to the control feed.

Table 19: Average consumption of food containing the plant products (%)

Group	Baseline	Week 4	Week 8	Week 12	Week 16
K. wilmsii 2.14g/kg 25 x daily dose	100.00	97.7	100.00	100.00	100.00
Control Maintenance diet	100.00	100.00	100.00	100.00	100.00

4.6.2 Biochemistry analysis

A summary of the biochemical parameters found in the test and control monkeys is shown in Table 20 and the corresponding graph and raw data are found in Appendices D and G including the means and standard deviations.

All the results for biochemical parameters were compared to the control group and the changes that occurred were recorded for the specific times as indicated in Table 21. The treatment was not associated with clinically significant differences, except for the Anion Gap which showed statistically significant changes. The anion gap (difference between the positively charged and

negatively charged electrolytes) is used to check the levels of acid in the body and is measured through the tests for electrolytes. The test values are all low as compared to the control. A low anion gap may be caused by kidney or heart problems.

There was an observed difference for ALP at week 8 where the test value (152u/L was significantly lower than the control (193u/L). Overally all the test values are lower than the control values (Table 20). ALP is an enzyme that is found in cells with the highest concentrations being found in the bones and liver. High ALP values suggest the presence of liver or bone disorders (Robinson *et al.*, 2008). The *K. wilmsii* results suggests that the plant has beneficial effects on the body as the values are low.

Table 20: Summary of biochemical parameters

			K. wilmsii					Control		
	Baseline	Week 4	Week 8	Week 12	Week 16	Baseline	Week 4	Week 8	Week 12	Week 16
ALT (u/L)	40.00±22.18	30.75±15.95	30.75±14.52	32.25±8.26	41.50±17.94	82.50±18.8	81.50±27.31	120.25±53.90	94.00±35.92	68.75±29.98
AST (u/L)	79.50±23.10	75.75±19.19	70.75±14.93	83.50±13.50	84.25±15.35	105.50±33.17	88.75±20.16	112.50±34.68	110.00±48.85	104.25±28.77
ALP (u/L)	188.75±27.99	173.50±25.12	152.50±24.53	127.25±25.51	128.25±22.94	204.00±89.15	187.25±82.29	193.25±79.93	166.00±68.75	161.75±69.74
GGT (u/L)	77.75±30.70	91.00±24.43	77.50±27.81	83.25±36.76	86.25±30.18	68.00±18.57	78.00±22.52	96.25±17.54	85.50±20.16	86.25±20.95
Total Protein (g/L)	59.75±1.26	57.25±0.96	58.25±0.50	58.25±2.50	59.00±0.82	59.75±1.50	59.50±1.00	60.75±1.71	61.5±0.96	60.75±2.63
Total bilirubin (µmol/L)	7.75±0.96	6.25±1.26	8.25±2.36	700±2.45	6.00±1.63	6.00±1.41	6.75±1.50	5.75±1.71	6.50±1.73	5.75±2.22
Direct bilirubin (µmol/L)	2.00±0.00	1.50±0.58	1.75±0.50	1.50±0.58	1.75±0.50	1.50±0.58	1.25±0.50	1.50±0.58	1.25±0.50	1.25±0.50
Albumin (g/L)	41.25±2.75	38.00±2.16	39.50±2.38	38.25±3.20	39.00±4.08	40.50±1.29	40.25±1.26	40.75±1.50	40.50±1.00	41.25±1.71
Globulin (g/L)	18.50±3.51	19.25±2.22	18.75±2.75	20.00±2.16	20.00±3.56	19.25±0.50	19.25±0.50	20.00±1.15	21.25±0.96	19.50±2.89
HD lipoprotein (mmol/L)	2.13±.0.39	2.00§0.52	2.00±0.32	1.90±0.27	2.23±0.10	2.35±0.37	2.33±0.400	2.28±0.39	2.30±0.29	2.28±10.38
LD lipoprotein (mmol/L)	1.85±0.82	1.88±0.67	2.03±0.73	1.78±0.48	1.68±0.43	2.40±0.61	2.55±0.79	2.38±0.62	2.10±0.42	1.95±0.55
Urea (mmol/L)	4.40±0.36	2.70±0.50	3.25±0.70	2.73±0.21	2.88±0.57	5.28±0.50	3.98±0.49	3.68±0.88	3.43±10.67	4.08±0.96
Creatine (u/L)	66.75±9.18	70.75±9.74	67.00±11.17	65.50±10.91	69.25±8.46	84.00±15.85	84.50±15.11	87.25±20.84	79.75±13.25	85.25±19.81
Triglycerides (mmol/L)	0.46±0.12	0.57±0.09	0.62±0.15	0.67±0.15	0.52±0.15	0.42±0.13	0.74±0.26	0.66±0.23	0.61±0.15	0.74±0.26
Calcium (mmol/L)	2.21±0.08	2.16±0.07	2.19±0.05	2.23±0.07	2.27±0.03	2.14±0.09	2.14±0.02	2.12±0.06	2.17±0.08	2.20±0.09
Sodium (mmol/L)	147.75±0.50	148.25±0.96	148.75±0.50	148.00±1.41	149.00±1.15	148.75±0.50	149.25±0.96	148.50±1.29	149.25±0.96	152.00±1.63
Magnesium (mmol/L)	0.52±0.08	0.57±0.07	0.61±0.06	0.72±0.15	0.57±0.07	0.53±0.08	0.56±0.06	0.60±0.09	0.58±0.08	0.68±0.09
Choride (mmol/L)	107.00±0.82	107.75±1.73	106.25±0.50	106.75±0.50	107.75±0.96	108.50±1.00	108.50±1.73	107.50±0.58	108.50±1.73	107.50±1.29
Anion gap (mmol/L)	13.25±3.40	9.75±1.71	8.75±0.96	7.75±0.96	10.00±2.58	10.00±1.83	11.50±0.58	12.25±3.95	10.50±3.00	14.00±2.71
Phosphate (mmol/L)	0.90±0.21	0.73±0.22	0.85±.28	0.71±0.27	0.64±0.21	0.90±0.21	0.63±0.22	0.75±0.10	1.08±0.19	1.10±0.19
Glucose (mmol/L)	3.83±0.22	3.80±1.22	3.90±0.42	4.45±0.45	4.63±0.68	4.18±0.75	3.75±0.41	4.33±0.78	4.75±1.11	3.83±0.95
Potassium (mmol/L)	3.53±0.10	2.70±0.67	3.35±0.13	2.95±0.34	2.45±0.19	3.53±0.17	2.98±.0.34	2.85±0.37	2.83±0.30	2.38±0.10
Total Bicarbonate (mmol/L)	7.70±3.23	31.00±1.71	33.95±0.33	33.38±1.24	31.45±1.89	29.95±2.63	29.30±1.71	28.88±4.06	30.23±2.51	30.68±2.82
Cratine Kinase (µ/L)	661.75±177.05	750.75±404.49	629.50±167.70	996.75±246.12	608.75±438.66	2864.75±1018.01	661.00±128.54	1429±759.31	1409.50±290.22	1393.50±984.33

Table 21: Summary of biochemistry observation

Variable	*P*<0.05	Figure	Conclusion
ALT	No	D1	
AST	No	D2	
ALP	No	D3	Not clinically significant
GGT	No	DD4	
Total Proteins	No	D5	Not clinically significant
Total Bilirubin	No	D6	
Direct Bilirubin	No	D7	
Albumin	Yes	D8	Not clinically significant
Globulin	No	D9	
HDL-C	No	D10	
LDL-C	No	D11	
Urea	No	D12	
Creatinine	No	D13	Not clinically significant
Triglycerides	No	D14	
Ca	No	D15	
Na	No	D16	
Mg	No	D17	
Cl	No	D18	
Anionic gap	Yes	D19	Not clinically significant
P	No	D20	
Glucose	No	D21	
K	No	D22	
CO_2	Yes	D23	Not clinically significant
CK	No	D24	

In conclusion the consumption of *K. wilmsii* showed similar biochemical results that are similar to that of the control suggesting that it is safe for human consumption.

4.6.3 Haematological analysis

The haematological summary of results is shown in Table 22 and the corresponding graphs are shown in Appendix E and raw data in Appendix G.

All the results that were compared to the control did not reveal any significant differences (Table 23).

Table 22: Summary of haematology results

			K. wilmsii					Control		
	Baseline	Week 4	Week 8	Week 12	Week 16	Baseline	Week 4	Week 8	Week 12	Week 16
Red blood cells (x 10^9/L)	6.60±0.26	6.43±0.30	6.53±0.31	6.60±0.15	6.77±0.34	6.28±0.41	6.41±0.28	6.70±0.36	6.61±0.32	6.53±0.34
White blood cells (x 10^9/L)	10.68±4.28	6.58±2.67	6.18±3.62	4.38±1.47	9.53±3.31	6.95±2.93	4.48±1.81	7.43±3.05	8.03±3.29	4.45±0.54
Mean Corp.Volume (fl)	77.50±3.70	78.50±2.38	79.50±4.43	79.75±5.74	81.00±4.69	76.50±2.38	78.75±2.50	78.00±2.16	79.25±2.63	80.25±2.63
Mea Corp. Haemoglobin (pg)	25.50±1.00	24.75±0.96	25.25±1.26	24.752)1.50	25.00±1.41	25.00±0.82	24.75±1.26	24.25±0.96	25.00±0.82	25.00±0.82
Mea Corp. Haemoglobin concentration (g/dl)	32.75±0.50	31.25±0.50	31.50±0.58	31.50±0.58	29.25±2.87	32.75±0.50	31.25±0.50	31.50±0.58	31.00±0.00	31.75±0.50
Haematocrit (%)	0.51±0.003	0.50±0.03	0.52±0.03	0.53±0.03	0.55±0.03	0.48±0.003	0.51±0.02	0.52±0.03	0.53±0.03	0.52±0.04
Red blood Cell Distribution Width (%)	13.43±0.62	13.73±0.83	14.33±1.09	14.03±1.17	1425±0.58	13.95±1.16	14.85±0.73	13.88±0.68	13.90±0.83	15.43±0.93
Haemoglobin (g/dl)	16.70±0.82	15.85±0.62	16.35±0.67	16.55±0.68	16.95±0.79	15.73±1.14	15.73±0.59	16.33±0.73	16.28±0.93	16.50±1.06
Neutrophils (x 10^9/L)	9.64±4.24	5.35±2.58	5.05§3.46	3.25±1.27	8.73±3.78	6.15±2.93	4.03±1.34	6.38±3.02	6.92↑13.05	3.56±0.55
Basophils (x 10^9/L)	0.0024±0.0015	0.0104±0.0003	0.0051±0.0024	0.0125±0.0099	0.0054±0.0021	0.0051±0.0022	0.0075±0.0027	0.0081±0.0021	0.0021±0.0015	0.0125±0.0101
Lymphocytes (x 10^9/L)	0.70±0.15	0.98±0.10	0.88±0.15	0.78±0.10	0.70±0.14	0.55 2410.13	0.67 2410.14	0.68±0.15	0.71±0.17	0.71±0.09
Monocytes (x 10^9/L)	0.34±0.11	0.28±0.13	0.28±0.13	0.33±0.15	0.35±0.10	0.28±0.10	0.48±0.13	0.33±0.19	0.39±0.26	0.20±0.08
Platelete count (x 10^9/L)	188±78.50	166.50±62.24	187.75±74.38	190.75±72.52	193.00±59.55	190.50±35.91	182.25±40.54	187.75±35.48	214.75±55.07	203.00±36.17

Table 23: Summary of haematological observation

Variable	*P*<0.05	Figure	Conclusion
RBC	No	E1	Not clinically significant
WBC	No	E2	Not clinically significant
MCV	No	E3	
MCH	No	E4	
MCHC	No	E5	
RDW	No	E6	
Hb	No	E7	
Neutrophils	No	E8	
Basophils	No	E9	
Lymphocytes	No	E10	
Monocytes	No	E1 1	
Platelets	No	E1 2	
Hct	No	E1 3	

4.6.4 Physical and physiological variable analysis

A summary of the physical and physiological parameters found in the test and control monkeys is shown in Table 24 and the corresponding graph and raw data are found in Appendices F and G including the means and standard deviations.

A summary of the observations is shown in Table 25. There was no clinically significant change and no statistically significant changes for *K. wilmsii* as compared to the control diet.

Table 24: Summary of physical and physiological variables

			K. wilmsii					Control		
	Baseline	Week 4	Week 8	Week 12	Week 16	Baseline	Week 4	Week 8	Week 12	Week 16
Weight (kg)	5.40±0.59	5.31±0.50	5.42±0.55	5.5±0.61	5.33±0.65	5.52±0.04	5.55±0.05	5.47±0.04	5.49±0.02	5.57±0.08
Body temperature (°C)	38.9±0.28	39.25±0.33	38.88±0.43	39.98±0.38	38.73±0.29	38.63±0.62	39.25±0.37	39.23±048	39.05±0.49	39.3±1.13
Repsiratory rate (breaths/min)	26.00±2.31	28±3.27	25.00±3.83	31.00±6.83	32.00±6.53	27.00±2.00	28.00±3.27	29.00±2.00	32.50±4.12	32.00±0.00
Systolic Pressure (mmHg)	102.75±21.06	100.00±25.83	101.25±12.74	102.50±8.54	143.25±40.73	92.75±29.34	108.25±16.11	106.25±8.025	105.25±2.74	109.00±11.52
Diastolic Pressure (mmHg)	42.00±5.60	44.50±8.85	45.50±4.80	44.50±9.61	106.50±20.53	54.75±15.17	48.00±2.58	55.25±3.30	51.25±9.74	47.25±11.87
Mean Arteral pressure (beats/min)	58.50±13.38	66.00±12.70	63.25±8.18	65.00±17.61	76.00±0.00	70.25±19.65	71.50±5.00	82.25±4.99	76.25±15.22	58±14.46
Pulse (beats/min)	135.75±23.92	126.25±28.17	124.50±28.59	136.75±24.88	77.00↑10.00	107.75±18.93	125.50±27.31	138.50±23.44	125.25±29.08	133.50±20.92

Table 25: Summary of physiological observations

Variable	*P*<0.05	Figure	Conclusion
Body weight	No	F1	No difference
Body temperature	No	F2	
Respiratory rate	No	F3	
Systolic pressure	No	F4	
Diastolic pressure	No	F5	
MAP	No	F6	
Pulse	No	F7	

It is interesting to take note of the blood pressure results which are shown if Figures 15 and 16. The systolic and diastolic pressure measurements are an indicator of the health of an individual. Several ethnobotanical surveys indicated that *K. wilmsii* is used to treat hypertension (Moeng and Potgieter, 2011; Semenya, Potgieter and Erasmus, 2012; Semenya and Potgieter, 2015; Maroyi, 2016). This assertion is supported by the results shown in Figures 15 and 16. The graphs show that for both pressures, monkey who were fed on *K. wilmsii* experienced lower pressures from the baseline week to week 12. Week 16 was the washout week whereby the use of *K. wilmsii* was discontinued. Although there were no clinically significant differences between the test and control results, the graphs clearly show that both pressures increased in the test results. This could have been caused by the involuntary dependence on *K. wilmsii* by the monkeys to control their blood pressures. This evidence is in direct support of the ethnobotanical surveys cited above which state that *K. wilmsii* can be used to treat hypertension.

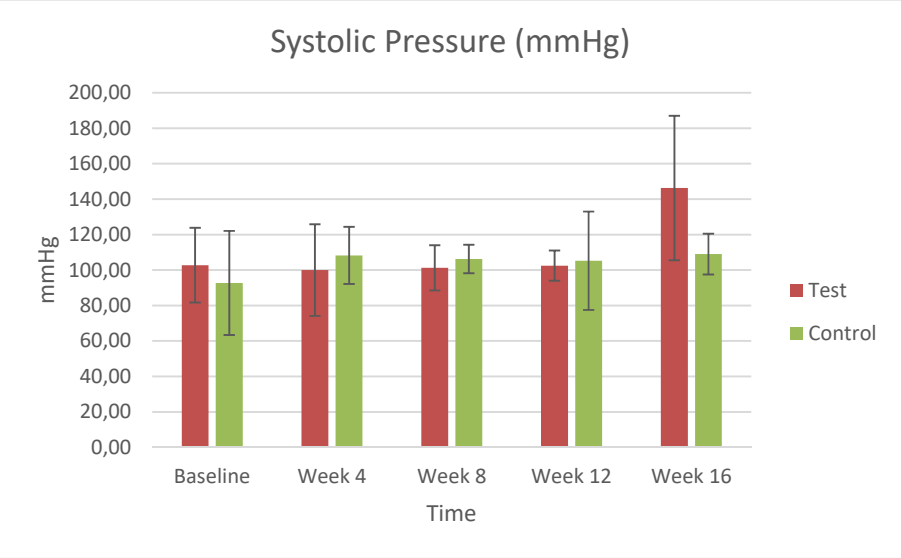

Figure 15: Systolic pressure measurement results

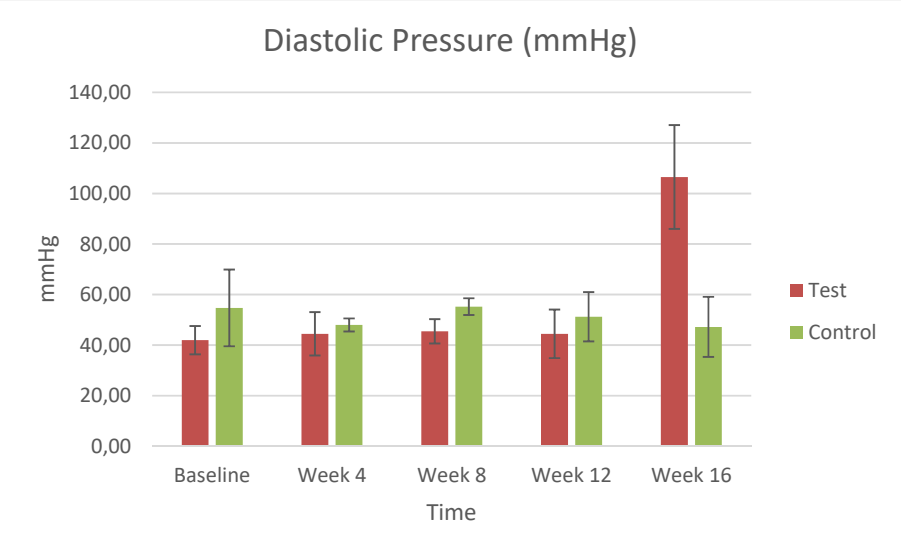

Figure 16: Diastolic pressure measurement results

All the sub-chronic toxicity studies that showed that all the monkeys that were fed with a *K. wilmsii* meal did not die and did not show signs of poisoning agree with the elemental analysis of the plant that showed that there are no harmful elements especially heavy metal available in the plant.. They also agree with the acid soluble ash which were low, showing that the amount of heavy metals was also low.

4.7 Chromatography

4.7.1 Column Chromatography

The extracts that showed encouraging antimicrobial test results were taken further for purification namely methanol, 50% acetone, MCW and 80% methanol extracts whose IC_{50} values are lower than 1.00mg/ml , a threshold for potency (Mabona *et al.*, 2013). The purity of all the following extracts were ascertained by TLC and UV-Vis spectrophotometry. The extracts that were not purified enough were shown by TLC to have 2 spots or more and were re-run on the column. The purified extracts that were collected in beakers before solvent removal and an example is shown in Figure 17. The extracts with same retention factors were mixed.

Figure 17: Purified extracts collected before TLC analysis and solvent removal

The purified extracts were run through the UV-Vis Spectrophotometer to check the maximum absorption peaks.

The solubility of compounds in certain solvents gives a rough indication of the functional groups present , for instance, solubility in polar solvents such as water could be enhanced due to hydrogen bonding (Markom *et al.*, 2007) . Our extracts are soluble in water and methanol and this could suggest the presence of carboxylic acids or hydroxyl groups.

4.7.1.1 50% Acetone Extracts

After purification, 8 purified extracts whose weights were 0.021g, 3.387g, 0.035g, 0.014g, 0.014g, 0.032g, 0.063g and 0.165g were obtained..

The purified extracts were run through the UV-Vis spectrophotometer, GC-MS and NMR.

4.7.1.2 80% Methanol Extracts

Seven 7 purified extracts were obtained weighing 0.017g, 0.014g, 2.196g, 0.010g, 0.203g, 0.040g and 0.0003g.

The purified extracts were run through the UV-Vis spectrophotometer, GC-MS and NMR.

4.7.1.3 Methanol Extracts

Purification yielded 12 purified extracts whose weights were 0.152g, 0.284g, 0.0214g, 0.021g, 0.007g, 5.084g, 0.017g, 0.012g, 0.056g, 0.182g, 0.005g and 0.003g.

The purified extracts were run through the UV-Vis spectrophotometer, GC-MS and NMR.

4.7.1.4 MCW Extracts

Eight purified extracts were obtained whose weights were 0.017g, 0.156g, 0.009g, 2.376g, 0.345g, 0.121g, 0.022g and 0.073g.

The purified extracts were run through the UV-Vis spectrophotometer, GC-MS and NMR.

Some of the TLC plates showing the CC purification process are shown in Figure 18.

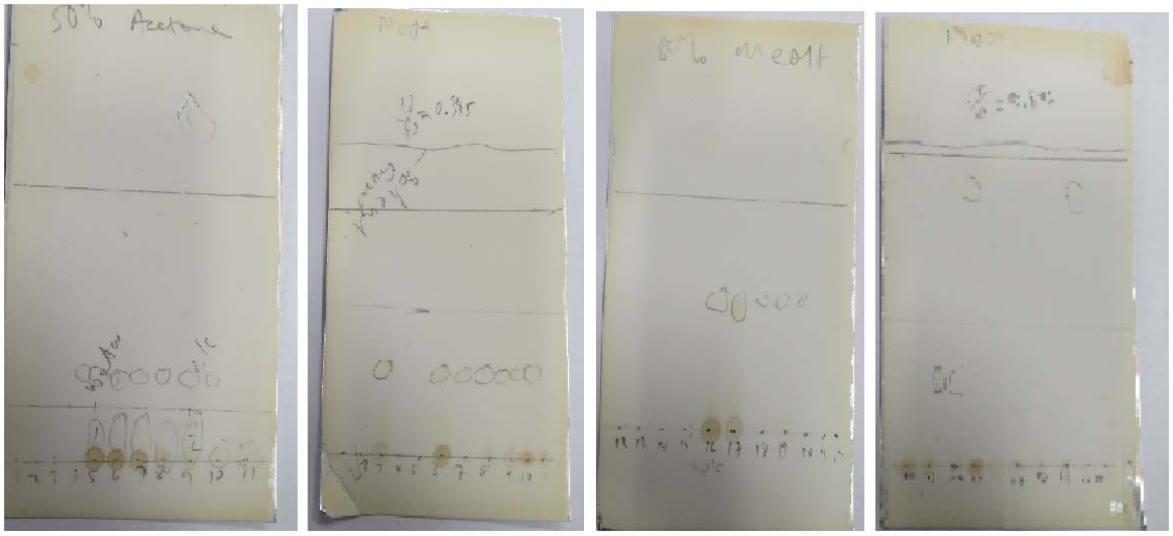

Figure 18: Picture of some of the TLC plates used to check the purity of extracts

The UV-Vis spectra of the 3 extracts finally sent for GC_MS and NMR analysis are exhibited different maximum absorption wavelength as shown I Figure 19. Extract 1 (KTC 3) exhibited a maximum at 247nm, extract 2 (KTC 4) at 290, 295 and 358nm and extract 3 (KTC 5) at 287nm.

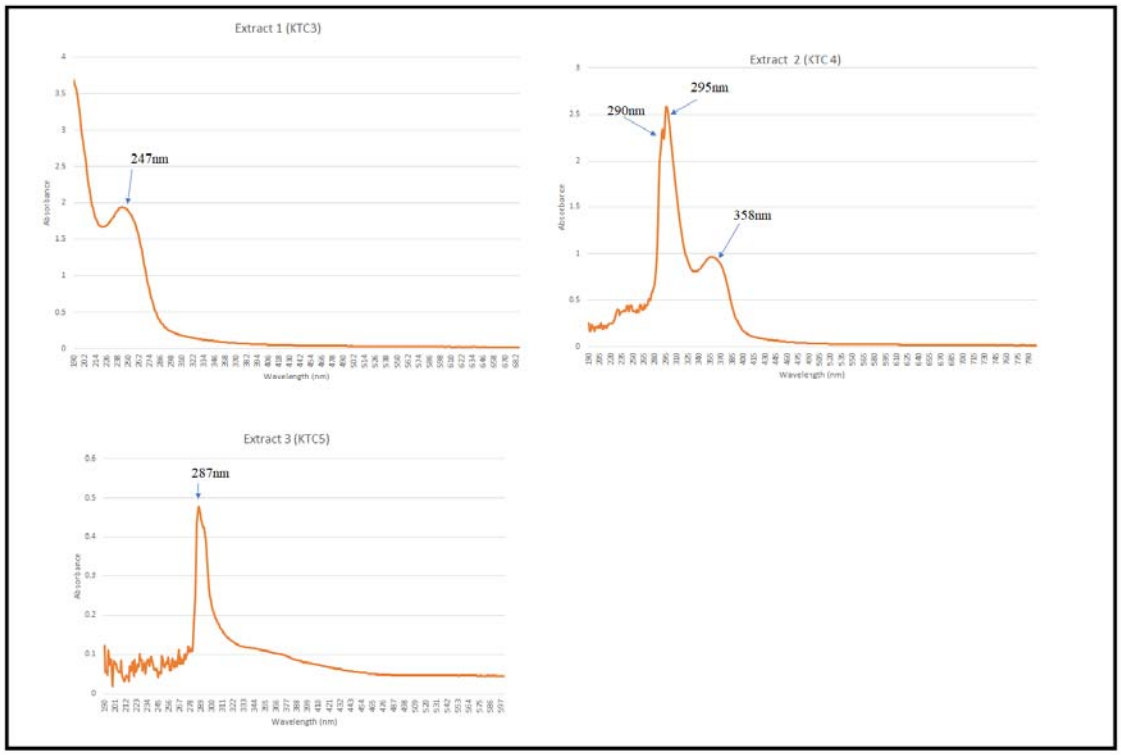

Figure 19: UV-Vis spectra of final extracts of interest

The extracts are highly soluble in water and hence all extracts were dissolved in deionised water and the UV-Vis experiments were run with deionised water as a blank. The spectra above show that the extracts are purified as few peaks were observed (Giridhar and Mahavidyalaya, 2015). The maximum 247nm (KTC3) could be indicative of the presence of flavonoids or quercetins, 295 and 358nm (KTC4) could indicate the presence of flavonoids and 287nm (KTC5) could be a phenolic compound (Porto *et al.*, 2016; Dhivya and Kalaichelvi, 2017; Kalaichelvi, 2017).

4.7.2 High Performance Liquid Chromatography

There are no known HPLC methods for the analysis of *K. wilmsii* as far as we know. The method developed in this research showed a good resolution. Several methods were developed but emphasis will be put on the 3 extracts that showed marked antimicrobial activity as well as

gave good NMR signals. The other examples of the chromatograms developed for the other extracts will be shown.

Some of the HPLC chromatograms developed and their conditions are described here. The HPLC method development procedures were undertaken for many different extracts before the purification process. The resolution of the peaks was good, and some chromatograms need further development.

The water extract was run on the HPLC with a PDA detector (200to 800nm) and the best chromatogram (Figure 20) was extracted at 254nm. The chromatogram shows several peaks.

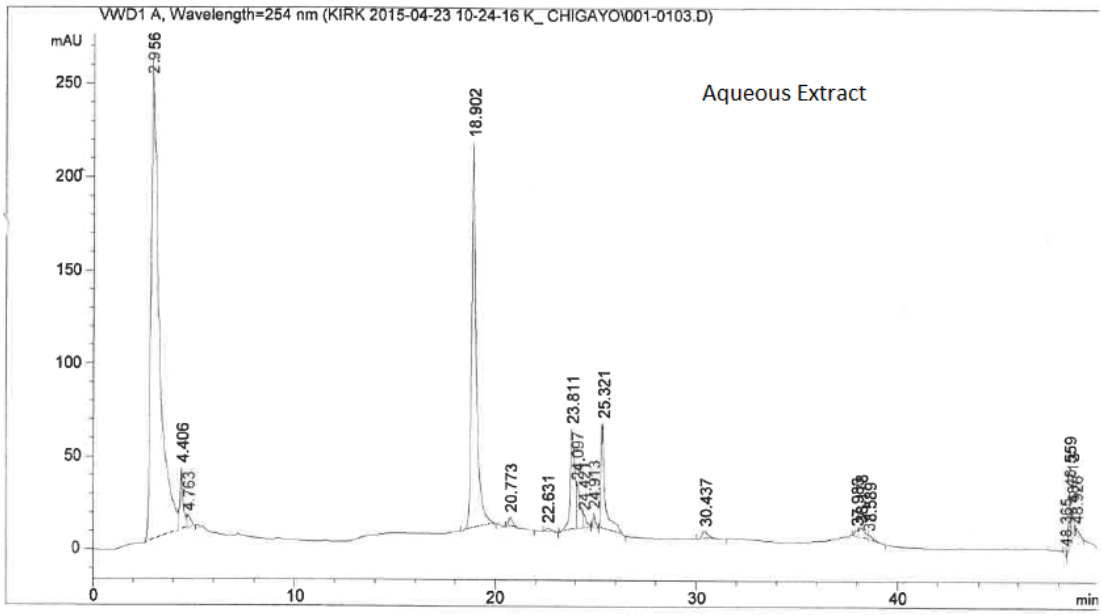

Figure 20 : HPLC chromatogram of aqueous extract at 254nm

with some resolved peaks and some peaks that need further development. This chromatogram was produced after several adjustments to improve the resolution. There were 4 major peaks at 3, 19 24 and 25 minutes. There are several small peaks after the major peak at 3 minutes which need further resolution through method development. The same should be done from 23 to 26 minutes, around 38 minutes and around 48minutes.

Method development was also carried for more extracts that is, methanol, ethanol, MCW, 80% methanol, 60% methanol and 80% ethanol extracts some of whose chromatograms are shown in Figure 21. The methanol chromatogram shows that the extract has many components at 3, 3.3, 4.8, 5, 5.3, 6.3 7, 7.8 13, 15 and 18 minutes. Further separation of the peaks was needed. The ethanol and 60% methanol chromatograms produced fewer peaks as shown below.

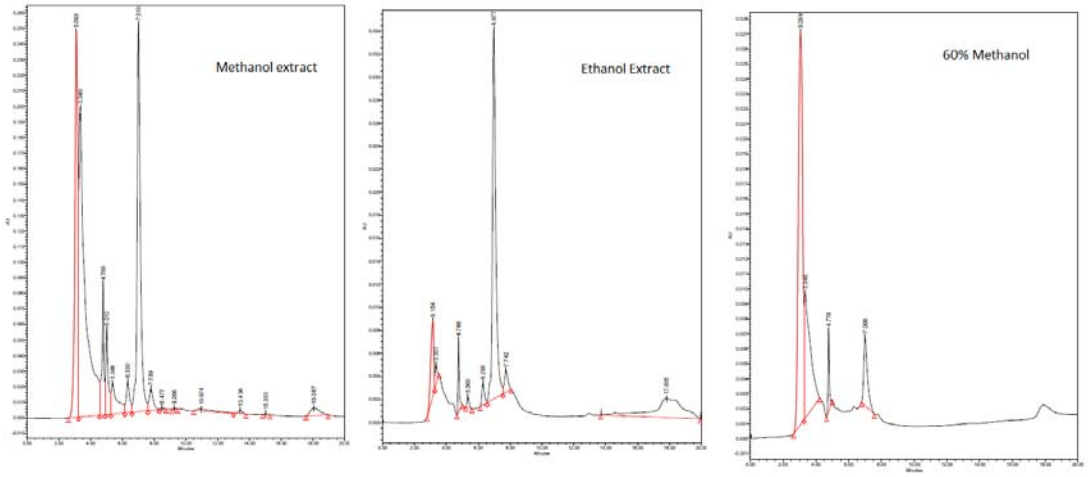

Figure 21: Chromatograms of other extracts (methanol, ethanol and 60% methanol) investigated

The developed chromatogram for KTC3 is shown in Figure 22. Major peaks were observed at 2.5, 3.8, 10.6, 12.3, 18.7 and 34.3minutes. The peak at 34 minutes was the broadest and the resolution was good for this peak and the peak at 118.7minuts. Better resolution was needed between the peaks at 2.5 and 3.9 minutes. Although the resolution between the peaks at 10.6 and 12.3 minutes was good, it could be improved. Purification could have been performed the HPLC as the peaks were well resolved as they appeared at different retention time. The purification process was however carried out using the traditional column chromatography.

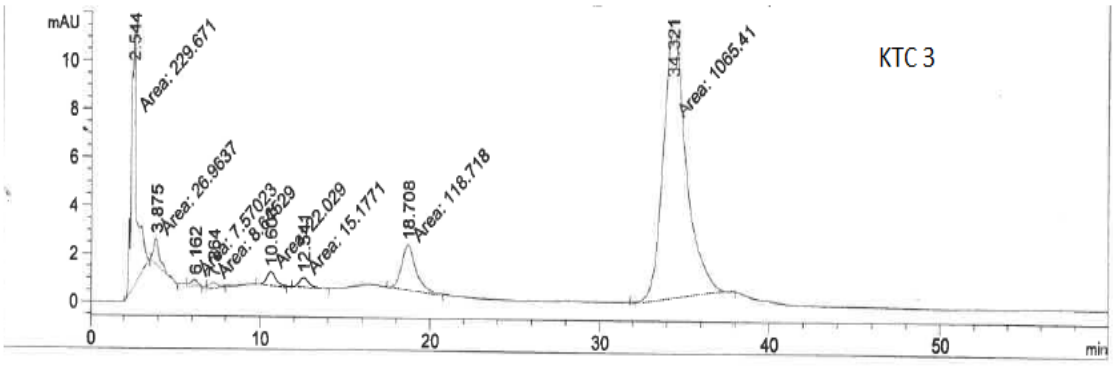

Figure 22: Chromatogram for extract KTC3 before purification

KTC4 produced a chromatogram with two major peaks at 8.7 and 14.3 minutes (Figure 23). However, there are many small peaks at 5.7, 6.7, 7.8, 10.5, 16.1, 20.4, 23.4 and an unresolved peaks in the region between 2 and 4 minutes.

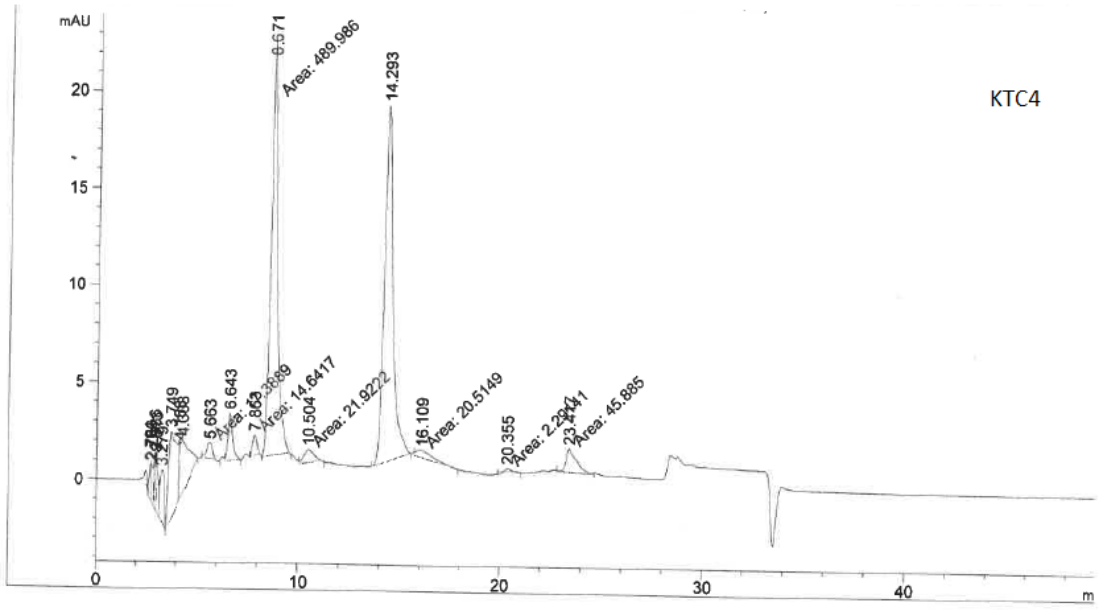

Figure 23: Chromatogram for extract KTC4

The peaks at 5.7, 6.7, 7.8, 8.7, 10.5, 14.3, 16.1, 20.4 and 23.4 minutes could have been collected using the fraction collector without any further method development. Further resolution would be needed between 2 and 4 minutes.

The chromatogram for KTC5 (Figure 24) was characterised by peaks at 3.3, 4.5, 5.1, 5.4, 5.6, 6.4, 7.3, 8.1, 8.9, 9.6, 10.4, 15 and 18 minutes. The resolution was also generally good in this chromatogram and fraction collection could have proceeded smoothly for the majority of peaks. Resolution of the peaks at 3.3 and 3.3minutes as well as the peaks at 5.1, 5.4 and 5.5 could be needed.

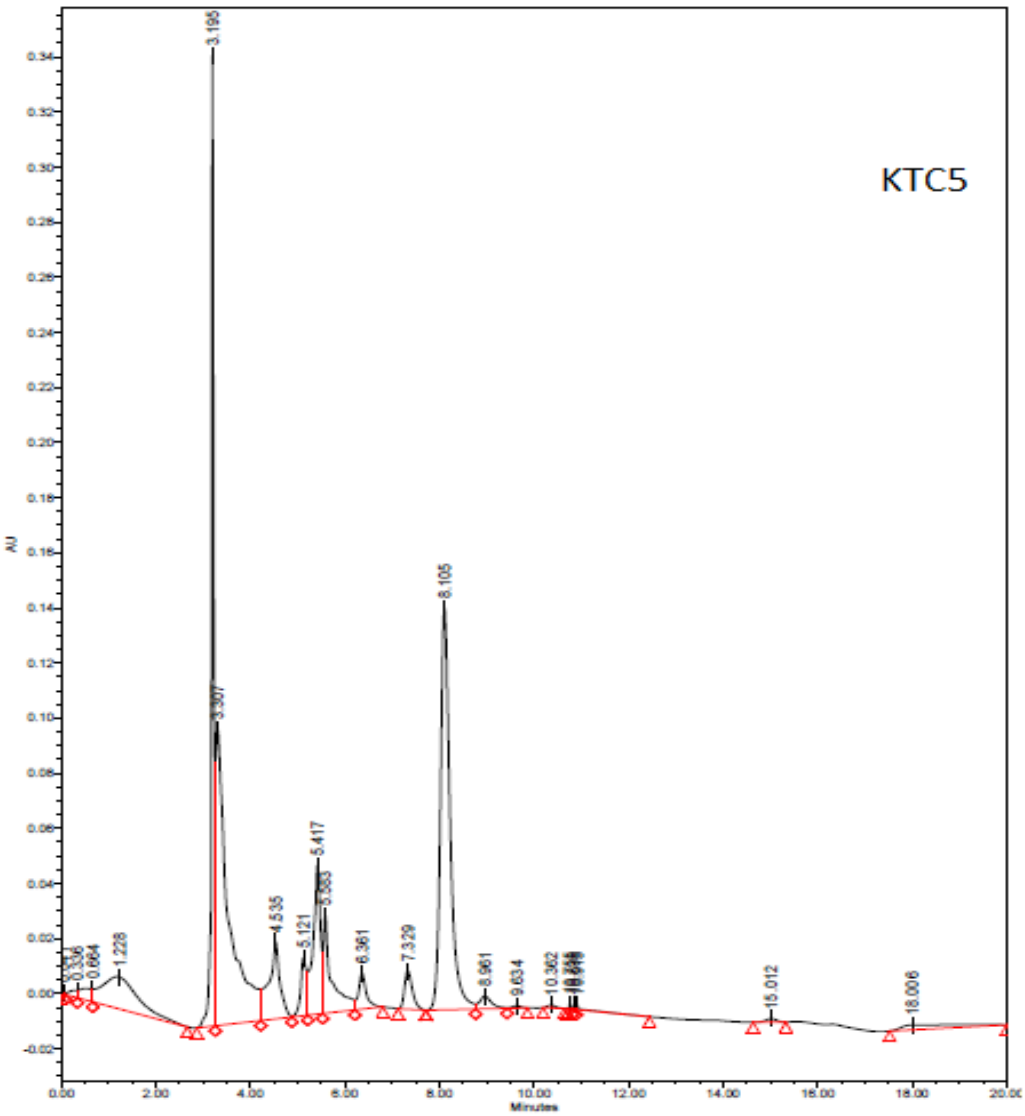

Figure 24: Chromatogram for extract KTC5

The chromatographic finger prints of the extracts obtained can then be used in drug discovery processes especially for the bioactive identification of the compounds of interest (Xu *et al.*, 2015)(Xu *et al.*, 2015). Through the fingerprint studies it can be deduced from the HPLC profile of the extracts, how many peaks and which peaks in particular are responsible for the bioactivity of the plant. Many different models have been proposed for such studies and they have been proven to give reliable information. These models include the sure independence screening and interval partial least squares (Xu *et al.*, 2015), the orthogonal partial least squares discriminant analysis (Rajalahti, Arneberg, Berven, *et al.*, 2009) and the discriminating

variable (DIVA) test and the selectivity ratio (SR) plot (Rajalahti, Arneberg, Kroksveen, *et al.*, 2009). These aspects will need to be investigated further.

4.8 Fourier Transform Infrared Spectroscopy

The data for the FTIR spectra for KTC3, KTC4 and KTC 5 (Figure 25 – 27) was extracted and an analysis of the 3 compounds are shown in table 26. The FTIR spectra are similar but showing slight differences and the similarities and differences are shown in the table. The differences are confirmed by the UV-Vis spectra.

The FTIR spectrum is normally divided into 2 regions; the fingerprint region (up to about 1450cm^{-1}) and the functional group region above 1500cm^{-1}. The 3 compounds have the same similar functional group spectra implying that their functional groups which is confirmed by the analysis of the FTIR spectra depicted in all below are the same with differences in the fingerprint region.

Table 26: FTIR results and interpretation

	(wave number cm^{-1})		
	KTC3	**KTC4**	**KTC5**
1	3254; broad -OH carboxylic acid or alcohol	3263; broad -OH carboxylic acid or alcohol	3297; broad -OH carboxylic acid or alcohol
2	2923; C-H alkyl	2928; C-H alkyl	2935; C-H alkyl
3	2100; C≡C terminal alkyne	2100; C≡C terminal alkyne	2082; C≡C terminal terminal alkyne
4		1716; C=O conjugated acid halide, aliphatic ketone or aldehyde	1718; C=O conjugated acid halide, aliphatic ketone or aldehyde
5	1611; C=C alkenyl or aromatic or N-H amine	1605; C=C alkenyl or aromatic or N-H amine	1617; C=C alkenyl or aromatic or N-H amine
6			1396; phenolic -OH
7	1365; C-H gem dimethyl	1352; C-H gem dimethyl	1352; C-H gem dimethyl
8	1245; S=O or C-O or aromatic C-H	1222; S=O or C-O or aromatic C-H	1212; S=O or C-O or aromatic C-H
9	1195; C-O		

10	1145; C-O		
11	1027; C-O or C-F	1029; C-O or C-F	1027; C-O or C-F
12		998; C-H stretch	
13	929; C=C		918; C=C
14	893; C=C		
15		866; C=C	
16	816; C=C	818; C=C	817; C=C

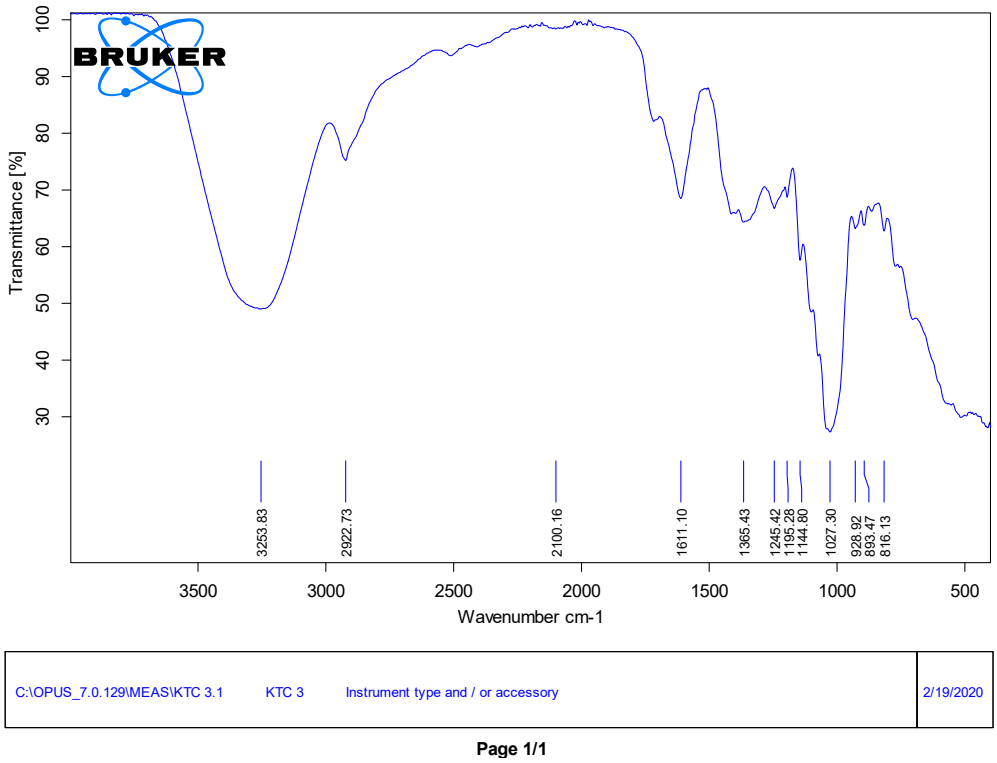

Figure 25: FTIR spectrum of KTC3

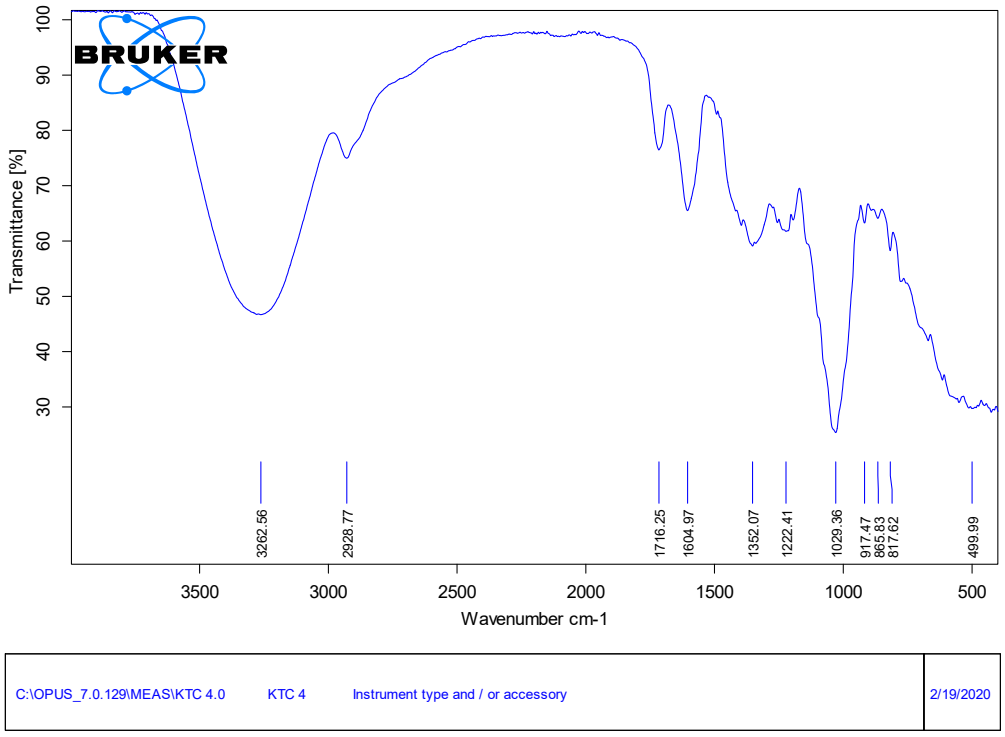

Figure 26: FTIR spectrum of KTC4

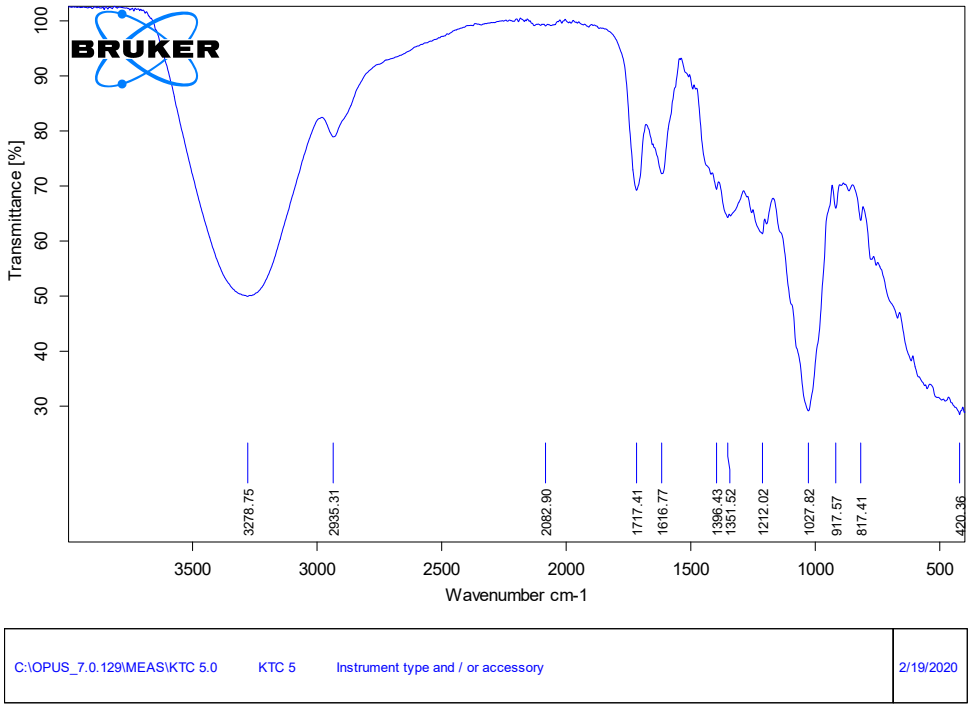

Figure 27: FTIR spectrum of KTC5

The suggested functional groups could show the presence of the following: S=O – organic sulphates; P=O – organic phosphates; C-O – ketone, ester and carboxylic acids. All the IR stretches were assigned according to literature (Coates, 2000).

The functional groups found in the three compounds will be confirmed by GC-MA and NMR.

4.9 Gas Chromatography – Mass Spectrometry

The GC-MS chromatograms and spectra obtained from the Perkin Elmer Clarus 680 GC are shown in Figures 28-30. Out of the 32 final purified extracts, KTC 3, KTC 4 and KTC 5 were chosen as the concentrations were high enough to be easily picked up by NMR.

The GC produced a chromatogram with a peak at 5.77minutes for KTC3, whose MS produced the base peak with a mass to charge ratio of 97 and the parent peak gave a mass to charge ratio of 118. The base peak indicates either the most common fragment or the most stable fragment while the parent peak gives an indication of the heaviest fragment produced, which is most likely to be the molar mass of the compound under analysis. Therefore, KTC3 compound is likely to have a molar mass of 118g/mol. KTC4's main peak was at 5.75 minutes exhibiting a mass spectrum with a base peak of 97 and parent peak of 119.

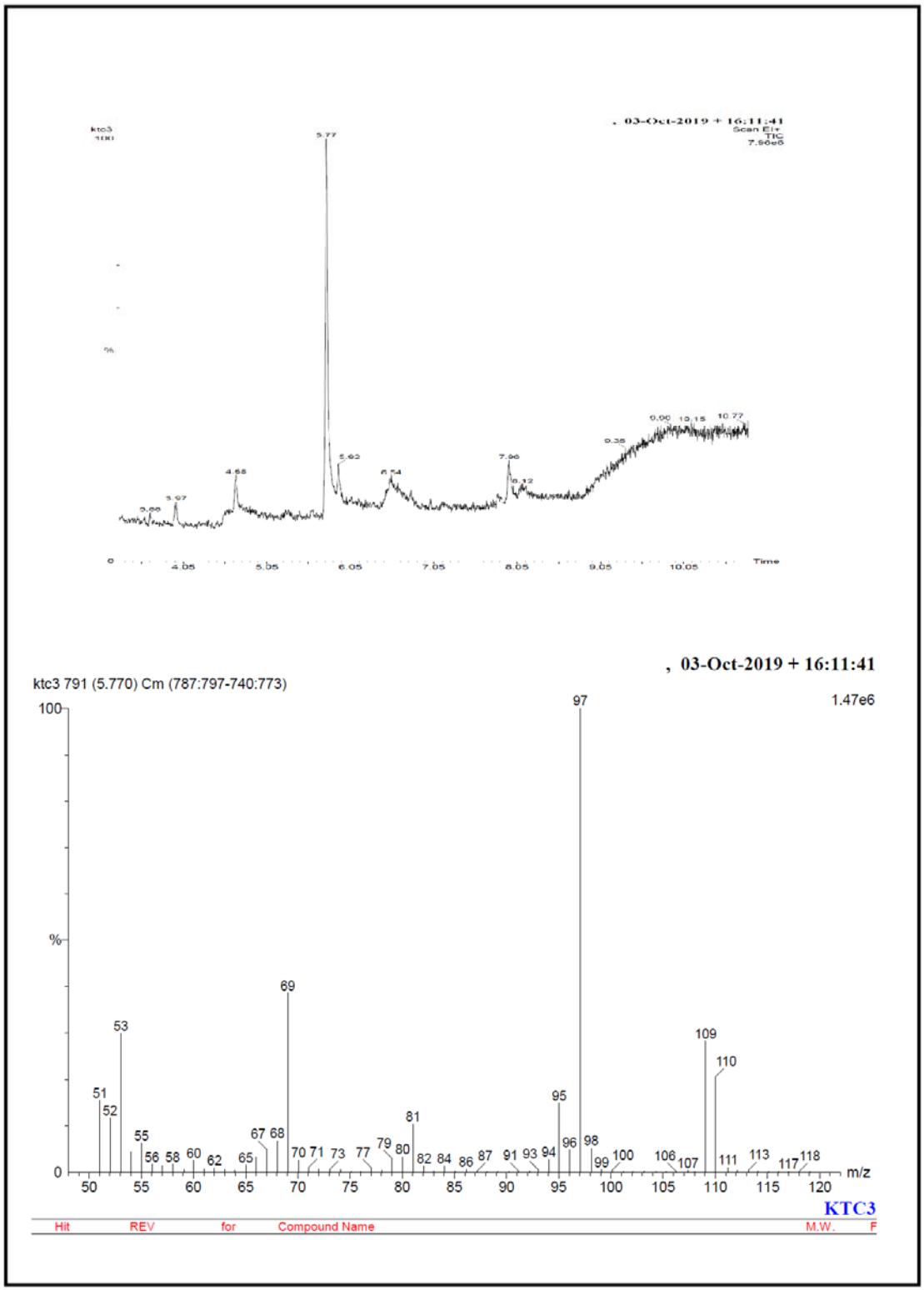

Figure 28: GC chromatogram of extract KTC3 and corresponding mass spectrum

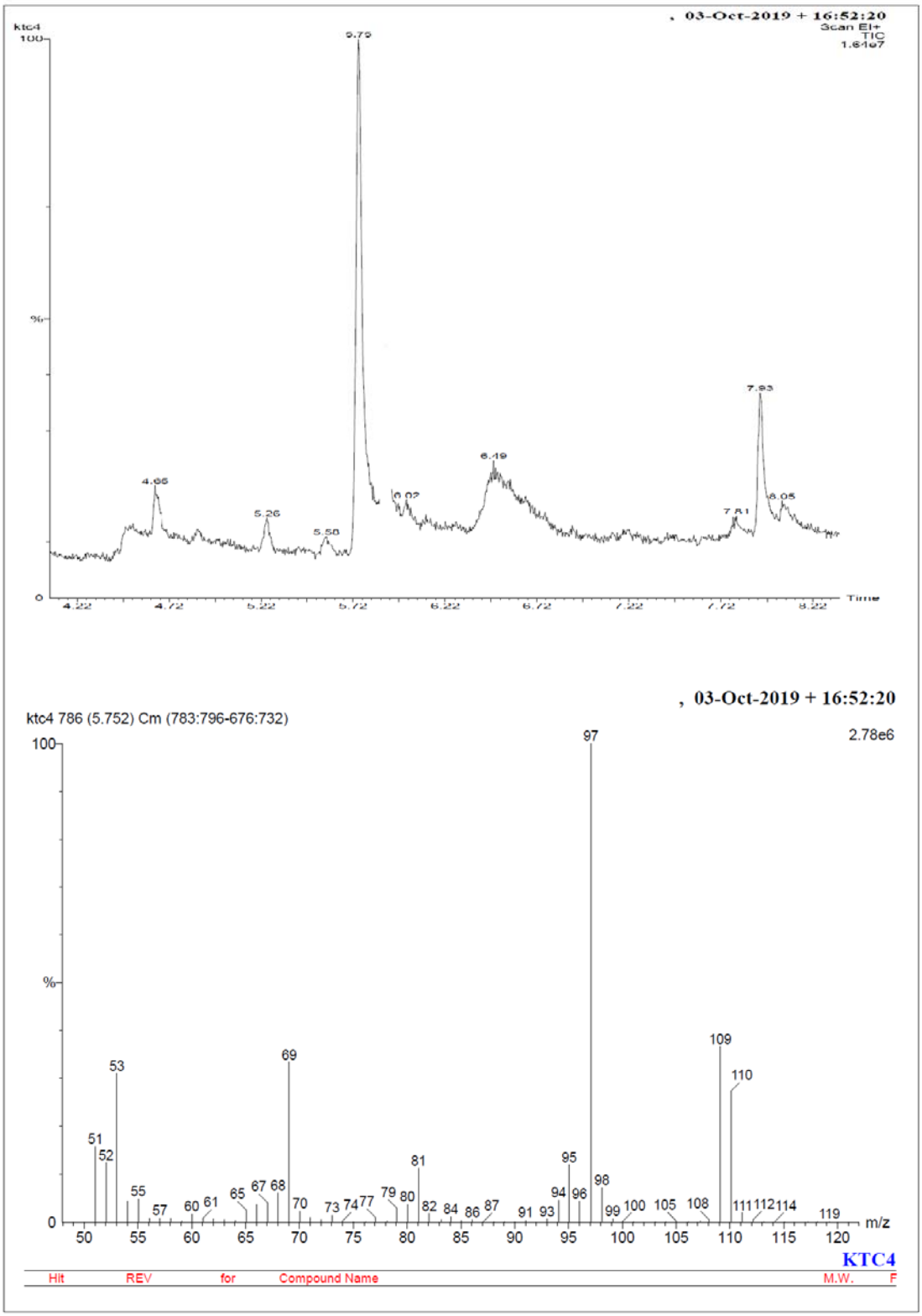

Figure 29: GC chromatogram of extract KTC4 and corresponding mass spectrum

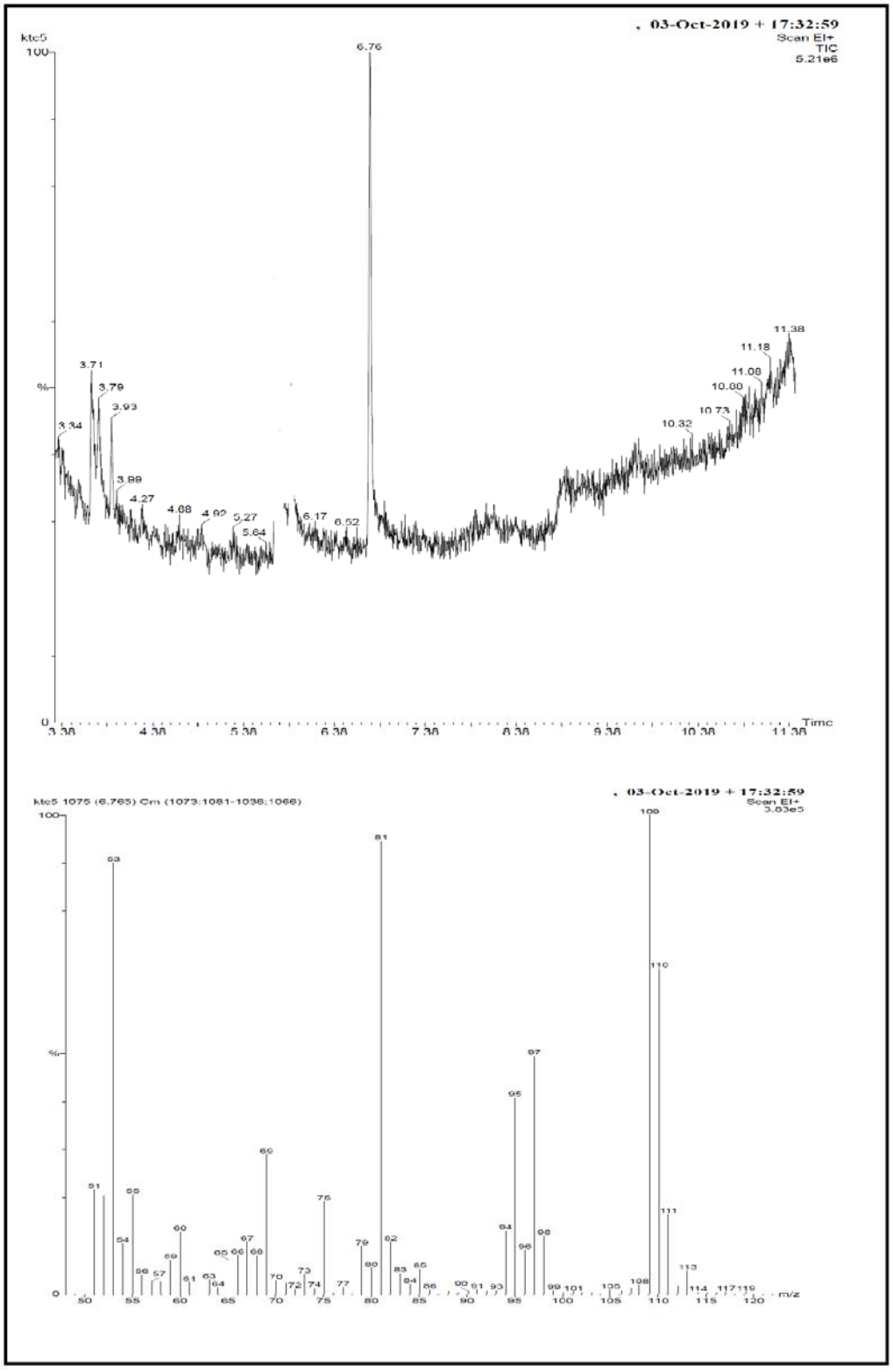

Figure 30: GC chromatogram of extract KTC5 and corresponding mass spectrum

The GC chromatograms for KTC3 and KTC4 differ very slightly in retention time which are 5.77 and 5.75minutes. The GC chromatograms for KTC5 had a retention time of 6.76minutes, a base peak of 109 and the parent molecule of molar mass 119.

The mass spectra are also very closely related with slight differences. However, their UV-Vis spectra are clearly different (Figure 17) as KTC 3 had a maximum at 247nm only whereas KTC 4 had 3 maxima at 290, 295 and 358nm.

According to the nitrogen rule for MS, an organic compound with an even number molecular weight is likely to contain C, H, O and halogens in the structure, which is the case for KTC3 whose parent peak is 118. According to the same rule, KTC4 and KTC5 might contain a nitrogen atom as the molecular weight is an odd number, the presence of an odd number of N atoms is anticipated. Therefore, KTC4 (parent peak 119) and KTC5 (parent peak 119) are likely to have an odd number of N atoms.

It should be noted that not all MS spectra give a parent peak as some are not stable enough to endure the whole process until detection. Therefore the parent peak rule is not 100% fool proof. Some compounds do not produce it at all!

The presence of isotopes in compounds is also detected by the M, M+1 and M+2 lines in the mass spectra. KTC3 has the lines 117 and 118 (M and M+1) and KTC5 has117 and 119 (M and M+2). This could mean that only KTC5 contains the isotopes ^{79}Br and ^{81}Br while KTC3 might contain the ^{13}C isotope.

4.10 Nuclear Magnetic Resonance

There are challenges that are met during drug discovery research from natural products and one of them is structural elucidation (Kaneko, Cooper and Mdluli, 2011). Although chemistry has been blessed with development in instrumental methods, the challenge remains. Improvement have been seen after the development of new methods such as HMBC and HSQC experiments. These new methods help in identifying interactions between neighbouring atoms. 1D and 2D tests were performed in this research. The signals obtained for KTC3, KTC4 and KTC are shown in Appendix E.

4.10.1 KTC3 Extract

An analysis of the NMR results for KTC3 are summarised in table 27. The H in the region 2.5-4.5 are connected to an electronegative atom such as N, O or Cl. The H atoms between 4.5

and 6.5 signify the presence of C=C-H protons. The protons between 6.5to 8 indicate the presence of aromatic protons. The signals between 9 and 10 signify the presence of O=C-H.

Table 27: NMR data for purified extract KTC3

Pos	^{13}C NMR	^{1}H NMR
1	29.31	
2	48.06	3.16q (dd) (0.95)
3	61.31	3.33s (3.49)
4	63.18	3.38 m (1.81)
5	64.35	3.47m (0.42)
6	67.89	3.50s (0.81)
7	70.41	3.70multiplet (3.61)
8	72.40	3.72d (1.26)
9	73.43	3.80 t (2.94)
10	74.86	3.88t (1.44)
11	76.16	4.15t (1.18)
12	81.81	4.52d (0.63)
13	92.53	5.15d (1.00)
14	96.76	7.08(0.13)
15	97.88	7.56s (0.01)
16	101.74	9.55s (0.02)
17	144.96	

The ^{13}C experiment shows that there are 17 non-equivalent carbon atoms in the molecule KTC3. The DEPT135 experiment proves that peaks at 61.31, 63.18 and 64.35 are facing down showing that the C atoms are connected to 2 H atoms. The chemical shifts indicate the presence of -CH2 (20-65), ≡C (70-90) and =C (100-150) and aromatic (120-170) (Zenebe *et al.*, 2015)

The final structure (Figure 31) as predicted from this data and computers assisted structural elucidation was:

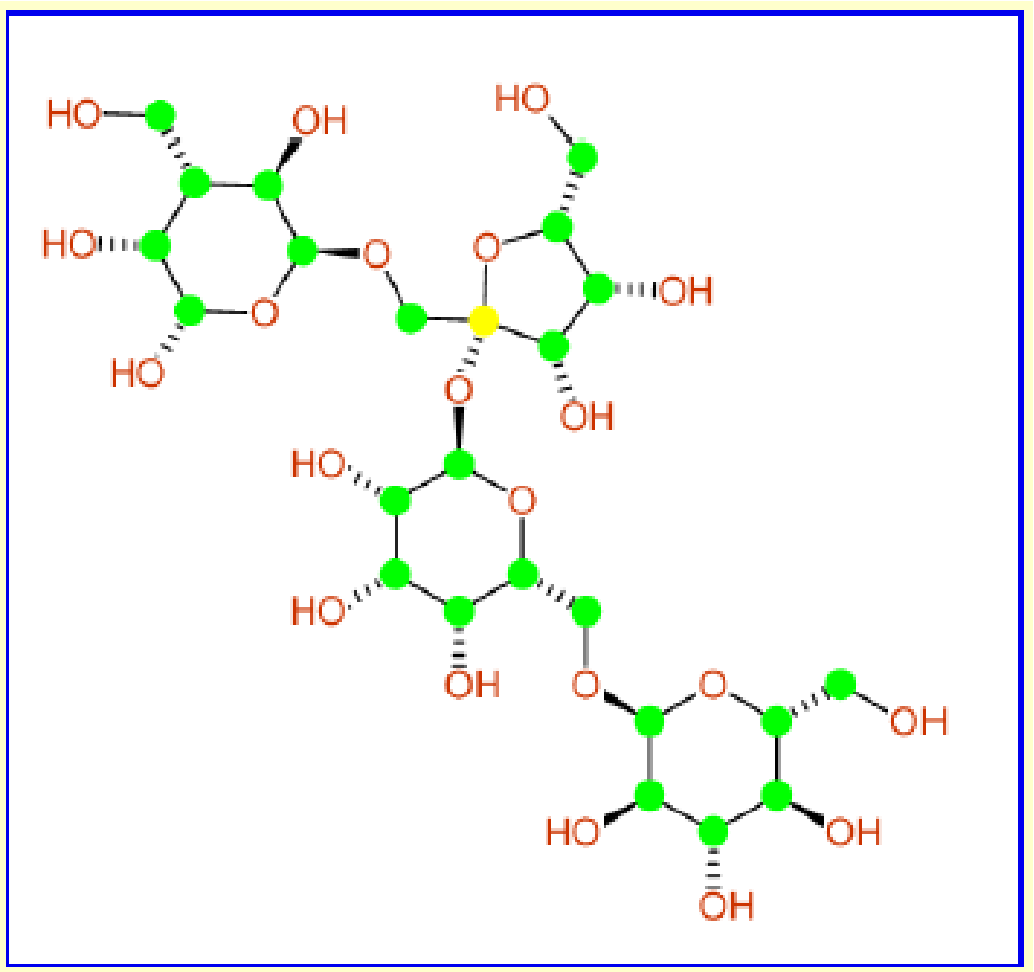

Figure 31: Proposed structure for KTC3

4.10.2 KTC4 Extract

An analysis of the NMR results for KTC4 are summarised in table 28. The chemical shifts of the protons were recorded in ppm and the shifts show that protons in the region 2.5-4.5 are connected to an electronegative atom such as N, O or Cl. The H atoms between 4.5 and 6.5 signify the presence of C=C-H protons. The protons between 6.5 to 8 indicate the presence of aromatic protons. The signals between 9 and 10 signify the presence of O=C-H.

Table 28: NMR data for purified extract KTC4

Pos	^{13}C NMR	^{1}H NMR
1	48.26	3.04quintet (32.83)
2	61.32	3.21d (137.72)
3	63.13	3.26t(64.49)

4	63.69	3.36s (15.75)
5	64.41	3.39d (31.85)
6	67.93	3.56multiplet (179.41)
7	70.42	3.69t (112.74)
8	71.57	3.75d (47.97)
9	72.42	3.87s (20.43)
10	72.69	3.92sd (20.53)
11	73.45	3.95d (32.18)
12	74.87	4.09s (11.22)
13	75.04	4.39d (24.67)
14	75.34	5.02d (41.59)
15	76.12	6.26s (2.09)
16	76.66	6.5d (2.00)
17	81.83	6.6d (2.61)
18	82.78	6.95s (6.47)
19	92.54	7.05d (3.81)
20	96.77	7.3q (2.10)
21	97.84	7.45d (3.63)
22	101.74	7.7s (1.30)
23	104.44	8.2s (0.72)
24	144.96	9.4s (1.00)

The ^{13}C experiment shows that there are 24 non-equivalent carbon atoms in the molecule KTC4. The chemical shifts indicate the presence of -CH2 (20-65), ≡C (70-90) and =C (100-150) and aromatic (120-170)

The DEPT135 experiment proves that peaks at 61.32, 63.69 and 64.41 are negative and the C atoms are connected to 2 H atoms.

The final structure (Figure 32) as predicted from this data and computers assisted structural elucidation was:

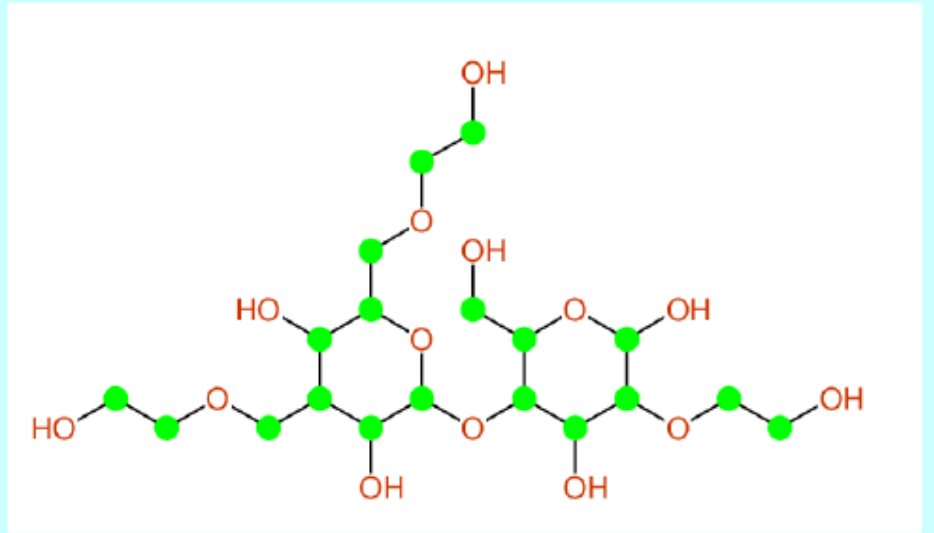

Figure 32: Proposed structure for KTC4

4.10.3 KTC5 Extract

An analysis of the NMR results for KTC5 are summarised in table 29. The H in the region 2.5-4.5 are connected to an electronegative atom such as N, O or Cl. The H atoms between 4.5 and 6.5 signify the presence of C=C-H protons. The protons between 6.5 to 8 indicate the presence of aromatic protons. The signals between 9 and 10 signify the presence of O=C-H.

Table 29: NMR data for purified extract KTC5

Pos	^{13}C NMR	^{1}H NMR
1	29.02	2.6d(1.04)
2	47.46	3.2p (1.39)
3	61.37	3.3s(2.48)
4	63.64	3.4t (1.84)
5	64.35	3.45d (1.33)
6	67.93	3.7multiplet (4.59)
7	70.05	3.85d (2.47)
8	71.58	3.90d (1.44)
9	72.34	4.1q (1.85)
10	72.95	4.2s (0.91)
11	74.33	4.5d (1.24)
12	75.31	6.31d (0.18)
13	76.59	6.41d (0.14)

14	81.88	6.62ds (0.09)
15	92.50	6.83t (0.16)
16	97.86	7.07 s (0.23)
17	98.06	7.16s (0.17)
18	101.72	7.47q (0.46)
19	144.93	7.71d (0.33)
20		9.54s (0.11)

The ^{13}C experiment shows that there are 19 non-equivalent carbon atoms in the molecule KTC5. The chemical shifts indicate the presence of -CH2 (20-65), ≡C (70-90) and =C (100-150) and aromatic (120-170)

The DEPT experiment proves that peaks at 29.02, 61.37, 63.64 and 64.35 are negative and the C atoms are connected to 2 H atoms.

The final structure (Figure 33) as predicted from this data and computers assisted structural elucidation was:

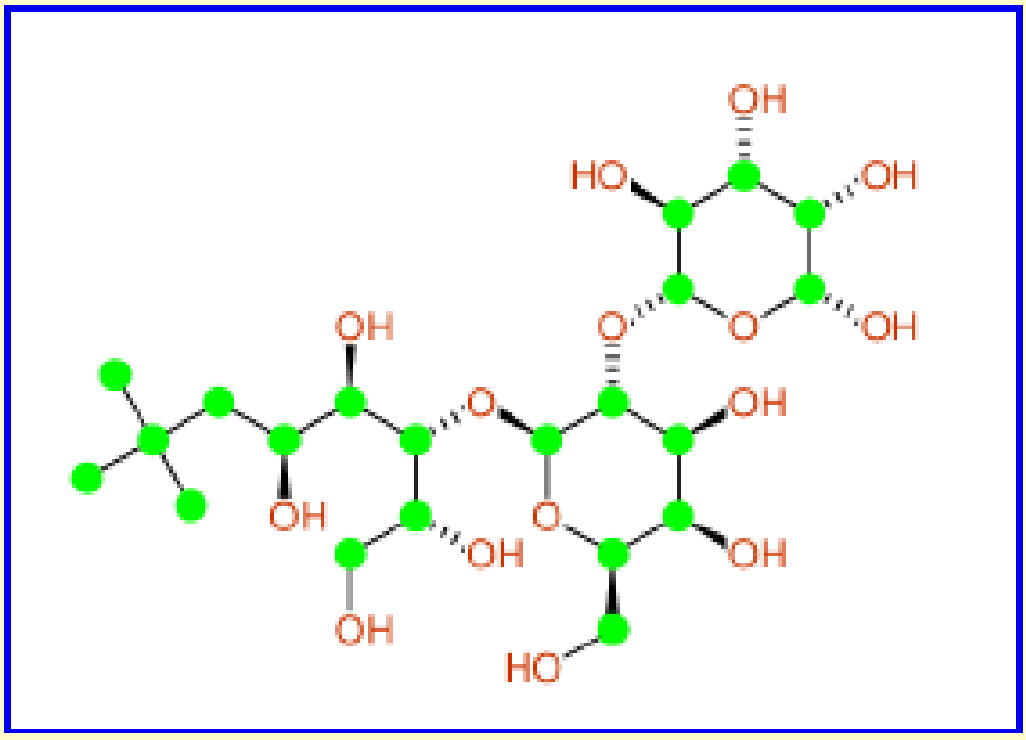

Figure 33: Proposed structure for KTC5

The three proposed structures that were isolated from *K. wilmsii* show the same tetrahdyopran core that was found in some bioactive compounds isolated from other plants. These compounds include a strong analgesic cis-(6-ethyl-tetrahydropyran-2-yl) formic acid isolated from the Brazilian plant *Vitex* (Miranda *et al.*, 2004) *and* antifungal and cytostatic macrolides, phorboxazoles A and B extracted from Indian Ocean marine sponge *Phorbas* (Searle and Molinski, 1995).

The proposed structures also confirm the presence of tretrahydropyrans which are also present in tannins and deoxy sugars found in the phytochemical screening tests.

CHAPTER FIVE: CONCLUSIONS

Ethanol, methanol, methanol/chloroform/water, 80% methanol, 60% methanol and water can be used as suitable phytochemical extraction solvents for *K. wilmsii* tubers. Total phenolic content and total flavonoid content analysis proved the presence of high levels of phenolic compounds as well as flavonoids. The presence of phenols and flavonoid could be responsible for the radical scavenging activities observed during the DPPH test.

MCW, 80% methanol, 50%acetone and 80% ethanol were recorded at the most potent extracts as they showed activity against the highest number of pathogens. *K. pneumoniae*, was resistant against all the extracts and the positive control. The results led to the conclusion that there could be some similarities between the *K. wilmsii* extracts and amoxycillin, a broad-spectrum antibiotic. The aqueous extracts tested negative for all micro-organisms under investigation. MIC values obtained for most of our extracts were also encouraging.

The powder may be considered suitable for use as a drug or a drug precursor. All parameters measured were within specifications and the metal content was low, which shows that the plant tubers do not pose a danger to human life. The measured parameters, moisture content, acid soluble ash, water soluble ash and heavy metal content all gave results that are well within specifications. The results also match the sub-chronic toxicity tests where no abnormal results were recorded on the monkeys fed on a *K. wilmsii* diet.

The consumption of *K. wilmsii* did not result in any unexpected issues with regards to animal welfare, morbidity or mortalities. No physiological or psychological signs of abnormalities indicative of unwell-being or distress were observed throughout the duration of the study. The

test did not deviate significantly in most of the tested parameters when compared to the control. Changes in all the parameters after consumption of the plant product were not associated with any clinically significant changes.

The overall finding of this study suggests that *K. wilmsii* tubers are safe to consume and can be regarded as non-toxic.

Of these 35 extracts only 3 were finally selected for structural elucidation mainly because the other extracts did not give signals on the NMR as their concentrations were too low. When the concentrations are too low, it takes too long to run all the experiments on the NMR.

KTC3, KT4 and KTC5 chromatograms were recorded and the resolution was generally satisfactory and a few components need to be separated. The HPLC profiles were meant to provide a tool for the purification of the extracts. The results obtained could further be used as the baseline information for further work.

KTC3, KT4 and KTC5 have similar FTIR traces with minor differences. The functional group region of the compounds is very similar and there are minor differences in the fingerprint region which shows that the 3 compounds are closely related but different as conformed by the UB-Vis spectra which sow distinct differences. The peaks above 1450cm-1 indicate that all the compounds could either be alcohols or carboxylic acids.

GC-MS results could not be used for the structural elucidation as there is a possibility that the compounds disintegrated and could not give collaborative results.

The NMR of the 3 compounds were recorded and the information obtained was deciphered. The DEPT135 experiment proves that the compounds are closely related. The structures of the compounds KTC3, KTC4 and KTC5 were proposed.

CHAPTER SIX: RECOMMENDATIONS

The HPLC method development tests are to be pursued as all the different extracts methods of analysis need to be developed and validated.

The antimicrobial activity tests are to be expanded. The individual purified extracts are to be tested to check whether the antimicrobial activities obtained before purification are maintained after splitting of extracts.

An investigation of synergic effects of the purified extracts would be informative.

Structure-activity relationship tests to be undertaken which shows which parts of the molecule cause the biological activity and which parts do not.

NMR analysis of the other extracts that were not investigated in this study are to be pursued.

Human clinical studies should be conducted to support the in-vitro studies and toxicology results obtained in the current study.

Synthesis, theoretical and computational studies of the isolated natural products that generate antimicrobial activity results of importance could be pursued. These studies could be used to confirm the results obtained from the natural extracts under investigation.

Tests on the extracts could be to determine bioavailability, adverse effects and metabolic half-life of the active ingredients in the extracts.

Fractionation using the HPLC will still be pursued once the fraction collector is functional.

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Appendices

Appendix A: Raw Data for Solvent extraction

Table A1: Results of the solvent extraction experiments

Solvent	15 minutes		30 minutes		60 minutes	
	% yield		% yield	Std dev	% yield	Std dev
Methanol	8.6 ± 0.9		13.9 ± 0.4		36.6 ± 1.3	
Ethanol	5.2 ± 0.4		7.1 ± 0.4		7.3 ± 0.6	
Dichloromethane	0.1		0.3		0.4	
Chloroform	0.4		0.4		0.5	
Acetone	0.8		1.4		1.5	
M/C/W	10.7 ± 0.6		11.0 ± 0.4		48.2 ± 2.4	
80% Methanol	12.9 ± 0.4		18.8 ± 0.5		52.9 ± 3.6	
60% Methanol	7.3 ± 0.4		34.9 ± 0.9		46.9 ± 2.5	
Water	5.9 ± 0.3		13.9 ± 0.4		20.9 ± 2.5	
Hexane	0.2		0.4		0.8	
Diethyl ether	0.3		0.3		0.4	
Ethyl acetate	0.2		0.4		0.6	

Appendix B: Raw Data Quantitative Tests

Table B1: Antioxidant activity test results performed through the DPPH test

	%Inhibition						
Conc (µg/ml)	**Ascorbic**	**Ethanol**	**Methanol**	**MCW**	**80%MeOH**	**60% MeOH**	**Water**
10.00	1.41 ± 1.15	0.49 ± 1.15	2.41± 2.08	2.55 ± 1.80	1.58 ±3.80	2.38 ± 2.08	-6.11 ±3.00
20.00	1.99 ± 8.78	6.16 ± 3.92	4.47 ± 3.93	3.25 ± 0.92	5.56 ± 1.62	3.52 ± 3.40	-1.11 ± 2.31
30.00	16.14 ± 1.15	6.92 ± 1.62	9.02 ± 4.19	5.45 ± 3.20	6.00 ± 1.85	5.96 ± 3.83	-0.36 ± 3.32
40.00	35.72 ± 1.60	7.11 ± 3.00	13.22 ± 1.39	7.68 ± 3.00	9.63 ± 3.00	6.17 ± 1.85	0.65 ± 2.19
50.00	42.06 ± 1.29	8.18 ± 3.23	15.00 ± 5.52	11.37 ± 1.39	9.94 ± 2.60	6.61 ± 3.00	2.96 ± 20.8
60.00	51.80 ± 0.92	9.09 ± 3.34	21.45 ± 3.33	13.42 ± 3.45	12.06 ± 3.67	7.23 ± 0.00	5.87 ± 1.62
70.00	68.09 ± 5.97	10.23 ± 2.60	22.15 ± 1.85	13.68 ± 2.54	15.59 ± 4.59	10.27 ± 0.00	10.30 ± 2.60
80.00	82.98 ± 0.42	12.72 ± 1.39	25.68 ± 4.54	16.36 ± 1.79	14.08 ± 1.15	12.82 ± 1.39	10.44 ± 6.38
100.00	94.28 ± 0.70	18.71 ± 5.03	33.66 ± 1.83	31.31 ± 1.62	31.01 ± 1.01	26.16 ± 2.40	26.87 ± 2.01
150.00	96.62 ± 0.50	38.43 ± 2.31	69.08 ± 3.82	45.36 ± 3.99	40.04 ± 7.61	48.36 ± 3.10	33.72 ±1.01
200.00	96.85 ± 0.20	53.74 ± 1.06	83.67 ± 4.20	63.96 ± 4.66	52.83 ± 2.77	65.11 ± 1.15	44.63 ± 1.01
250.00	97.33 ± 0.70	60.26 ± 3.21	93.20 ± 3.33	70.05 ± 0.92	62.69 ± 2.41	78.02 ± 3.86	55.12 ± 0.61

Table B2: Calibration data for Garlic Acid

Gallic Acid	
Conc(µg/ml)	Absorbance
0	0.0005
20	0.2381
40	0.5394
60	0.6010
80	0.9571
100	1.2558

Table B3: Calibration data for Quercetin

Quercetin	
Conc(µg/ml)	Absorbance
0	-0.0001
50	0.0082
100	0.0112
150	0.0153
200	0.0182
250	0.0235

Appendix C: Raw Data for Antimocrobial Activity

Table C1: Summary of all anti-microbial activity results

Extract	**Micro-organism inhibition zones (mm)**						
	E. coli	***S. aureus***	***S. saprophycticus***	***C. albicans***	***S. epidermidis***	***S. pyogenes***	***K. pneumon***
Amoxycillin	30.23	29.96	39.19	26.02	41.08	9.92	---
80% Acetone	---	6.38	11.37	---	10.81	---	---
MCW	7.18	15.28	22.26	---	18.45	7.09	---
Water	---	---	---	---	---	---	---
80% Methanol	10.74	8.78	6.48	5.08	11.04	22.18	---
60% Methanol	---	---	7.01	8.37	---	7.61	---
Methanol	---	---	7.82	7.96	---	---	---
50% Acetone	8.36	13.31	7.34	10.17	18.18	17.80	---
80% Ethanol	8.46	14.80	---	5.06	8.92	18.97	---
Ethanol	---	---	6.60	---	---	8.33	---

Appendix D: Biochemistry

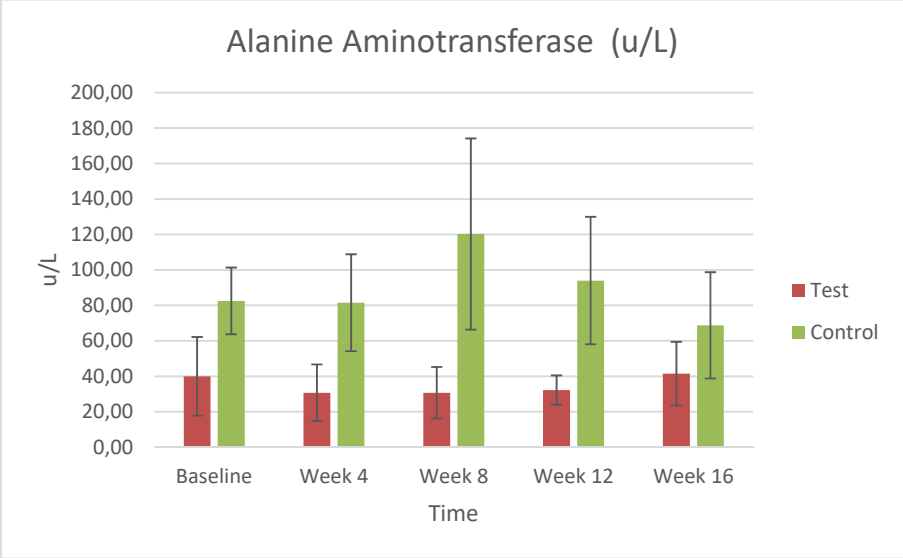

Figure D1: Alanine aminotransferase (ALT)

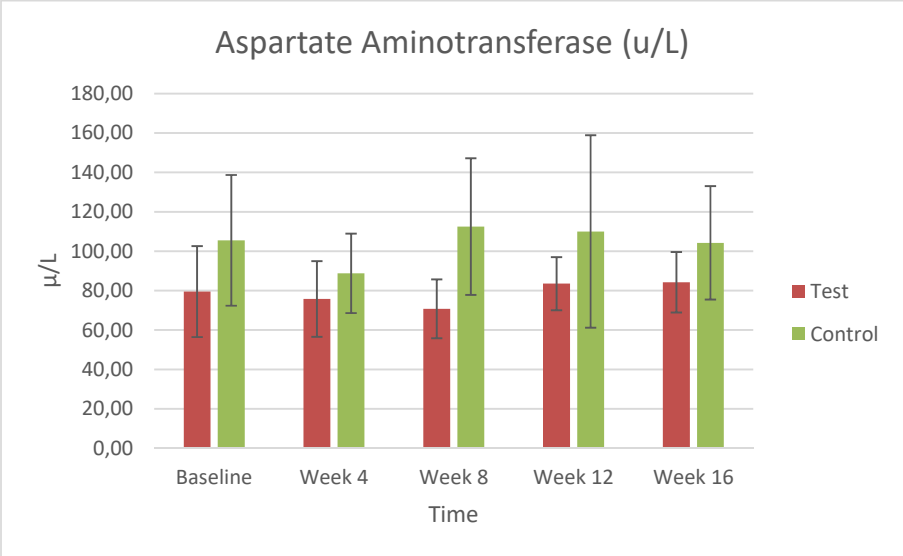

Figure D2: Aspartate aminotransferase (AST)

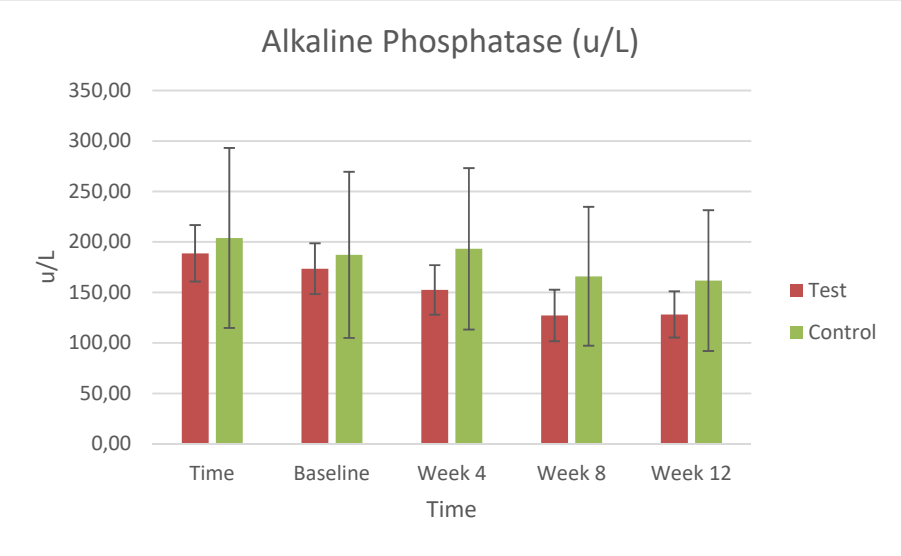

Figure D3: Alkaline phosphatase (ALP)

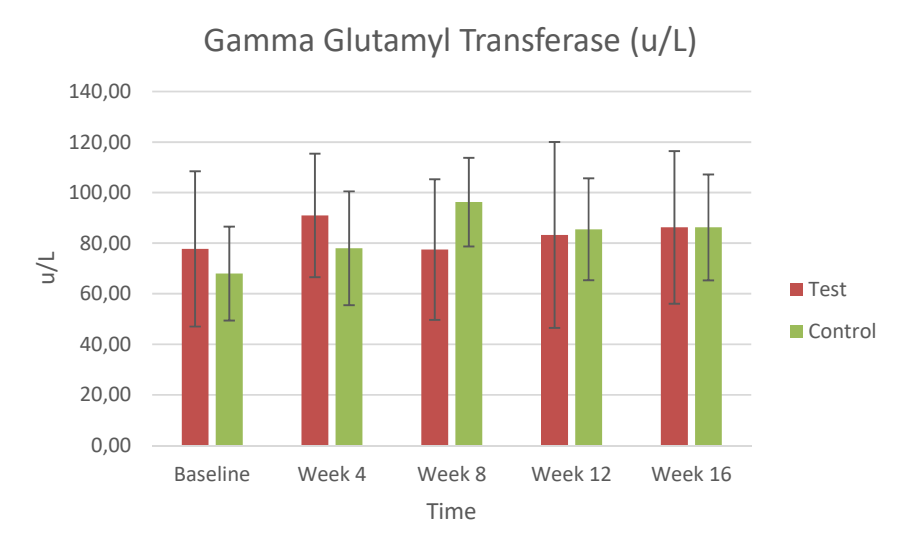

Figure D4: Gamma-glutamyl transferase (GGT)

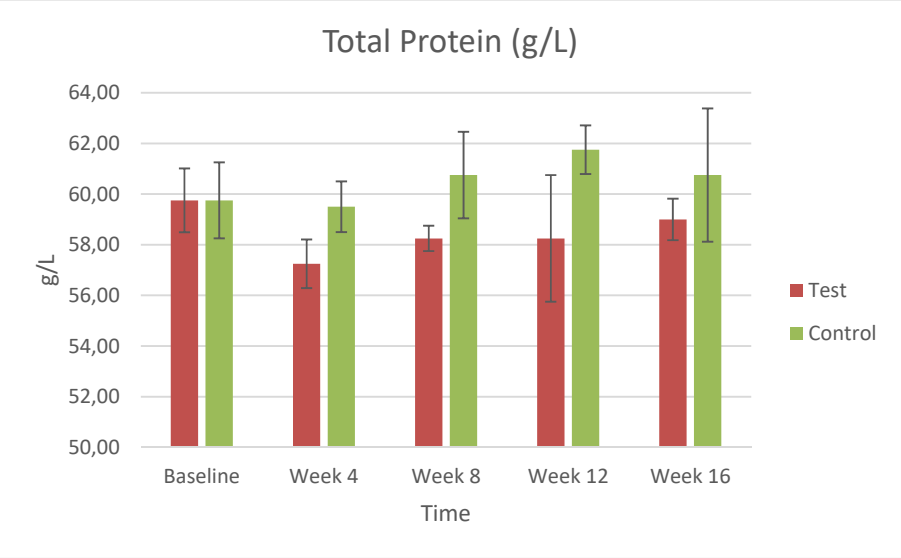

Figure D5: Total protein

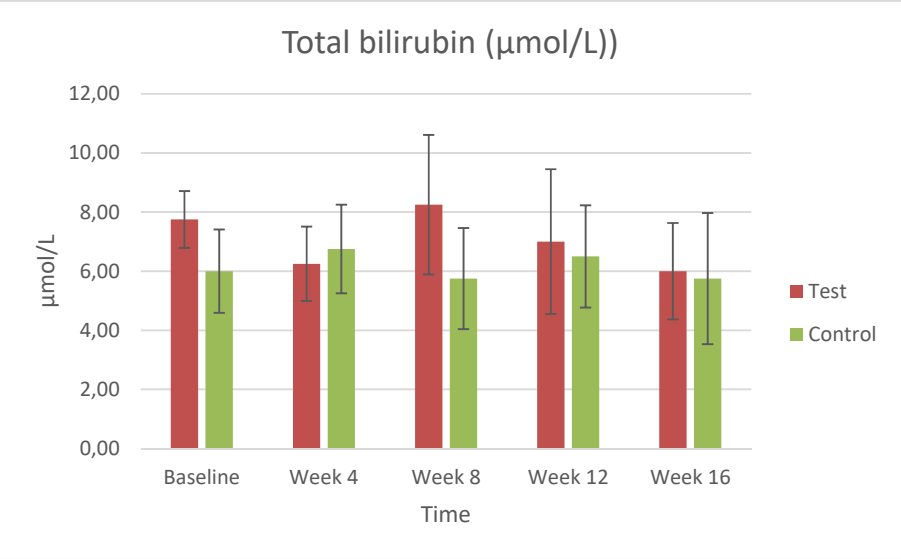

Figure D6: Total bilirubin

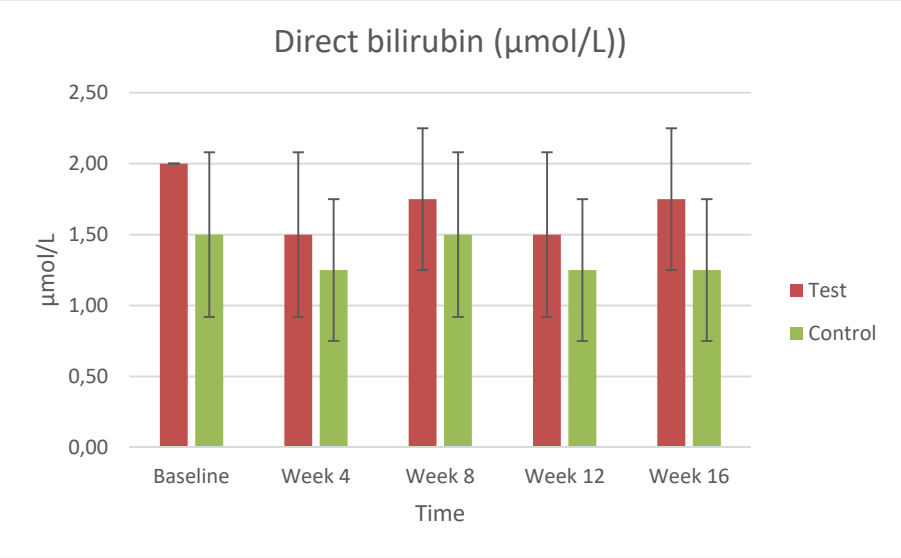

Figure D7: Direct bilirubin

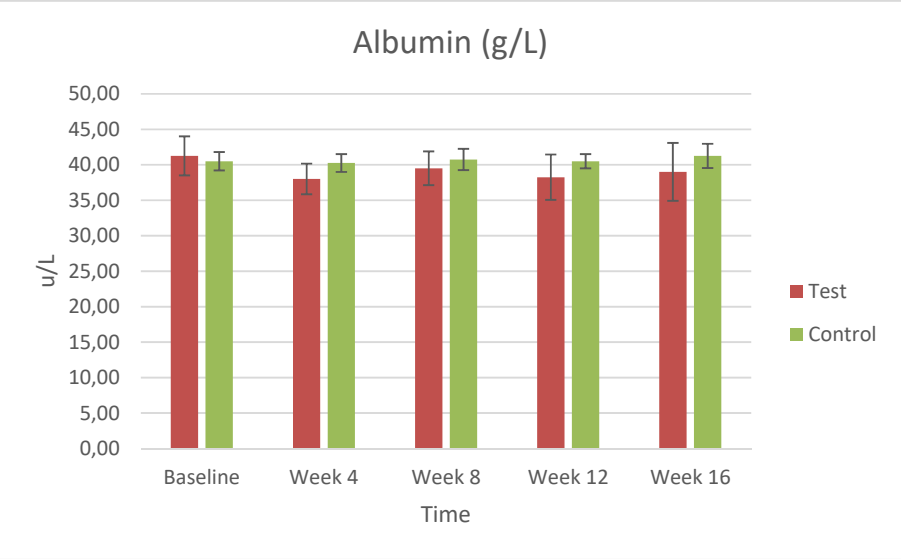

Figure D8: Albumin

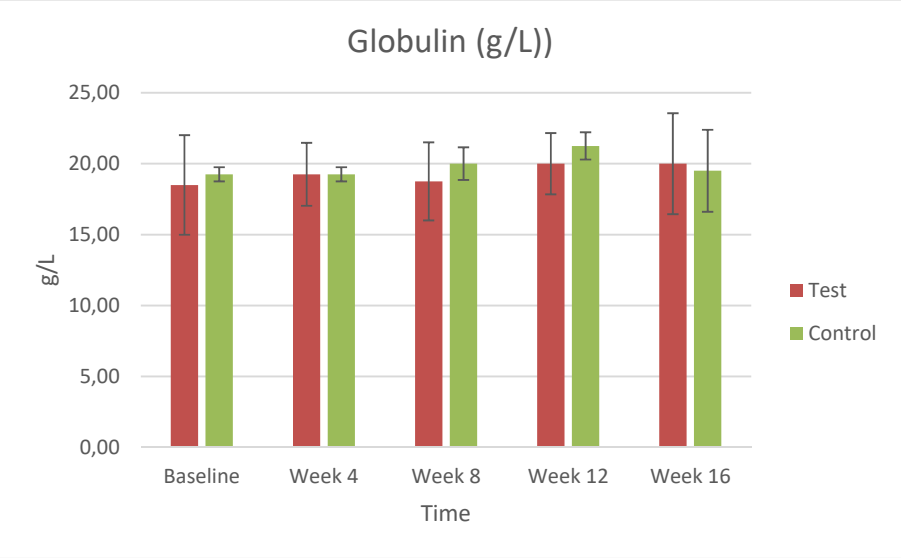

Figure D9: Globulin

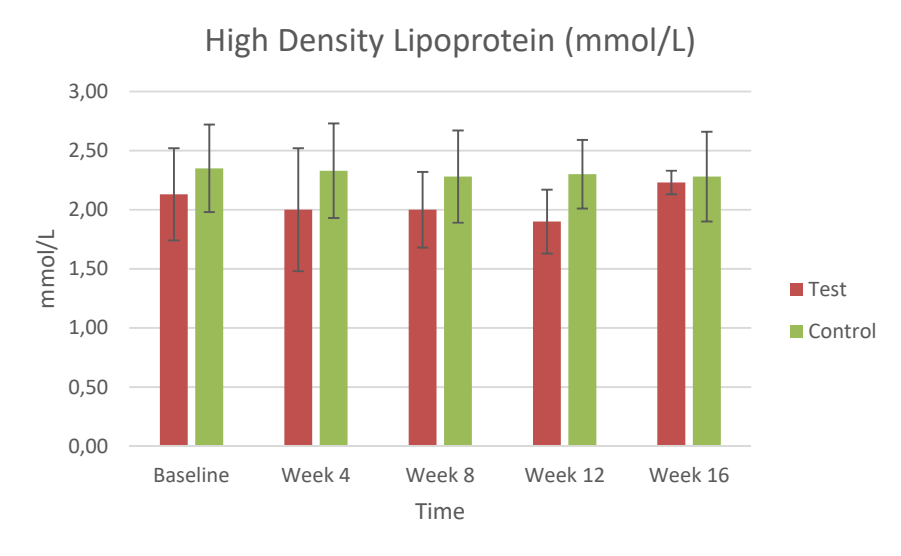

Figure D10: High density lipopreotein-cholesterol (HDL-C)

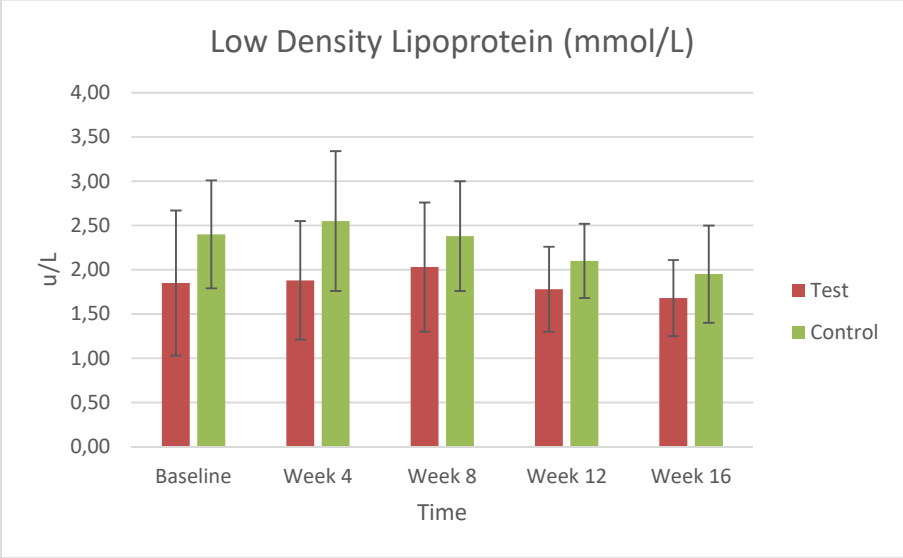

Figure D11: Low density lipopreotein-cholesterol (LDL-C)

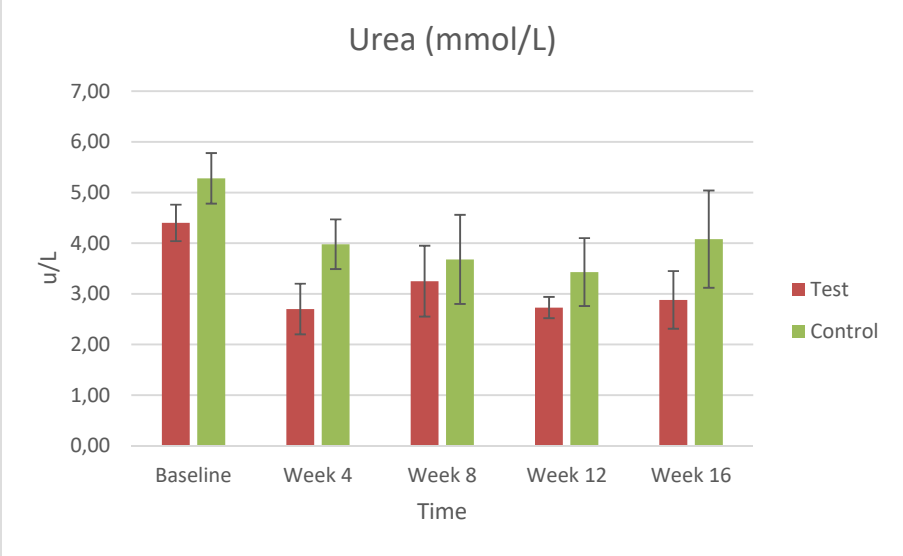

Figure D12: Urea

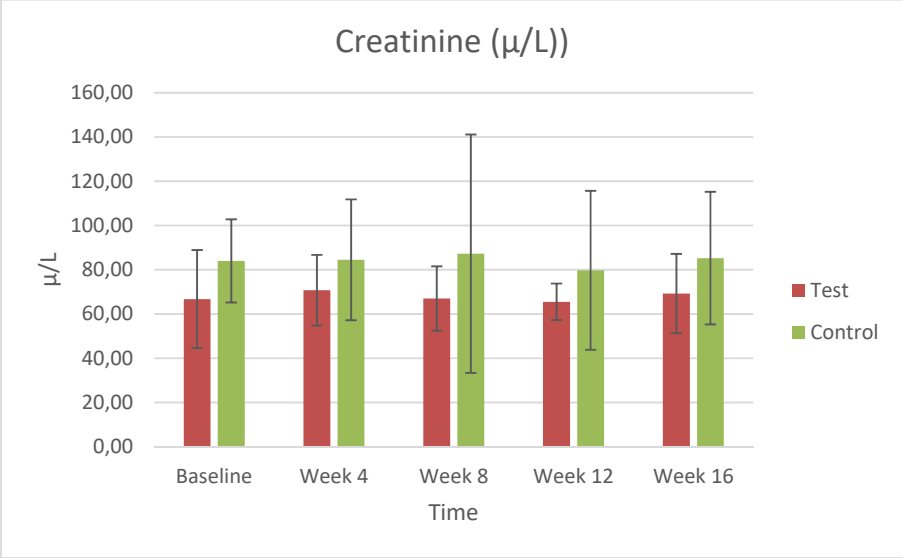

Figure D13: Creatinine

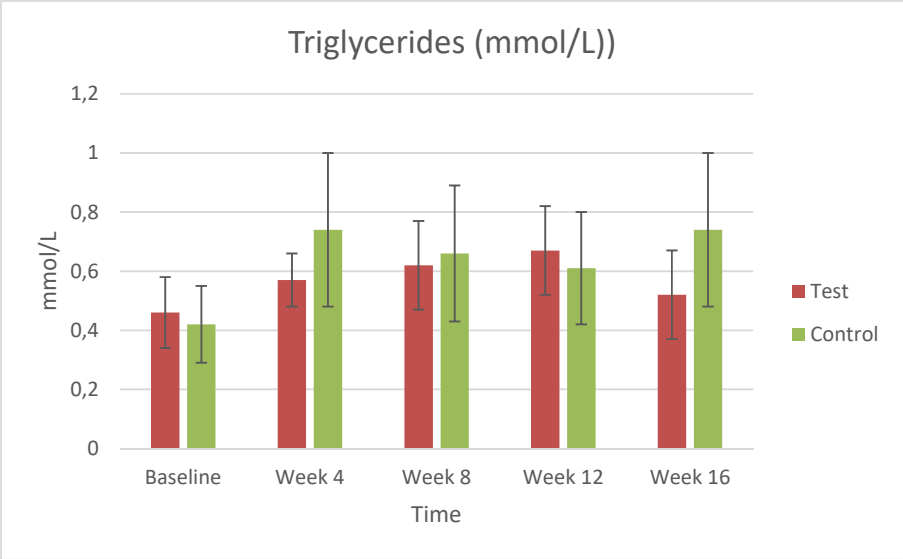

Figure D14: Triglycerides

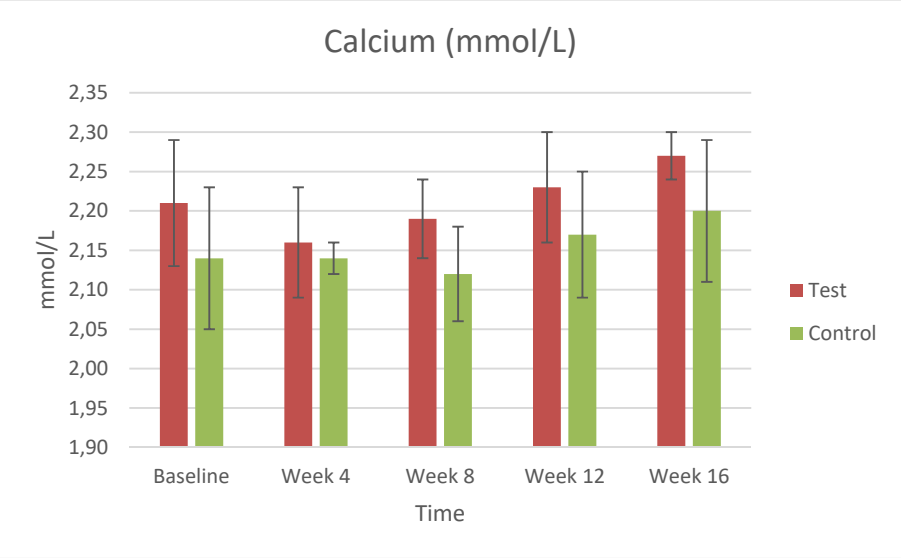

Figure D15: Calcium

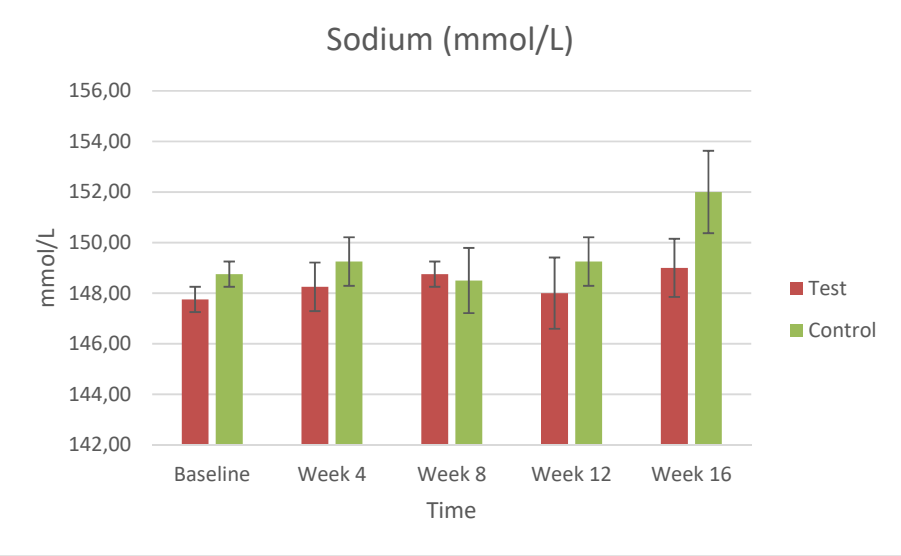

Figure D16: Sodium

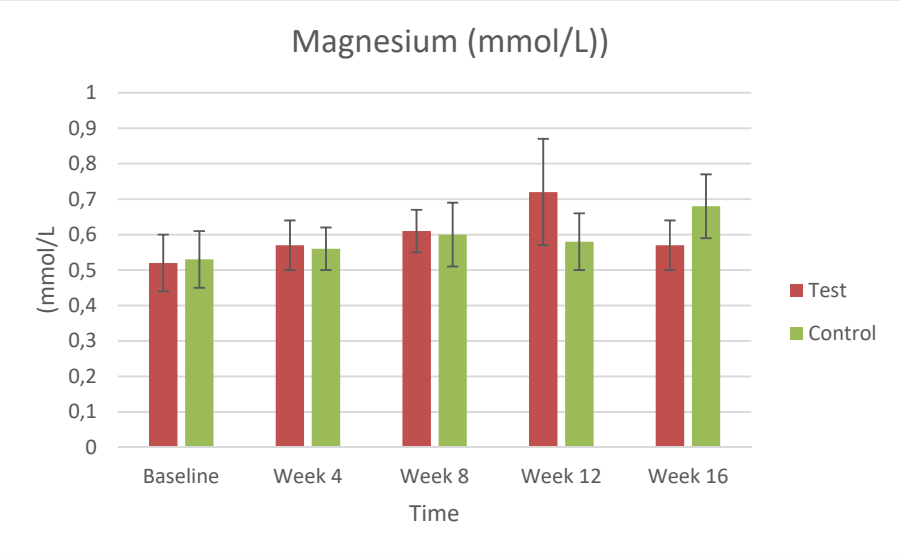

Figure D17: Magnesium

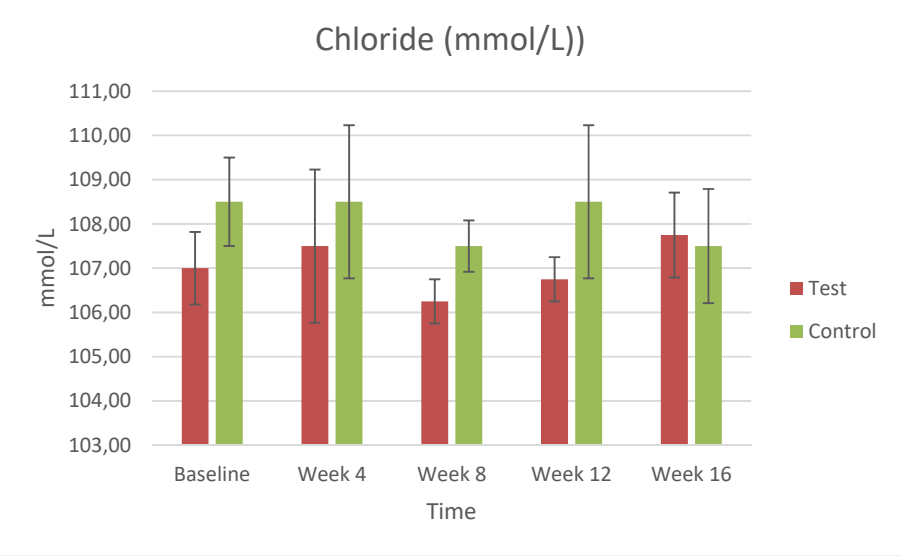

Figure D18: Chloride

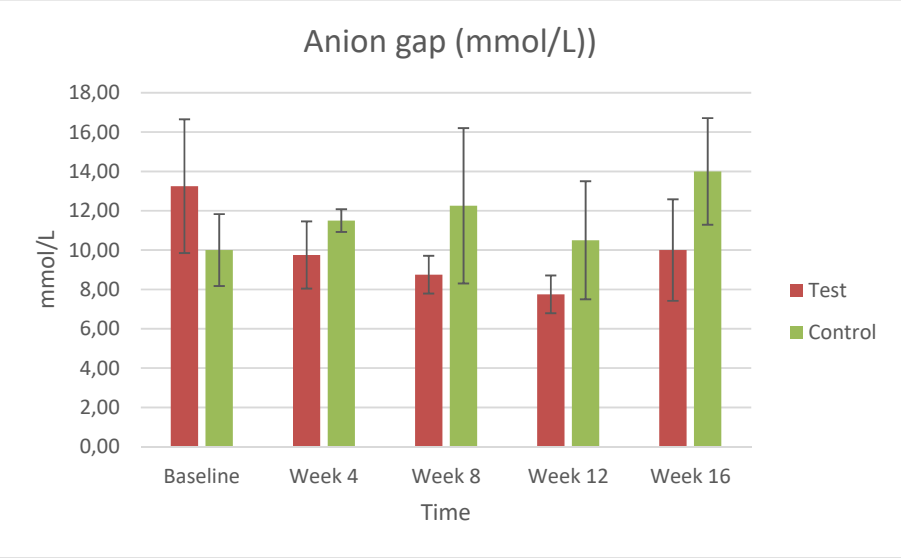

Figure D19: Anion gap

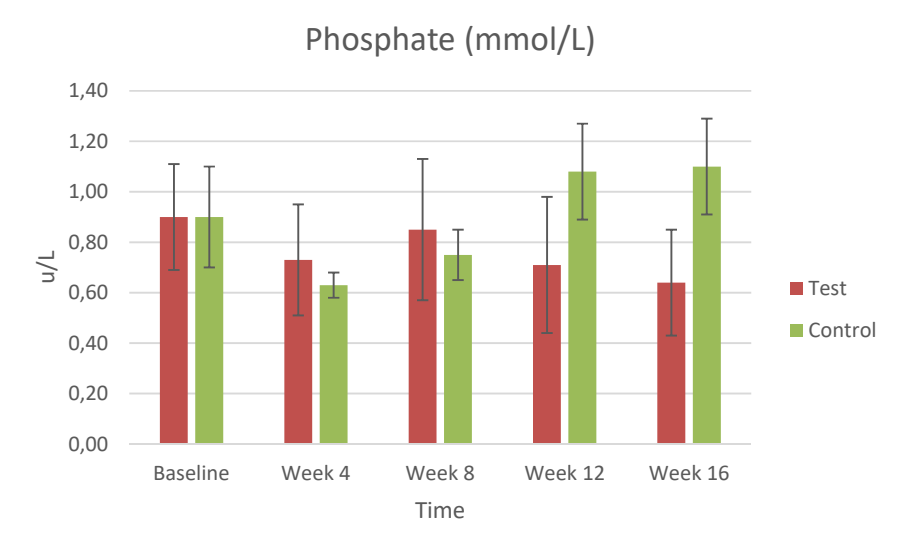

Figure D20: Phosphate

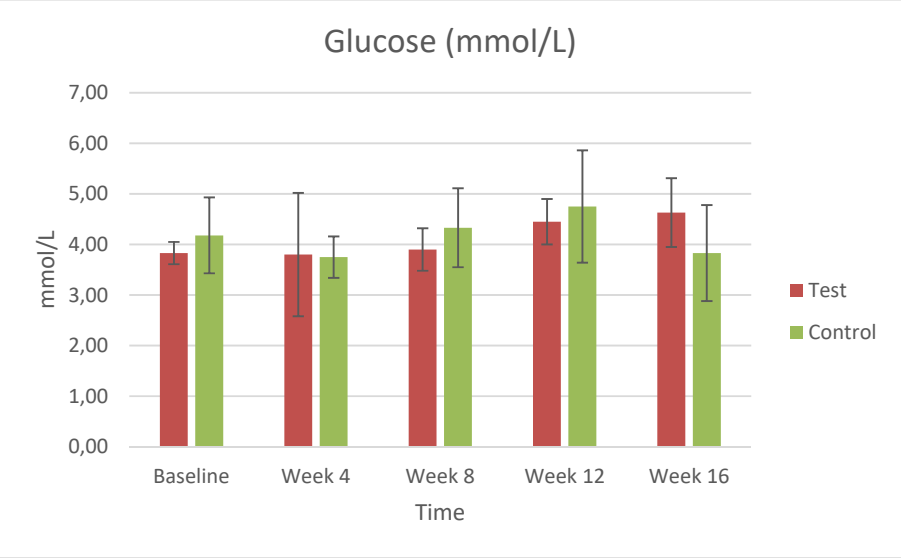

Figure D21: Glucose

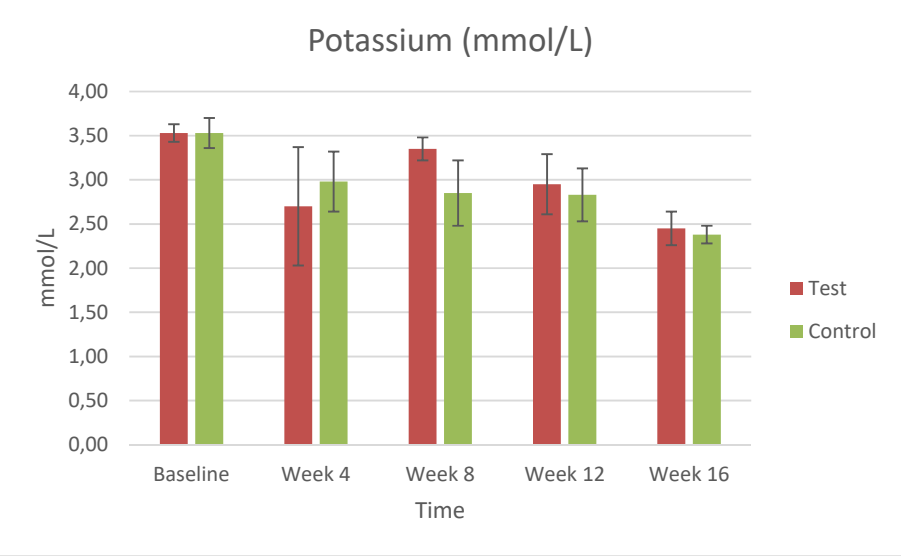

Figure D22: Potassium

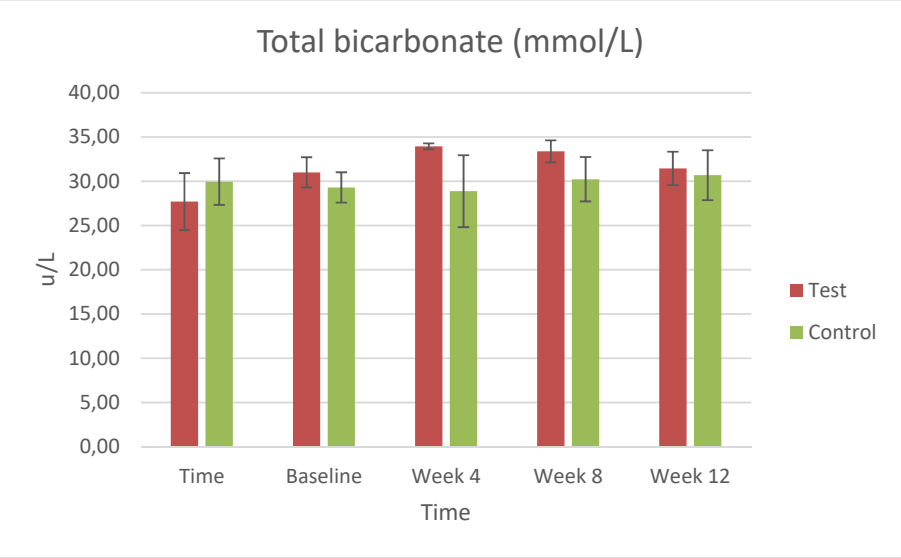

Figure D23: Total bicarbonate (CO_2)

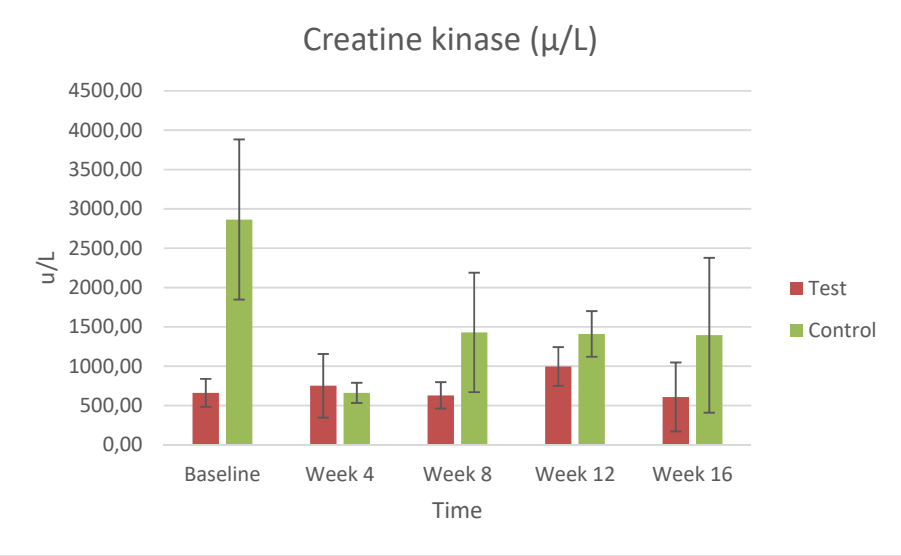

Figure D24: Creatine kinase (Ck)

Appendix E: Haematology

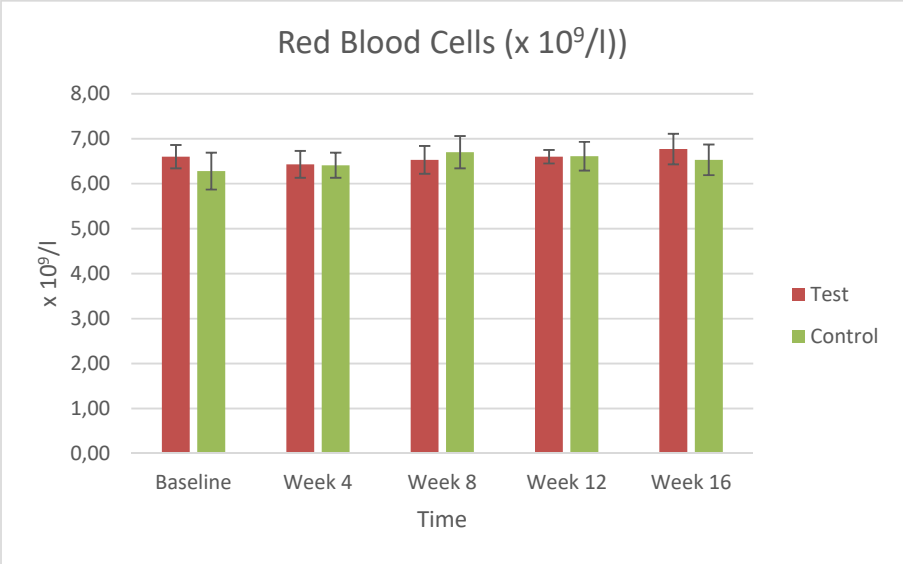

Figure E1: Red blood cells (RBCs)

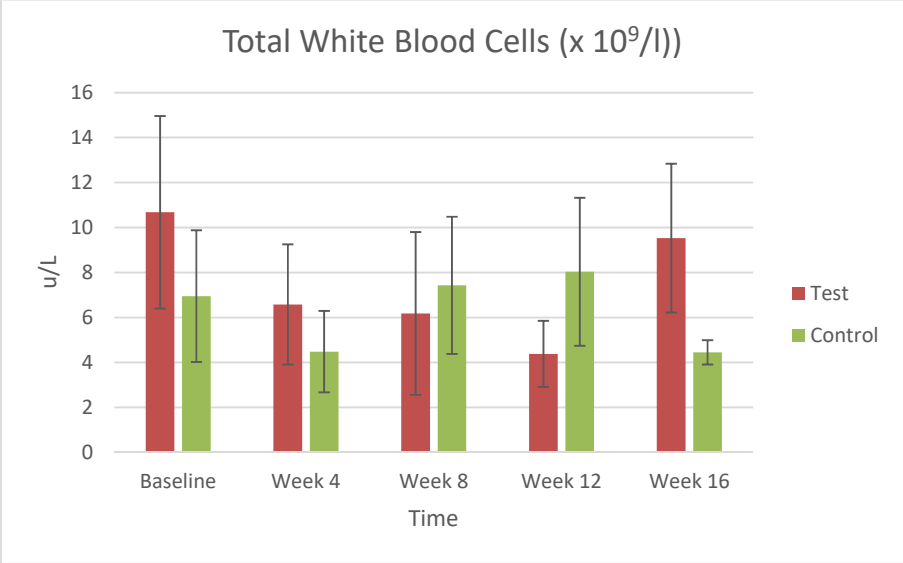

Figure E2: Total white blood cells (WBC)

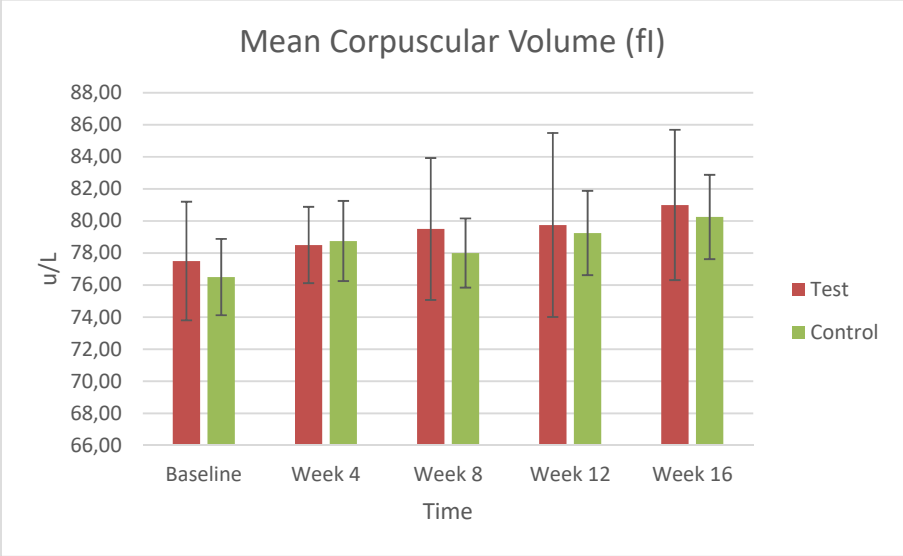

Figure E3: Mean corpuscular volume (MCV)

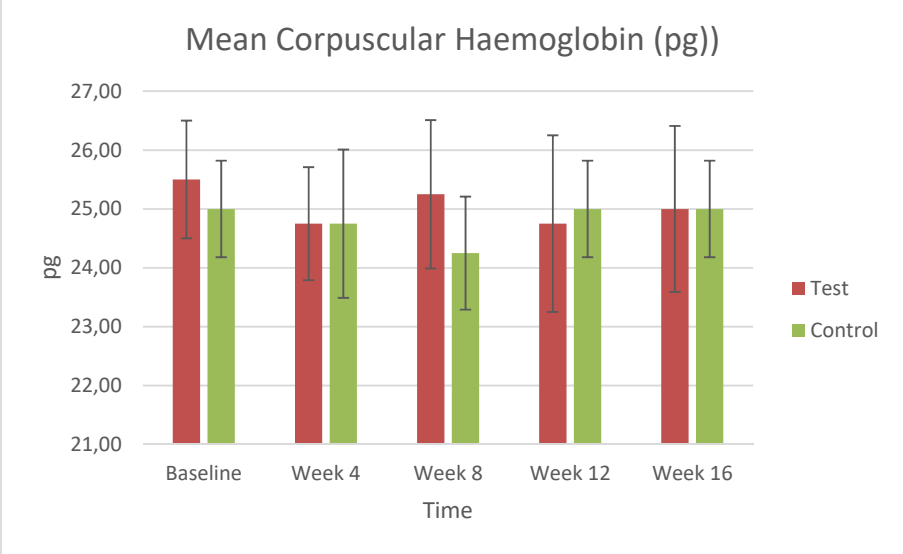

Figure E4: Mean corpuscular haemoglobin (MCH)

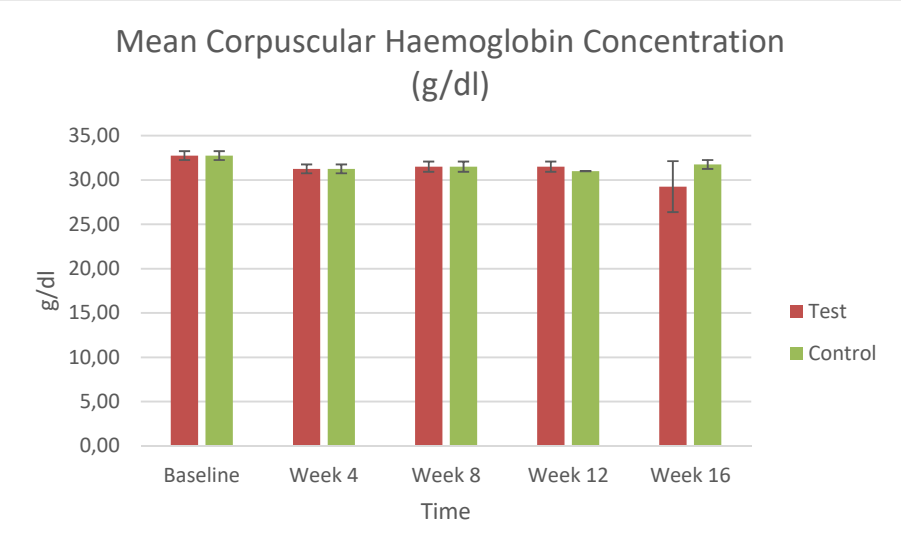

Figure E.5: Mean corpuscular haemoglobin concentration (MCHC)

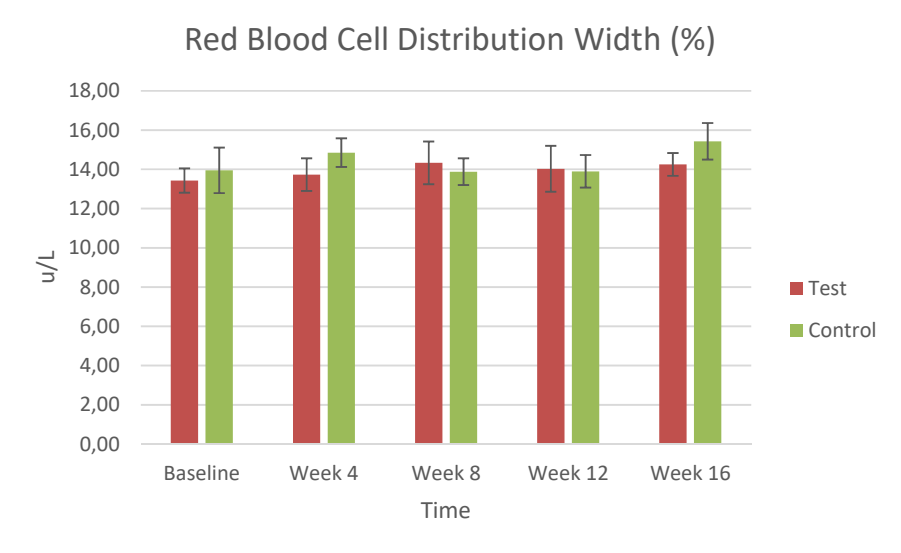

Figure E6: Red cell distribution width (RDW)

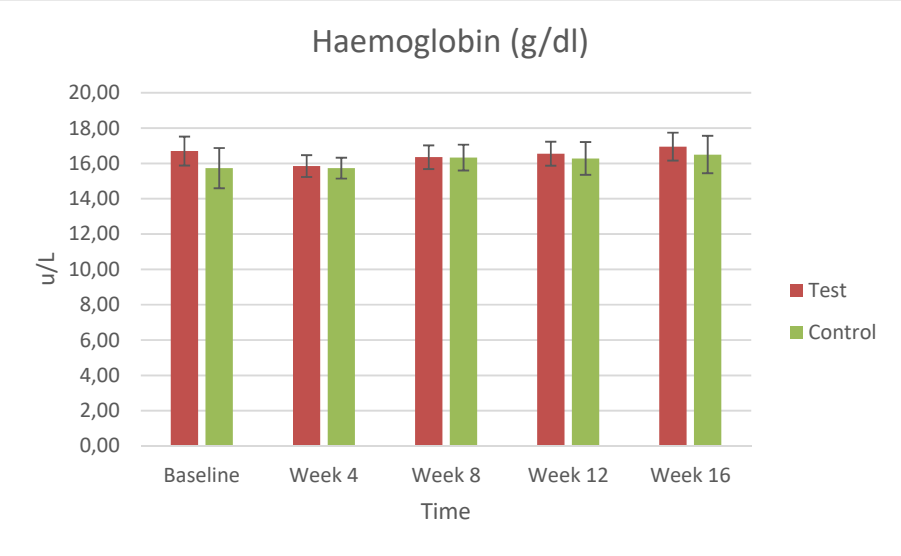

Figure E7: Haemoglobin (Hb)

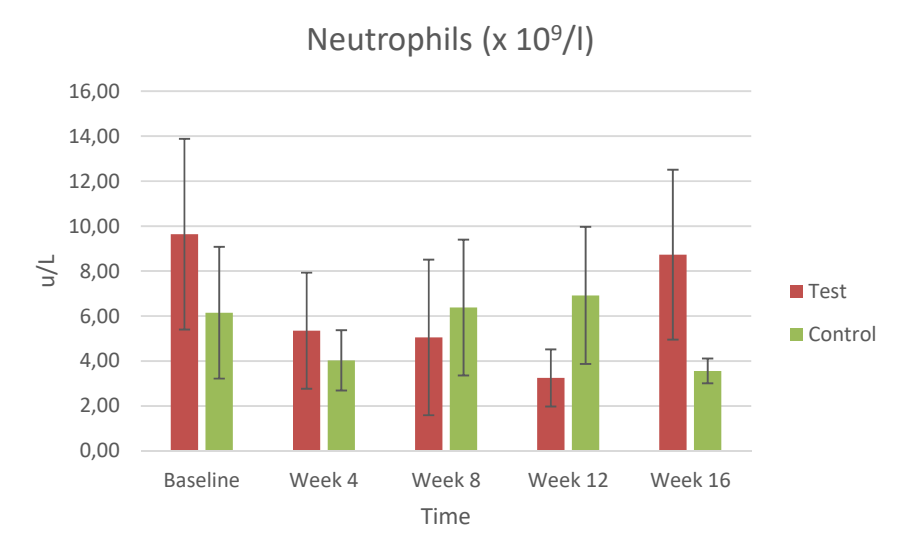

Figure E8: Neutrophils

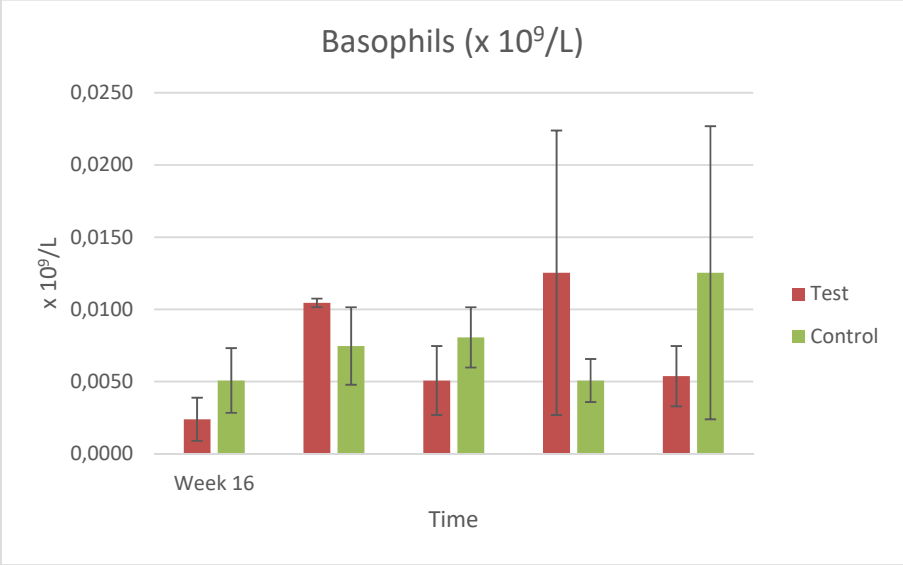

Figure E9: Basophils

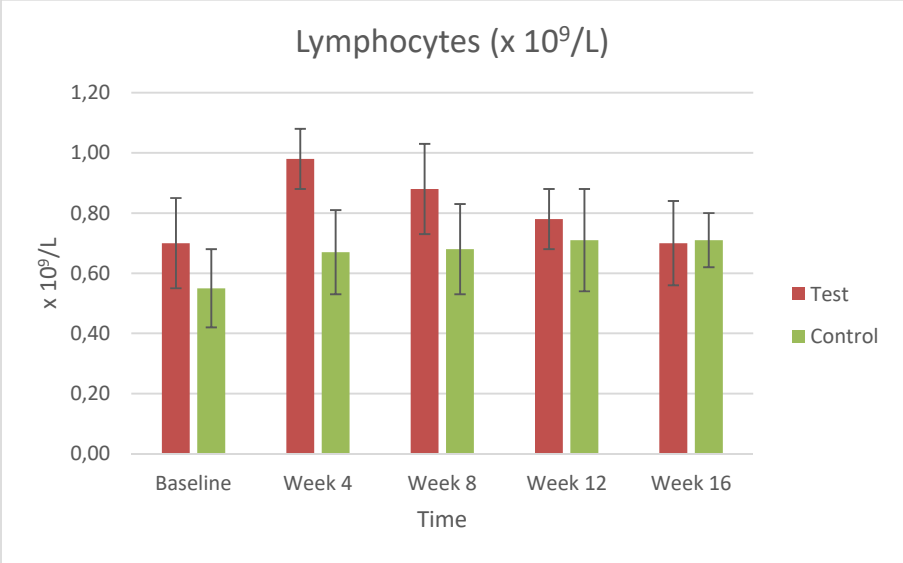

Figure E10: Lymphocytes

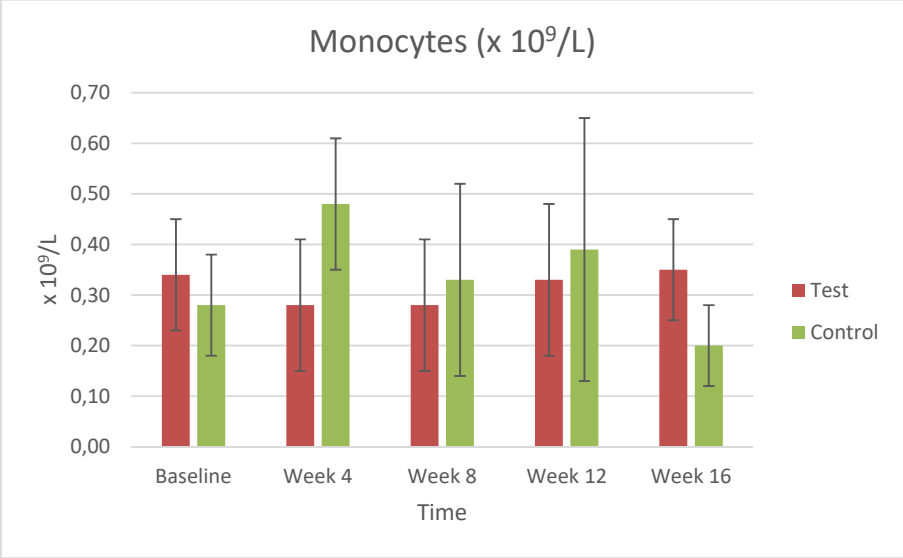

Figure E11: Monocytes

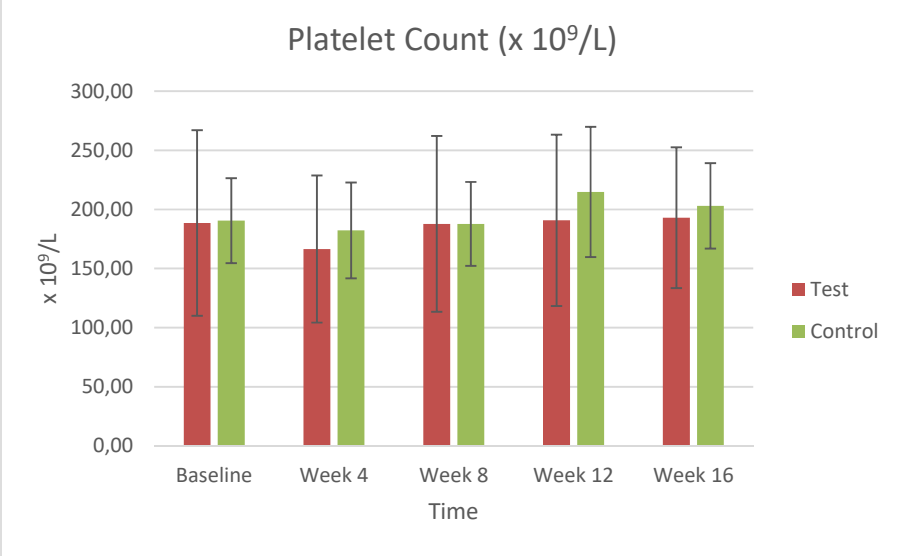

Figure E12: Platelet count

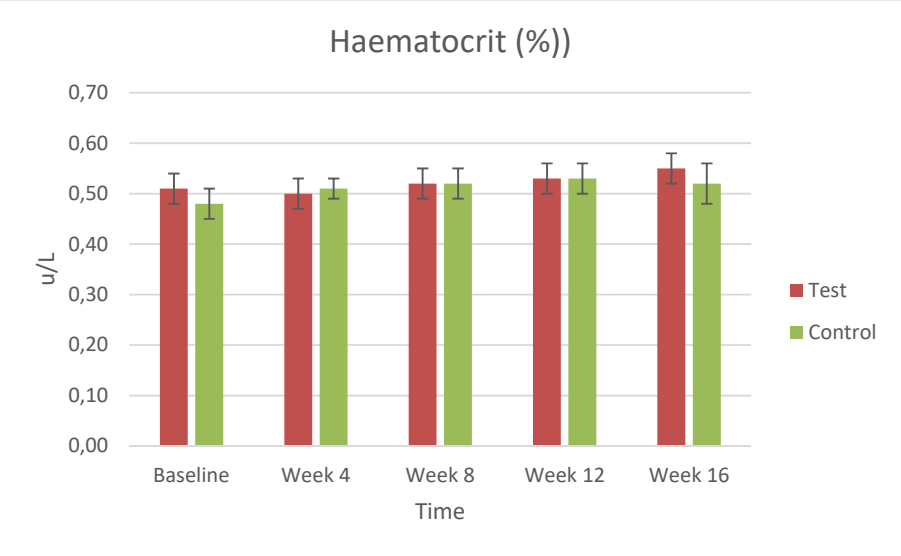

Figure E13: Haematocrit (Hct)

Appendix F: Physical and Physiological variables

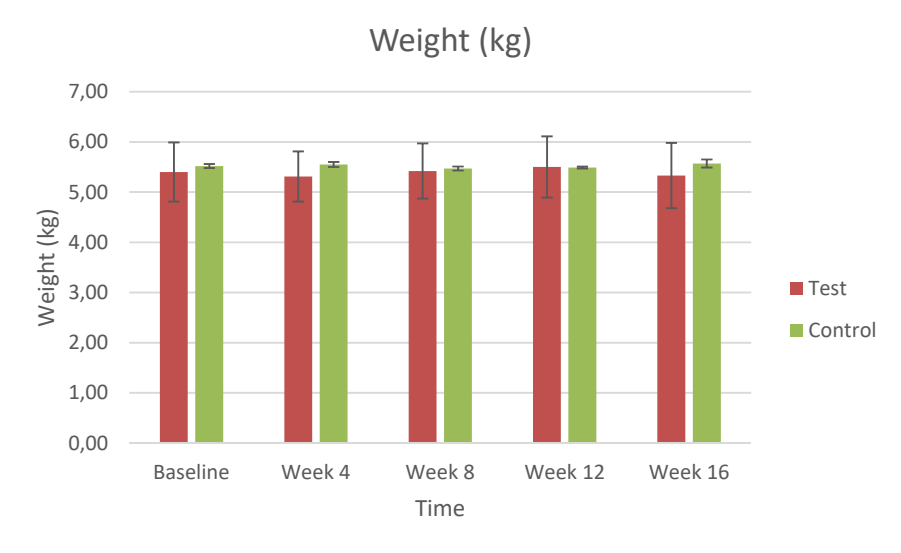

Figure F1: Body weight

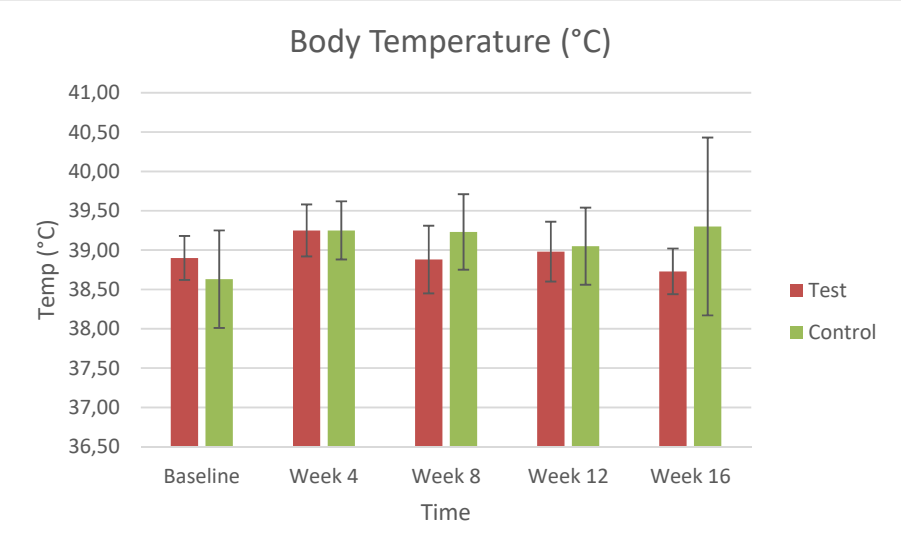

Figure F2: Body temperature

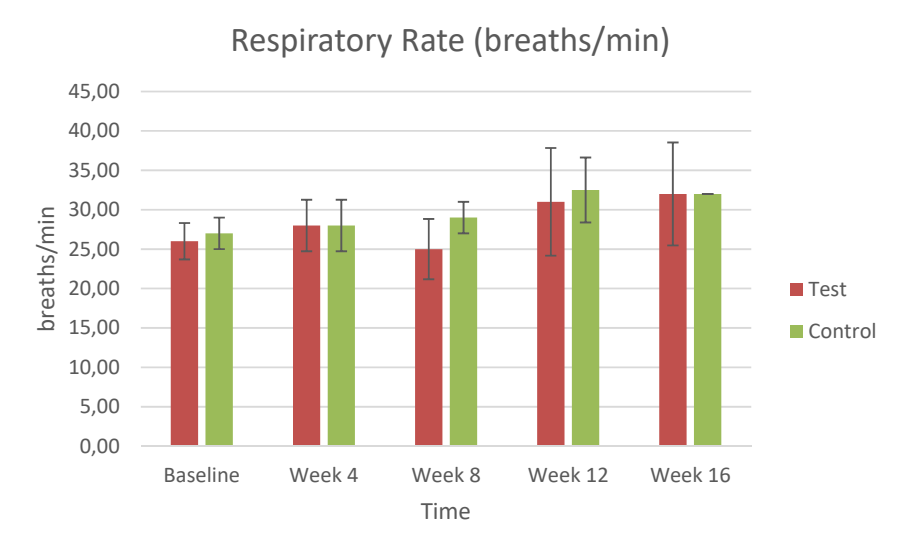

Figure F3: Respiratory rate

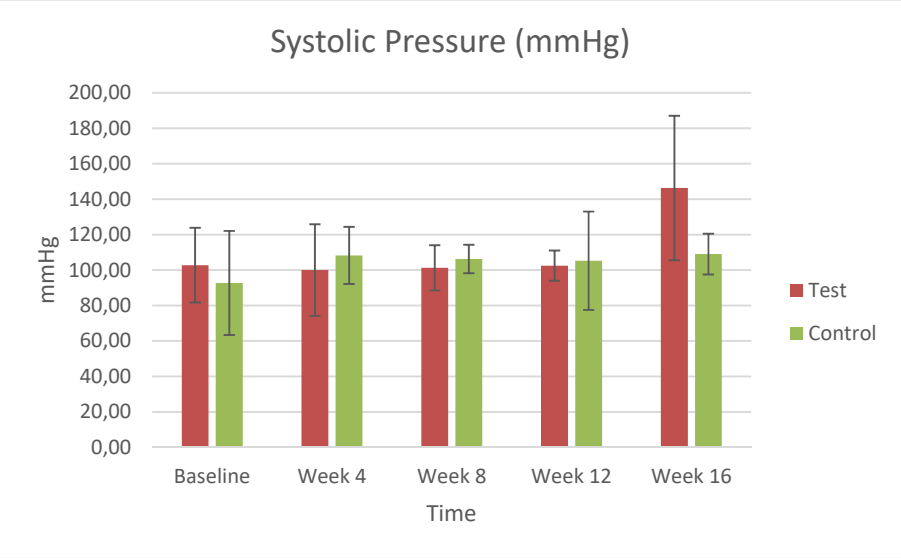

Figure F4: Systolic pressure

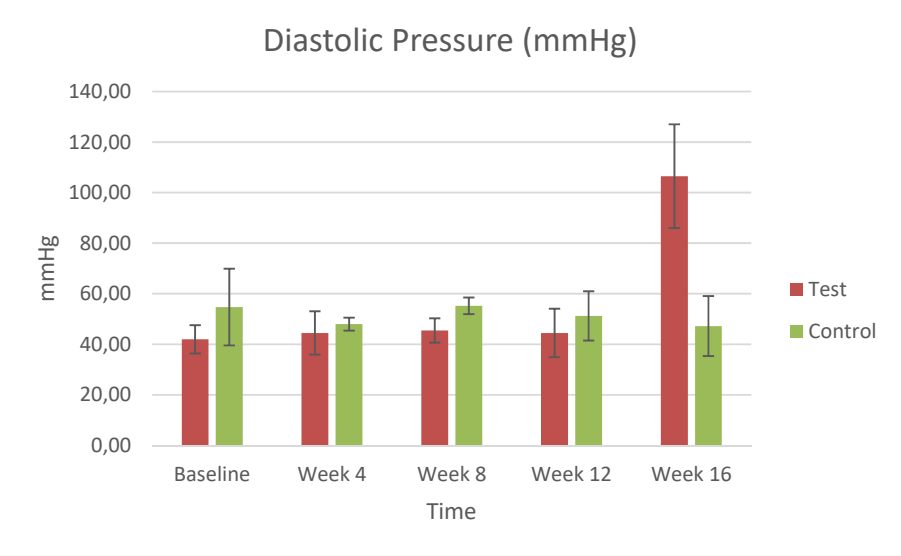

Figure F5: Diastolic pressure

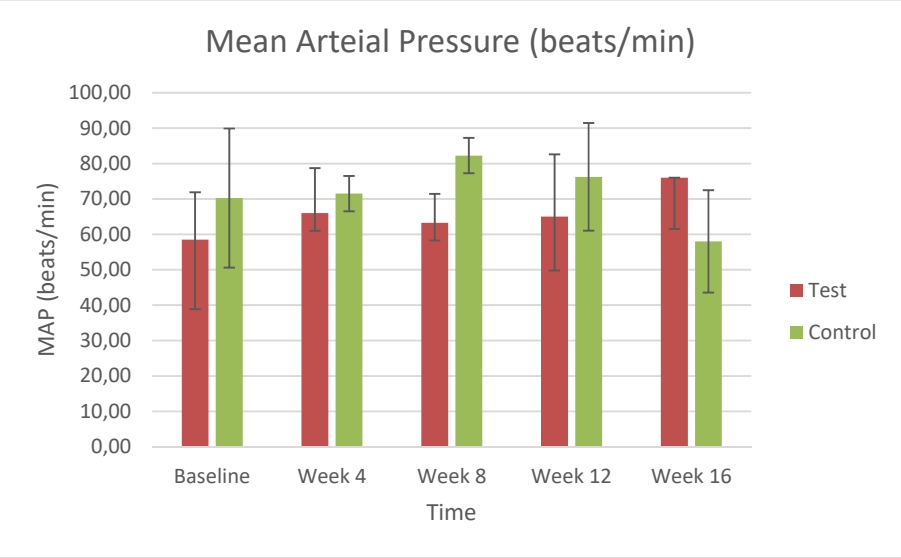

Figure F6: Mean arterial pressure

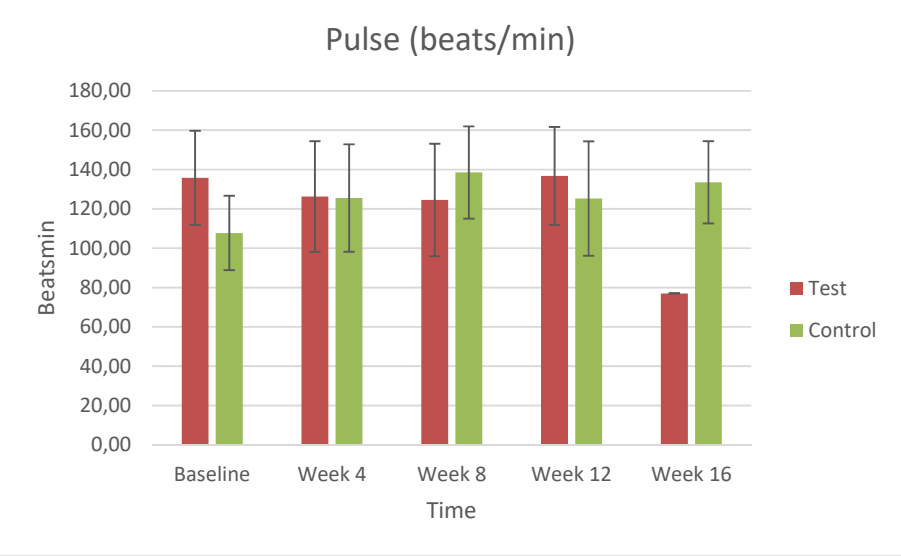

Figure F7: Pulse

Appendix G: Raw Data for Toxicology Studies

Biochemistry

Table G1: ALT (u/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 12 23.12.14	Week 21.01.15
Gr.1 *K. wilmsii* 2.14g/kg	374	33.00	28.00	30.00	32.00	46.00
	292	73.00	54.00	51.00	44.00	65.00
	397	27.00	22.00	25.00	27.00	28.00
	387	27.00	19.00	17.00	26.00	27.00
	Mean	**40.00**	**30.75**	**30.75**	**32.25**	**41.50**
	SD	**22.18**	**15.95**	**14.52**	**8.26**	**17.94**
Gr.4 Control	390	69.00	47.00	97.00	66.00	60.00
	389	64.00	73.00	71.00	60.00	47.00
	244	102.00	98.00	196.00	123.00	113.00
	130	95.00	108.00	117.00	127.00	55.00
	Mean	**82.50**	**81.50**	**120.25**	**94.00**	**68.75**
	SD	**18.81**	**27.31**	**53.90**	**35.92**	**29.98**

Table G2: AST (u/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	88.00	84.00	85.00	95.00	101.00
	292	108.00	99.00	81.00	95.00	93.00
	397	65.00	60.00	64.00	75.00	68.00
	387	57.00	60.00	53.00	69.00	75.00
	Mean	**79.50**	**75.75**	**70.75**	**83.50**	**84.25**
	SD	**23.10**	**19.19**	**14.93**	**13.50**	**15.35**
Gr.4 Control	390	144.00	74.00	112.00	114.00	109.00
	389	63.00	73.00	67.00	62.00	67.00
	244	109.00	92.00	120.00	88.00	137.00
	130	106.00	116.00	151.00	176.00	104.00
	Mean	**105.50**	**88.75**	**112.50**	**110.00**	**104.25**
	SD	**33.17**	**20.16**	**34.68**	**48.85**	**28.77**

Table G3: ALP (u/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	210.00	199.00	181.00	153.00	140.00
	292	151.00	144.00	127.00	97.00	101.00
	397	184.00	162.00	138.00	116.00	153.00
	387	210.00	189.00	164.00	143.00	119.00
	Mean	**188.75**	**173.50**	**152.50**	**127.25**	**128.25**
	SD	**27.99**	**25.12**	**24.53**	**25.51**	**22.94**
Gr.4 Control	390	255.00	204.00	230.00	193.00	200.00
	389	151.00	148.00	144.00	127.00	124.00
	244	109.00	103.00	112.00	95.00	85.00
	130	301.00	294.00	287.00	249.00	238.00
	Mean	**204.00**	**187.25**	**193.25**	**166.00**	**161.75**
	SD	**89.15**	**82.29**	**79.93**	**68.75**	**69.74**

Table G4: GGT (u/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	57.00	94.00	62.00	64.00	69.00
	292	117.00	115.00	118.00	137.00	128.00
	397	50.00	57.00	57.00	56.00	60.00
	387	87.00	98.00	73.00	76.00	88.00
	Mean	**77.75**	**91.00**	**77.50**	**83.25**	**86.25**
	SD	**30.70**	**24.43**	**27.81**	**36.76**	**30.18**
Gr.4 Control	390	65.00	68.00	87.00	77.00	80.00
	389	94.00	103.00	105.00	103.00	108.00
	244	50.00	52.00	77.00	61.00	60.00
	130	63.00	89.00	116.00	101.00	97.00
	Mean	**68.00**	**78.00**	**96.25**	**85.50**	**86.25**
	SD	**18.57**	**22.52**	**17.54**	**20.16**	**20.95**

Table G5: Total-protein (g/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	60.00	58.00	58.00	59.00	60.00
	292	61.00	58.00	58.00	55.00	58.00
	397	60.00	57.00	59.00	58.00	59.00
	387	58.00	56.00	58.00	61.00	59.00
	Mean	**59.75**	**57.25**	**58.25**	**58.25**	**59.00**
	SD	**1.26**	**0.96**	**0.50**	**2.50**	**0.82**
Gr.4 Control	390	61.00	59.00	63.00	63.00	63.00
	389	59.00	61.00	59.00	61.00	57.00
	244	61.00	59.00	61.00	62.00	61.00
	130	58.00	59.00	60.00	61.00	62.00
	Mean	**59.75**	**59.50**	**60.75**	**61.75**	**60.75**
	SD	**1.50**	**1.00**	**1.71**	**0.96**	**2.63**

Table G6: Total bilirubin (µmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 12 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	7.00	5.00	8.00	5.00	4.00
	292	9.00	6.00	5.00	5.00	6.00
	397	7.00	8.00	10.00	10.00	8.00
	387	8.00	6.00	10.00	8.00	6.00
	Mean	**7.75**	**6.25**	**8.25**	**7.00**	**6.00**
	SD	**0.96**	**1.26**	**2.36**	**2.45**	**1.63**
Gr.4 Control	390	5.00	5.00	5.00	6.00	4.00
	389	6.00	8.00	4.00	6.00	5.00
	244	5.00	6.00	6.00	5.00	5.00
	130	8.00	8.00	8.00	9.00	9.00
	Mean	**6.00**	**6.75**	**5.75**	**6.50**	**5.75**
	SD	**1.41**	**1.50**	**1.71**	**1.73**	**2.22**

Table G7: Direct bilirubin (µmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	2.00	1.00	2.00	1.00	1.00
	292	2.00	1.00	1.00	1.00	2.00
	397	2.00	2.00	2.00	2.00	2.00
	387	2.00	2.00	2.00	2.00	2.00
	Mean	**2.00**	**1.50**	**1.75**	**1.50**	**1.75**
	SD	0.00	0.58	0.50	0.58	0.50
Gr.4 Control	390	2.00	1.00	2.00	1.00	1.00
	389	1.00	1.00	1.00	1.00	1.00
	244	1.00	1.00	1.00	1.00	1.00
	130	2.00	2.00	2.00	2.00	2.00
	Mean	**1.50**	**1.25**	**1.50**	**1.25**	**1.25**
	SD	**0.58**	**0.50**	**0.58**	**0.50**	**0.50**

Table G8: ALB (g/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	44.00	40.00	41.00	41.00	43.00
	292	40.00	38.00	38.00	36.00	36.00
	397	38.00	35.00	37.00	35.00	35.00
	387	43.00	39.00	42.00	41.00	42.00
	Mean	**41.25**	**38.00**	**39.50**	**38.25**	**39.00**
	SD	2.75	2.16	2.38	3.20	4.08
Gr.4 Control	390	41.00	40.00	42.00	41.00	43.00
	389	40.00	42.00	40.00	41.00	41.00
	244	42.00	40.00	42.00	41.00	42.00
	130	39.00	39.00	39.00	39.00	39.00
	Mean	**40.50**	**40.25**	**40.75**	**40.50**	**41.25**
	SD	**1.29**	**1.26**	**1.50**	**1.00**	**1.71**

Table G9: Globulin (g/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	16.00	18.00	17.00	18.00	17.00
	292	21.00	20.00	20.00	19.00	22.00
	397	22.00	22.00	22.00	23.00	24.00
	387	15.00	17.00	16.00	20.00	17.00
	Mean	**18.50**	**19.25**	**18.75**	**20.00**	**20.00**
	SD	**3.51**	**2.22**	**2.75**	**2.16**	**3.56**
Gr.4 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**

Table G10: HDL (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	2.50	2.60	2.40	2.30	2.30
	292	2.10	1.80	1.80	1.80	2.30
	397	2.30	2.20	2.10	1.70	2.20
	387	1.60	1.40	1.70	1.80	2.10
	Mean	**2.13**	**2.00**	**2.00**	**1.90**	**2.23**
	SD	**0.39**	**0.52**	**0.32**	**0.27**	**0.10**
Gr.4 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**

Table G11: LDL (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	1.60	2.00	1.80	1.80	1.60
	292	2.50	2.30	2.50	2.20	2.10
	397	2.50	2.30	2.70	2.00	1.90
	387	0.80	0.90	1.10	1.10	1.10
	Mean	**1.85**	**1.88**	**2.03**	**1.78**	**1.68**
	SD	**0.82**	**0.67**	**0.73**	**0.48**	**0.43**
Gr.4 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**

Table G12: Urea (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	4.40	2.30	4.00	2.90	2.50
	292	4.70	2.70	2.70	2.50	2.50
	397	3.90	3.40	3.70	2.60	3.70
	387	4.60	2.40	2.60	2.90	2.80
	Mean	**4.40**	**2.70**	**3.25**	**2.73**	**2.88**
	SD	**0.36**	**0.50**	**0.70**	**0.21**	**0.57**
Gr.4 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**

Table G13: Creatinine (u/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	56.00	57.00	55.00	52.00	60.00
	292	63.00	73.00	62.00	63.00	66.00
	397	77.00	80.00	81.00	78.00	71.00
	387	71.00	73.00	70.00	69.00	80.00
	Mean	**66.75**	**70.75**	**67.00**	**65.50**	**69.25**
	SD	9.18	9.74	11.17	10.91	8.46
Gr.4 Control	390	94.00	99.00	108.00	93.00	107.00
	389	70.00	70.00	66.00	66.00	67.00
	244	71.00	73.00	73.00	71.00	70.00
	130	101.00	96.00	102.00	89.00	97.00
	Mean	**84.00**	**84.50**	**87.25**	**79.75**	**85.25**
	SD	**15.85**	**15.11**	**20.84**	**13.25**	**19.81**

Table G14: Triglycerides (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	0.44	0.47	0.50	0.67	0.42
	292	0.31	0.65	0.55	0.56	0.37
	397	0.59	0.64	0.84	0.87	0.67
	387	0.48	0.53	0.58	0.56	0.62
	Mean	**0.46**	**0.57**	**0.62**	**0.67**	**0.52**
	SD	**0.12**	**0.09**	**0.15**	**0.15**	**0.15**
Gr.4 Control	390	0.59	0.88	0.80	0.71	0.97
	389	0.28	0.56	0.37	0.39	0.37
	244	0.36	1.03	0.88	0.82	0.82
	130	0.46	0.48	0.57	0.53	0.81
	Mean	**0.42**	**0.74**	**0.66**	**0.61**	**0.74**
	SD	**0.13**	**0.26**	**0.23**	**0.19**	**0.26**

Table G15: Calcium (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	2.19	2.16	2.22	2.22	2.26
	292	2.20	2.14	2.16	2.30	2.25
	397	2.32	2.24	2.23	2.26	2.31
	387	2.14	2.08	2.13	2.14	2.25
	Mean	**2.21**	**2.16**	**2.19**	**2.23**	**2.27**
	SD	**0.08**	**0.07**	**0.05**	**0.07**	**0.03**
Gr.4 Control	390	2.20	2.15	2.16	2.16	2.23
	389	2.00	2.12	2.04	2.07	2.06
	244	2.15	2.16	2.16	2.23	2.22
	130	2.20	2.13	2.12	2.23	2.27
	Mean	**2.14**	**2.14**	**2.12**	**2.17**	**2.20**
	SD	**0.09**	**0.02**	**0.06**	**0.08**	**0.09**

Table G16: Na (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	147.00	148.00	149.00	147.00	148.00
	292	148.00	149.00	148.00	147.00	150.00
	397	148.00	149.00	149.00	150.00	148.00
	387	148.00	147.00	149.00	148.00	150.00
	Mean	**147.75**	**148.25**	**148.75**	**148.00**	**149.00**
	SD	**0.50**	**0.96**	**0.50**	**1.41**	**1.15**
Gr.4 Control	390	149.00	150.00	149.00	150.00	152.00
	389	149.00	148.00	148.00	148.00	150.00
	244	149.00	150.00	150.00	150.00	154.00
	130	148.00	149.00	147.00	149.00	152.00
	Mean	**148.75**	**149.25**	**148.50**	**149.25**	**152.00**
	SD	**0.50**	**0.96**	**1.29**	**0.96**	**1.63**

Table G17: Mg (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	0.49	0.53	0.64	0.65	0.61
	292	0.53	0.59	0.61	0.59	0.58
	397	0.62	0.65	0.66	0.72	0.63
	387	0.42	0.50	0.53	0.93	0.47
	Mean	**0.52**	**0.57**	**0.61**	**0.72**	**0.57**
	SD	**0.08**	**0.07**	**0.06**	**0.15**	**0.07**
Gr.4 Control	390	0.55	0.57	0.63	0.59	0.70
	389	0.43	0.49	0.49	0.48	0.54
	244	0.52	0.53	0.57	0.56	0.70
	130	0.63	0.64	0.70	0.67	0.76
	Mean	**0.53**	**0.56**	**0.60**	**0.58**	**0.68**
	SD	**0.08**	**0.06**	**0.09**	**0.08**	**0.09**

Table G18: Chl (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	107.00	110.00	106.00	107.00	108.00
	292	108.00	107.00	106.00	106.00	109.00
	397	106.00	107.00	106.00	107.00	107.00
	387	107.00	106.00	107.00	107.00	107.00
	Mean	**107.00**	**107.50**	**106.25**	**106.75**	**107.75**
	SD	**0.82**	**1.73**	**0.50**	**0.50**	**0.96**
Gr.4 Control	390	109.00	110.00	107.00	109.00	108.00
	389	107.00	106.00	107.00	106.00	107.00
	244	109.00	109.00	108.00	109.00	106.00
	130	109.00	109.00	108.00	110.00	109.00
	Mean	**108.50**	**108.50**	**107.50**	**108.50**	**107.50**
	SD	**1.00**	**1.73**	**0.58**	**1.73**	**1.29**

Table G19: Anion gap (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	13.00	8.00	9.00	7.00	9.00
	292	10.00	9.00	8.00	7.00	7.00
	397	12.00	10.00	10.00	8.00	11.00
	387	18.00	12.00	8.00	9.00	13.00
	Mean	**13.25**	**9.75**	**8.75**	**7.75**	**10.00**
	SD	**3.40**	**1.71**	**0.96**	**0.96**	**2.58**
Gr.4 Control	390	9.00	12.00	18.00	14.00	16.00
	389	11.00	11.00	11.00	12.00	10.00
	244	8.00	11.00	9.00	8.00	15.00
	130	12.00	12.00	11.00	8.00	15.00
	Mean	**10.00**	**11.50**	**12.25**	**10.50**	**14.00**
	SD	**1.83**	**0.58**	**3.95**	**3.00**	**2.71**

Table G20: Phosphate (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	1.07	0.65	1.09	1.03	0.80
	292	1.01	0.90	1.10	0.83	0.73
	397	0.60	0.45	0.61	0.53	0.32
	387	0.93	0.90	0.60	0.46	0.69
	Mean	**0.90**	**0.73**	**0.85**	**0.71**	**0.64**
	SD	**0.21**	**0.22**	**0.28**	**0.27**	**0.21**
Gr.4 Control	390	0.92	0.67	0.85	1.26	1.23
	389	0.98	0.61	0.62	0.85	0.86
	244	1.09	0.67	0.82	1.22	1.26
	130	0.62	0.57	0.72	1.00	1.03
	Mean	**0.90**	**0.63**	**0.75**	**1.08**	**1.10**
	SD	**0.20**	**0.05**	**0.10**	**0.19**	**0.19**

Table G21: Glucose (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	3.70	5.50	3.40	4.50	5.60
	292	3.90	3.40	3.80	4.40	4.10
	397	4.10	3.70	4.40	3.90	4.60
	387	3.60	2.60	4.00	5.00	4.20
	Mean	**3.83**	**3.80**	**3.90**	**4.45**	**4.63**
	SD	**0.22**	**1.22**	**0.42**	**0.45**	**0.68**
Gr.4 Control	390	4.60	4.10	4.90	5.80	4.00
	389	5.00	4.10	5.10	5.60	5.10
	244	3.70	3.30	3.70	4.00	3.20
	130	3.40	3.50	3.60	3.60	3.00
	Mean	**4.18**	**3.75**	**4.33**	**4.75**	**3.83**
	SD	**0.75**	**0.41**	**0.78**	**1.11**	**0.95**

Table G22: Potassium (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	3.60	1.80	3.40	3.30	2.60
	292	3.60	3.10	3.50	3.10	2.60
	397	3.50	2.60	3.30	2.90	2.40
	387	3.40	3.30	3.20	2.50	2.20
	Mean	**3.53**	**2.70**	**3.35**	**2.95**	**2.45**
	SD	**0.10**	**0.67**	**0.13**	**0.34**	**0.19**
Gr.4 Control	390	3.60	2.70	2.40	2.50	2.40
	389	3.70	3.40	3.30	2.90	2.50
	244	3.30	2.70	2.80	2.70	2.30
	130	3.50	3.10	2.90	3.20	2.30
	Mean	**3.53**	**2.98**	**2.85**	**2.83**	**2.38**
	SD	**0.17**	**0.34**	**0.37**	**0.30**	**0.10**

Table G23: CO_2 (mmol/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	27.50	29.80	33.90	33.00	31.50
	292	30.20	32.80	34.20	34.10	34.10
	397	29.90	32.10	33.50	34.60	30.20
	387	23.20	29.30	34.20	31.80	30.00
	Mean	**27.70**	**31.00**	**33.95**	**33.38**	**31.45**
	SD	**3.23**	**1.71**	**0.33**	**1.24**	**1.89**
Gr.4 Control	390	30.70	27.60	23.70	26.90	28.40
	389	31.00	31.30	30.20	30.40	32.70
	244	32.00	30.10	33.40	33.00	33.50
	130	26.09	28.20	28.20	30.60	28.10
	Mean	**29.95**	**29.30**	**28.88**	**30.23**	**30.68**
	SD	**2.63**	**1.71**	**4.06**	**2.51**	**2.82**

Table G24: CK (µ/L), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	573.00	450.00	790.00	913.00	1000.00
	292	664.00	1202.00	711.00	1269.00	220.00
	397	908.00	371.00	615.00	1106.00	238.00
	387	502.00	980.00	402.00	699.00	977.00
	Mean	**661.75**	**750.75**	**629.50**	**996.75**	**608.75**
	SD	**177.05**	**404.49**	**167.70**	**246.12**	**438.66**
Gr.4 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**

Haematology

Table G25: RBC (x 10^9/l), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	6.42	6.31	6.62	6.72	7.19
	292	6.43	6.34	6.28	6.52	6.41
	397	6.98	6.87	6.93	6.72	6.87
	387	6.55	6.20	6.28	6.43	6.59
	Mean	**6.60**	**6.43**	**6.53**	**6.60**	**6.77**
	SD	**0.26**	**0.30**	**0.31**	**0.15**	**0.34**
Gr.4 Control	390	6.78	6.78	7.20	6.92	6.86
	389	6.01	6.29	6.41	6.45	6.20
	244	5.89	6.12	6.47	6.23	6.27
	130	6.45	6.43	6.73	6.83	6.79
	Mean	**6.28**	**6.41**	**6.70**	**6.61**	**6.53**
	SD	**0.41**	**0.28**	**0.36**	**0.32**	**0.34**

Table G26: WBC (x 10^9/l), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	12.90	7.40	4.30	4.00	8.90
	292	4.40	3.00	3.10	2.60	5.50
	397	13.80	9.40	11.30	6.10	13.50
	387	11.60	6.50	6.00	4.80	10.20
	Mean	**10.68**	**6.58**	**6.18**	**4.38**	**9.53**
	SD	**4.28**	**2.67**	**3.62**	**1.47**	**3.31**
Gr.4 Control	390	11.20	6.80	11.70	11.80	5.10
	389	6.40	4.20	7.50	9.60	3.80
	244	5.60	4.50	5.50	6.20	4.30
	130	4.60	2.40	5.00	4.50	4.60
	Mean	**6.95**	**4.48**	**7.43**	**8.03**	**4.45**
	SD	**2.93**	**1.81**	**3.05**	**3.29**	**0.54**

Table G27: MCV (fl), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	76.00	78.00	78.00	77.00	78.00
	292	75.00	77.00	76.00	75.00	79.00
	397	76.00	77.00	78.00	79.00	79.00
	387	83.00	82.00	86.00	88.00	88.00
	Mean	**77.50**	**78.50**	**79.50**	**79.75**	**81.00**
	SD	**3.70**	**2.38**	**4.43**	**5.74**	**4.69**
Gr.4 Control	390	75.00	76.00	76.00	77.00	78.00
	389	75.00	79.00	78.00	79.00	80.00
	244	76.00	78.00	77.00	78.00	79.00
	130	80.00	82.00	81.00	83.00	84.00
	Mean	**76.50**	**78.75**	**78.00**	**79.25**	**80.25**
	SD	**2.38**	**2.50**	**2.16**	**2.63**	**2.63**

Table G28: MCH (pg), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	25.00	24.00	25.00	24.00	24.00
	292	25.00	25.00	25.00	24.00	25.00
	397	25.00	24.00	24.00	24.00	24.00
	387	27.00	26.00	27.00	27.00	27.00
	Mean	**25.50**	**24.75**	**25.25**	**24.75**	**25.00**
	SD	**1.00**	**0.96**	**1.26**	**1.50**	**1.41**
Gr.4 Control	390	24.00	23.00	23.00	24.00	24.00
	389	25.00	25.00	25.00	25.00	25.00
	244	25.00	25.00	24.00	25.00	25.00
	130	26.00	26.00	25.00	26.00	26.00
	Mean	**25.00**	**24.75**	**24.25**	**25.00**	**25.00**
	SD	**0.82**	**1.26**	**0.96**	**0.82**	**0.82**

Table G29: MCHC (g/dl), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	33.00	31.00	32.00	32.00	31.00
	292	33.00	32.00	32.00	32.00	25.00
	397	33.00	31.00	31.00	31.00	31.00
	387	32.00	31.00	31.00	31.00	30.00
	Mean	**32.75**	**31.25**	**31.50**	**31.50**	**29.25**
	SD	**0.50**	**0.50**	**0.58**	**0.58**	**2.87**
Gr.4 Control	390	32.00	31.00	31.00	31.00	31.00
	389	33.00	31.00	31.00	31.00	32.00
	244	33.00	32.00	32.00	31.00	32.00
	130	33.00	31.00	32.00	31.00	32.00
	Mean	**32.75**	**31.25**	**31.50**	**31.00**	**31.75**
	SD	**0.50**	**0.50**	**0.58**	**0.00**	**0.50**

Table G30: HCT (%), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	0.49	0.47	0.52	0.52	0.56
	292	0.48	0.49	0.48	0.49	0.51
	397	0.53	0.53	0.54	0.53	0.55
	387	0.54	0.51	0.54	0.56	0.58
	Mean	**0.51**	**0.50**	**0.52**	**0.53**	**0.55**
	SD	**0.03**	**0.03**	**0.03**	**0.03**	**0.03**
Gr.4 Control	390	0.51	0.52	0.55	0.53	0.54
	389	0.45	0.50	0.50	0.51	0.49
	244	0.45	0.48	0.50	0.49	0.49
	130	0.51	0.53	0.54	0.57	0.57
	Mean	**0.48**	**0.51**	**0.52**	**0.53**	**0.52**
	SD	**0.03**	**0.02**	**0.03**	**0.03**	**0.04**

Table G31: RDW (%),values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	14.20	14.70	15.00	13.06	14.00
	292	12.70	12.90	13.30	13.05	14.10
	397	13.30	14.10	13.50	14.60	13.80
	387	13.50	13.20	15.50	15.40	15.10
	Mean	**13.43**	**13.73**	**14.33**	**14.03**	**14.25**
	SD	**0.62**	**0.83**	**1.09**	**1.17**	**0.58**
Gr.4 Control	390	15.50	14.90	14.70	14.80	16.10
	389	14.10	15.40	14.10	14.10	16.20
	244	12.80	13.80	13.10	12.80	14.20
	130	13.40	15.30	13.60	13.90	15.20
	Mean	**13.95**	**14.85**	**13.88**	**13.90**	**15.43**
	SD	**1.16**	**0.73**	**0.68**	**0.83**	**0.93**

Table G32: Hb (g/dl), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	15.90	15.30	16.40	16.40	17.50
	292	16.10	15.50	15.40	15.90	15.90
	397	17.30	16.70	16.90	16.40	16.80
	387	17.50	15.90	16.70	17.50	17.60
	Mean	**16.70**	**15.85**	**16.35**	**16.55**	**16.95**
	SD	**0.82**	**0.62**	**0.67**	**0.68**	**0.79**
Gr.4 Control	390	16.50	15.80	16.80	16.40	16.70
	389	14.90	15.50	15.70	15.90	15.50
	244	14.60	15.10	15.70	15.30	15.90
	130	16.90	16.50	17.10	17.50	17.90
	Mean	**15.73**	**15.73**	**16.33**	**16.28**	**16.50**
	SD	**1.14**	**0.59**	**0.73**	**0.93**	**1.06**

Table G33: Neutrophils (x 10^9/l), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	11.90	6.10	3.30	2.90	7.70
	292	3.40	2.00	2.00	1.70	4.50
	397	12.60	8.20	9.90	4.70	13.60
	387	10.66	5.10	5.00	3.70	9.10
	Mean	**9.64**	**5.35**	**5.05**	**3.25**	**8.73**
	SD	**4.24**	**2.58**	**3.46**	**1.27**	**3.78**
Gr.4 Control	390	10.40	6.03	10.60	10.49	4.20
	389	5.60	3.20	6.50	8.30	2.90
	244	4.80	3.60	4.40	5.20	3.40
	130	3.80	3.30	4.00	3.70	3.73
	Mean	**6.15**	**4.03**	**6.38**	**6.92**	**3.56**
	SD	**2.93**	**1.34**	**3.02**	**3.05**	**0.55**

Table G34: Eosinophils (x 10^9/l), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	0.00	0.00	0.00	0.00	0.00
	292	0.00	0.00	0.01	0.01	0.00
	397	0.00	0.00	0.00	0.00	0.00
	387	0.00	0.00	0.00	0.00	0.00
	Mean	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
	SD	**0.00**	**0.00**	**0.01**	**0.01**	**0.00**
Gr.4 Control	390	0.00	0.01	0.00	0.00	0.00
	389	0.00	0.01	0.01	0.01	0.01
	244	0.00	0.00	0.01	0.00	0.01
	130	0.00	0.00	0.00	0.00	0.00
	Mean	**0.00**	**0.01**	**0.01**	**0.00**	**0.01**
	SD	**0.00**	**0.01**	**0.01**	**0.01**	**0.01**

Table G35: Basophils (x 10^9/l), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 12 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	0.0000	0.0100	0.0100	0.0100	0.0000
	292	0.0100	0.0100	0.0100	0.0100	0.0100
	397	0.0000	0.0100	0.0000	0.0200	0.0000
	387	0.0000	0.0100	0.0000	0.0100	0.0100
	Mean	**0.0024**	**0.0104**	**0.0051**	**0.0125**	**0.0054**
	SD	**0.0015**	**0.0003**	**0.0024**	**0.0099**	**0.0021**
Gr.4 Control	390	0.0000	0.0000	0.0000	0.0000	0.0000
	389	0.0000	0.0100	0.0000	0.0000	0.0100
	244	0.0100	0.0100	0.0100	0.0100	0.0100
	130	0.0100	0.0100	0.0100	0.0100	0.0100
	Mean	**0.0051**	**0.0075**	**0.0081**	**0.0051**	**0.0125**
	SD	**0.0022**	**0.0027**	**0.0021**	**0.0015**	**0.0101**

Table G36: Lymphocytes (x 10^9/l), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	0.70	1.00	0.80	0.70	0.80
	292	0.80	0.90	1.00	0.80	0.80
	397	0.80	0.90	1.00	0.90	0.50
	387	0.49	1.10	0.70	0.70	0.70
	Mean	**0.70**	**0.98**	**0.88**	**0.78**	**0.70**
	SD	**0.15**	**0.10**	**0.15**	**0.10**	**0.14**
Gr.4 Control	390	0.40	0.48	0.50	0.55	0.60
	389	0.50	0.80	0.60	0.90	0.70
	244	0.70	0.70	0.80	0.80	0.80
	130	0.60	0.70	0.80	0.60	0.75
	Mean	**0.55**	**0.67**	**0.68**	**0.71**	**0.71**
	SD	**0.13**	**0.14**	**0.15**	**0.17**	**0.09**

Table G37: Monocytes (x 10^9/l), values during baseline, week 4, 8, 12 and 16. Means and SD

are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	0.30	0.40	0.30	0.40	0.40
	292	0.20	0.10	0.10	0.10	0.20
	397	0.40	0.30	0.40	0.41	0.40
	387	0.45	0.30	0.30	0.40	0.40
	Mean	**0.34**	**0.28**	**0.28**	**0.33**	**0.35**
	SD	**0.11**	**0.13**	**0.13**	**0.15**	**0.10**
Gr.4 Control	390	0.40	1.30	0.60	0.76	0.30
	389	0.30	0.20	0.30	0.40	0.20
	244	0.20	0.20	0.20	0.20	0.10
	130	0.20	0.20	0.20	0.20	0.21
	Mean	**0.28**	**0.48**	**0.33**	**0.39**	**0.20**
	SD	**0.10**	**0.55**	**0.19**	**0.26**	**0.08**

Table G38: Platelet count (x 10^9/l), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	304.00	258.00	293.00	295.00	281.00
	292	147.00	119.00	130.00	145.00	155.00
	397	170.00	141.00	187.00	185.00	178.00
	387	133.00	148.00	141.00	138.00	158.00
	Mean	**188.50**	**166.50**	**187.75**	**190.75**	**193.00**
	SD	**78.50**	**62.24**	**74.38**	**72.52**	**59.55**
Gr.4 Control	390	148.00	137.00	151.00	158.00	154.00
	389	231.00	226.00	222.00	283.00	238.00
	244	206.00	205.00	214.00	233.00	220.00
	130	177.00	161.00	164.00	185.00	200.00
	Mean	**190.50**	**182.25**	**187.75**	**214.75**	**148.55**
	SD	**35.91**	**40.54**	**35.48**	**55.07**	**103.44**

Physical and physiological variables

Table G39: Weight (Kg), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 12 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	4.55	4.62	4.70	4.70	4.55
	292	5.90	5.83	6.03	6.19	6.12
	397	5.56	5.44	5.41	5.47	5.47
	387	5.57	5.35	5.52	5.62	5.17
	Mean	**5.40**	**5.31**	**5.42**	**5.50**	**5.33**
	SD	**0.59**	**0.50**	**0.55**	**0.61**	0.65
Gr.4 Control	390	5.52	5.55	5.47	5.49	5.57
	389	5.45	5.54	5.50	5.44	5.55
	244	5.42	5.49	5.41	5.46	5.55
	130	5.46	5.44	5.51	5.47	5.40
	Mean	**5.46**	**5.51**	**5.47**	**5.47**	**5.52**
	SD	**0.04**	**0.05**	**0.04**	**0.02**	**0.08**

Table G40: Body temperature (°C), values during baseline, week 4, 8, 12 and 16. Means Taand SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	39.1	39.2	38.8	39.1	38.80
	292	38.5	38.9	38.3	38.5	38.30
	397	39.1	39.2	39.1	38.9	38.90
	387	38.9	39.7	39.3	39.4	38.90
	Mean	**38.90**	**39.25**	**38.88**	**38.98**	**38.73**
	SD	**0.28**	**0.33**	**0.43**	**0.38**	0.29
Gr.4 Control	390	39.5	39.7	39.9	39.7	40.8
	389	38.6	39.4	39.0	39.0	39.4
	244	38.3	38.9	39.2	38.5	38.1
	130	38.1	39.0	38.8	39.0	38.9
	Mean	**38.63**	**39.25**	**39.23**	**39.05**	**39.30**
	SD	**0.62**	**0.37**	**0.48**	**0.49**	**1.13**

Table G41: Respiratory rate (breaths/min), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	24	32	28	24	24.0
	292	28	24	24	40	40.0
	397	24	28	28	28	32.0
	387	28	28	20	32	32.0
	Mean	**26.00**	**28.00**	**25.00**	**31.00**	**32.00**
	SD	**2.31**	**3.27**	**3.83**	**6.83**	6.53
Gr.4 Control	390	28	32	28	32	32
	389	28	24	28	38	32
	244	28	28	32	28	32
	130	24	28	28	32	32
	Mean	**27.00**	**28.00**	**29.00**	**32.50**	**32.00**
	SD	**2.00**	**3.27**	**2.00**	**4.12**	**0.00**

Table G42: Systolic pressure (mmHg), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14		Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	134	126		120	112	198.0
	292	95	65		92	100	158.0
	397	94	110		95	106	123.0
	387	88	99		98	92	106.0
	Mean	**102.75**	**100.00**		**101.25**	**92.00**	**146.25**
	SD	**21.06**	**25.83**		**12.74**	**8.54**	40.73
Gr.4 Control	390	115	131		118	143	122
	389	50	105		103	105	111
	244	98	104		100	77	94
	130	108	93		104	96	109
	Mean	**92.75**	**108.25**		**106.25**	**105.25**	**109.00**
	SD	**29.34**	**16.11**		**8.02**	**27.74**	**11.52**

Table G43: Diastolic pressure (mmHg), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	41	55	43	51	98.0
	292	50	34	46	53	135.0
	397	40	45	41	32	106.0
	387	37	44	52	42	87.0
	Mean	**42.00**	**44.50**	**45.50**	**44.50**	98.0
	SD	**5.60**	**8.58**	**4.80**	**9.61**	135.0
Gr.4 Control	390	76	45	56	54	65
	389	42	47	55	55	42
	244	46	51	51	37	42
	130	55	49	59	59	40
	Mean	**54.75**	**48.00**	**55.25**	**51.25**	**47.25**
	SD	**15.17**	**2.58**	**3.30**	**9.74**	**11.87**

Table G44: MAP (beats/min), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 21.01.15
Gr.3 2.14g/kg	374	61	79	67	78	76.0
	292	76	49	71	81	0.0
	397	45	71	52	44	0.0
	387	52	65	63	57	0.0
	Mean	**58.50**	**66.00**	**63.25**	**65.00**	**19.00**
	SD	**13.38**	**12.70**	**8.18**	17.61	38.00
Gr.4 Control	390	95	65	85	79	85
	389	47	71	83	85	85
	244	71	77	75	54	59
	130	68	73	86	87	61
	Mean	**70.25**	**71.50**	**82.25**	**76.25**	**58.00**
	SD	**19.65**	**5.00**	**4.99**	**15.22**	**14.46**

Table G45: Pulse (beats/min), values during baseline, week 4, 8, 12 and 16. Means and SD are indicated in bold.

Groups	Monkey No.	Baseline 29.09.14	Week 4 29.10.14	Week 8 26.11.14	Week 23.12.14	Week 16 21.01.15
Gr.3 2.14g/kg	374	148	150	123	131	77.0
	292	100	86	85	105	0.0
	397	145	129	140	163	0.0
	387	150	140	150	148	0.0
	Mean	**135.75**	**126.25**	**124.50**	**136.75**	**19.25**
	SD	23.92	28.17	28.59	**24.88**	38.50
Gr.4 Control	390	133	160	166	166	163
	389	101	129	119	119	114
	244	88	119	150	97	131
	130	109	94	119	119	126
	Mean	**107.75**	**125.50**	**138.50**	**125.25**	**133.50**
	SD	**18.93**	**27.31**	**23.44**	**29.08**	**20.92**

Appendix H: Log Sheets for Toxicology Studies

LOG SHEET SAMPLE

Check list: behaviour

0 = absent, not observed, not displayed + = weak, poor, minimal

++ = normal, average, moderate +++ = excessive, strong

Date	Exp. No.	Monkey No.	alert	fearful	aggressive	confused	depressed

Check list: motor function and activity

0 = absent, not observed, not displayed + = weak, poor, minimal

++ = normal, average, moderate +++ = excessive, strong

Date	Exp. No.	posture	coordination	locomotion	active	Use of exercise cage

Check list: physical

0 = absent, not observed, not displayed + = weak, poor, minimal

++ = normal, average, moderate +++ = excessive, strong, copious

disc. = discoloured d = discharge s = soft w = watery

Date	Exp. No.	Monkey No.	coat	feces	urine	eyes	nose	ears	genitals	rectal

Appendix I: NMR Spectra

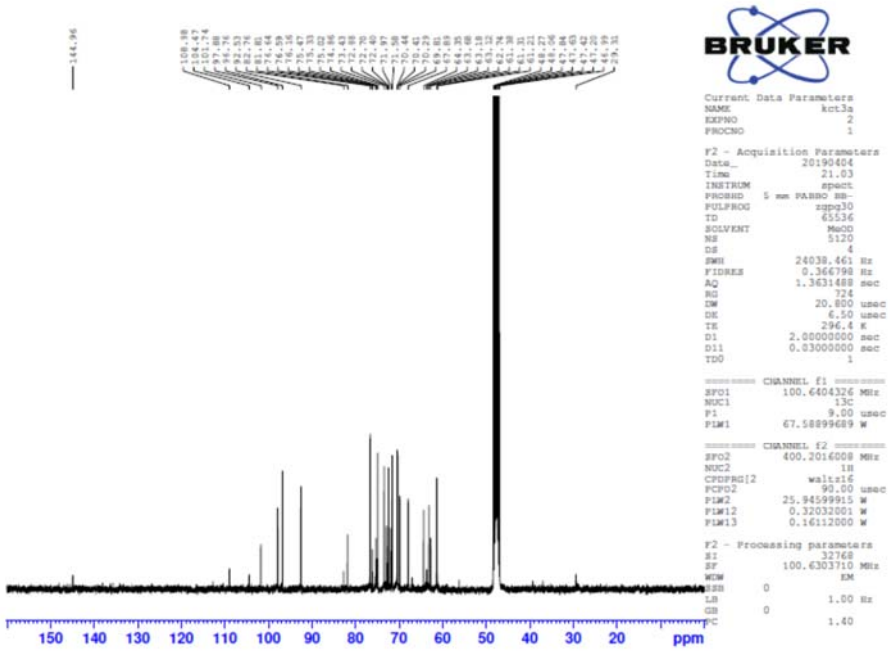

Figure I1.1: ^{13}C Spectrum of KTC3

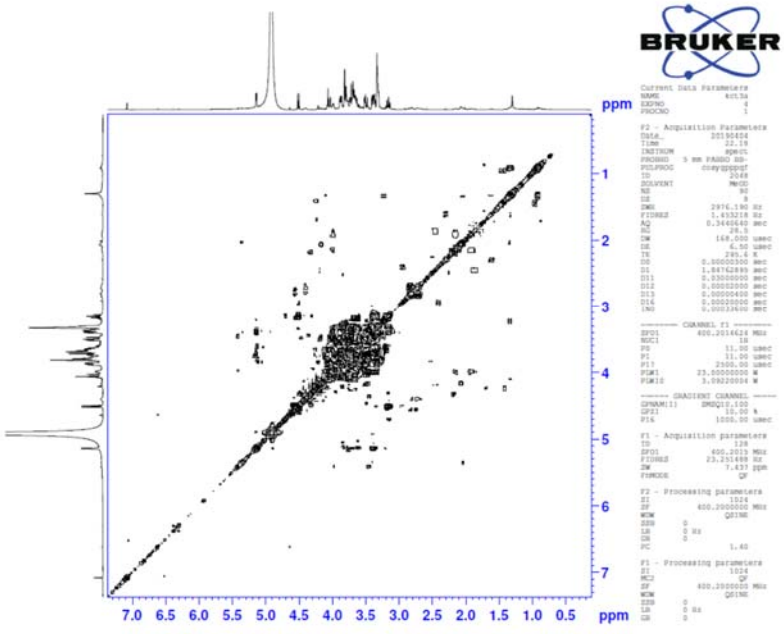

Figure 11.2: COSY Spectrum of KTC3

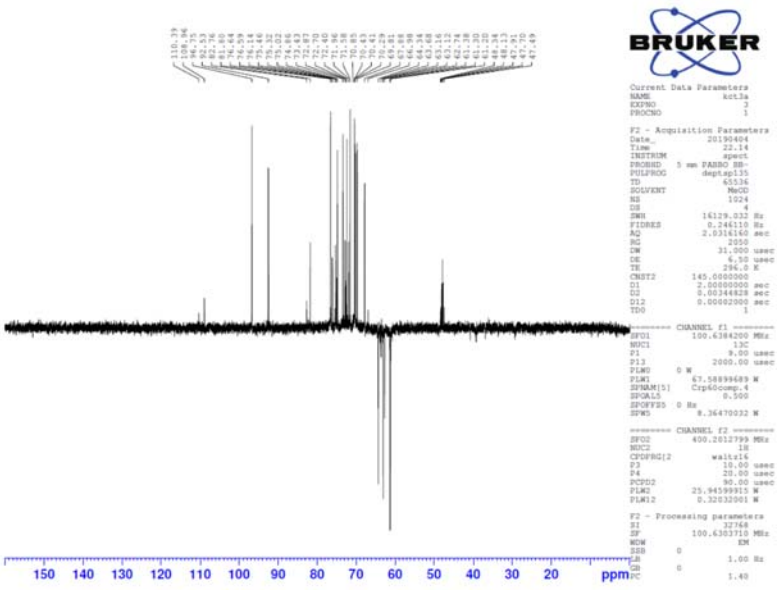

Figure I1.3: DEPT135 Spectrum of KTC3

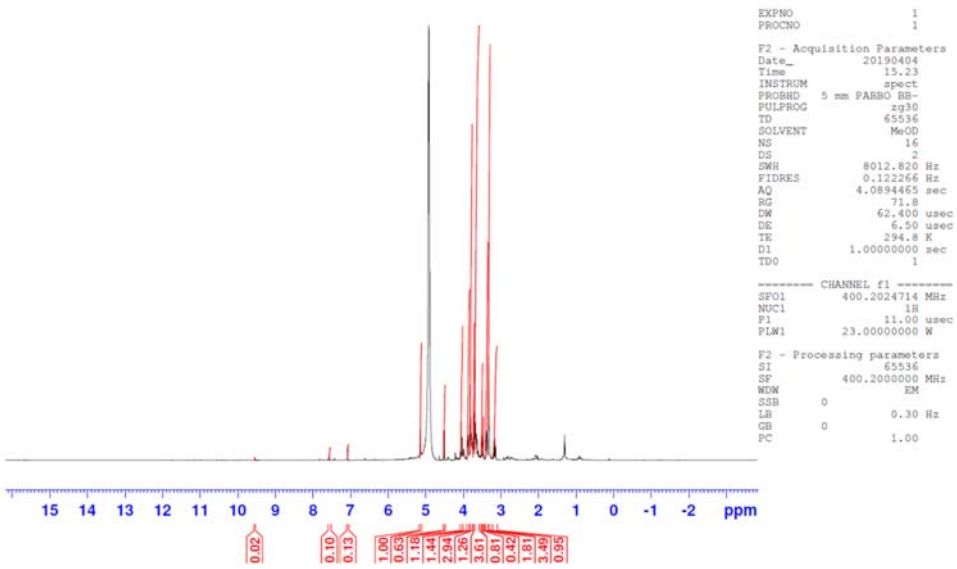

Figure I1.4: Proton Spectrum of KTC3

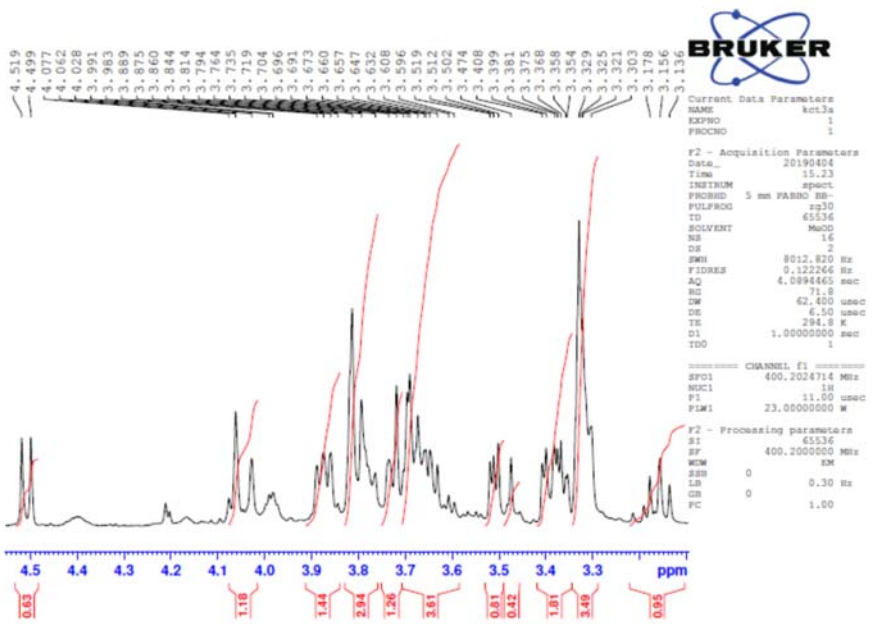

Figure I1.5: Proton NMR Spectrum of KTC3 (Expansion)

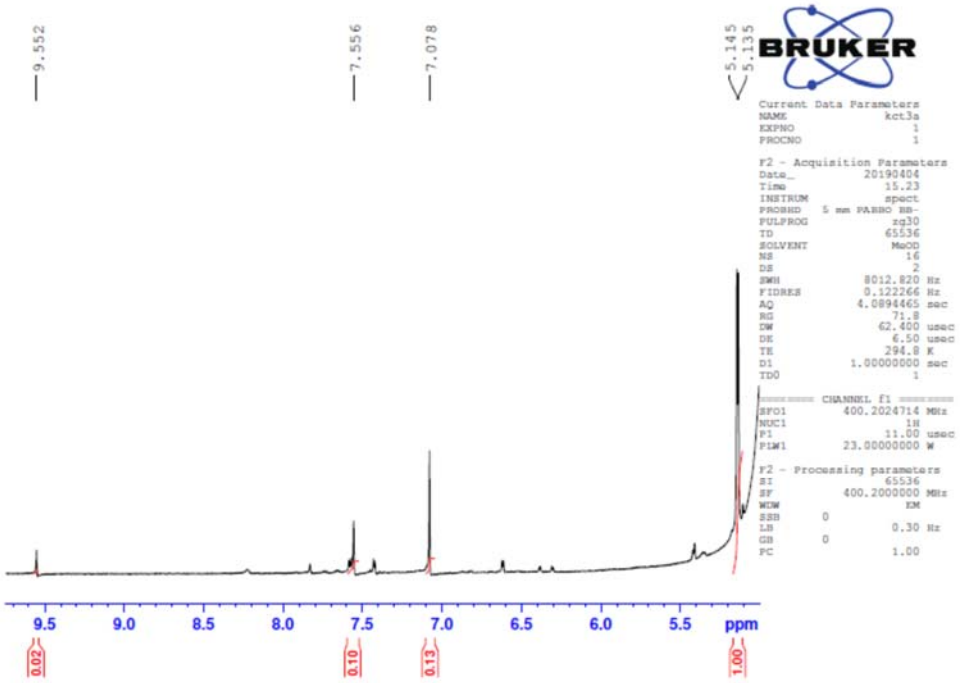

Figure I1.6: Proton NMR Spectrum of KTC3 (Expansion1)

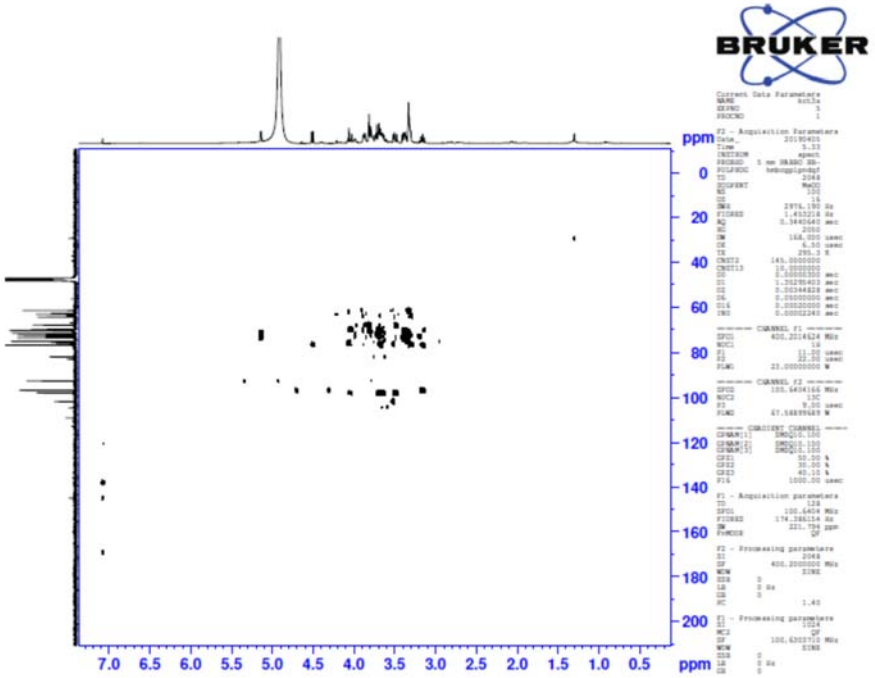

Figure I1.7: HMBC Spectrum of KTC3

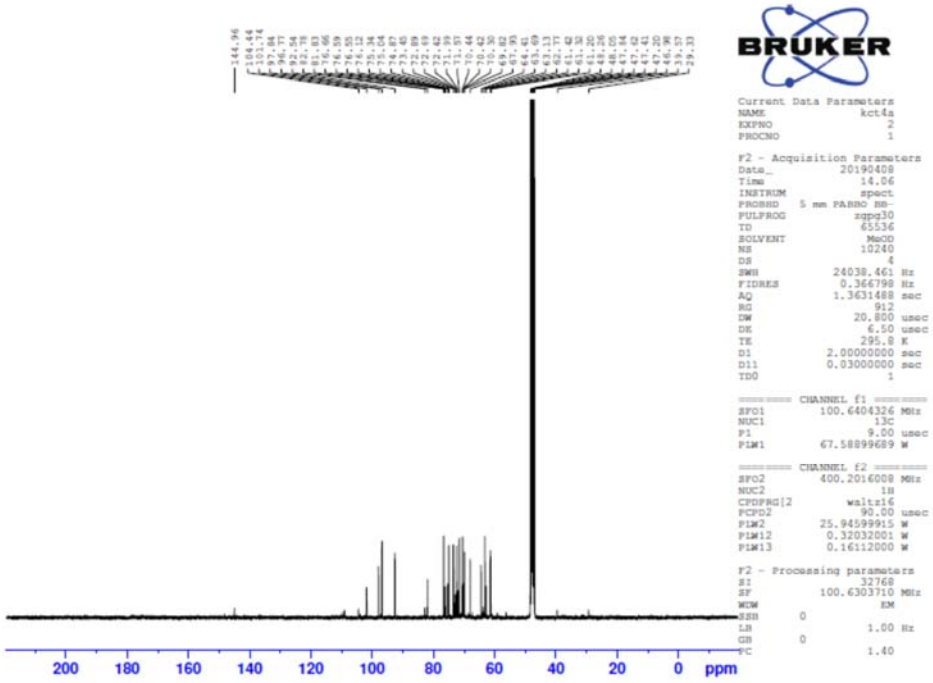

Figure I2.1: ^{13}C Spectrum of KTC4

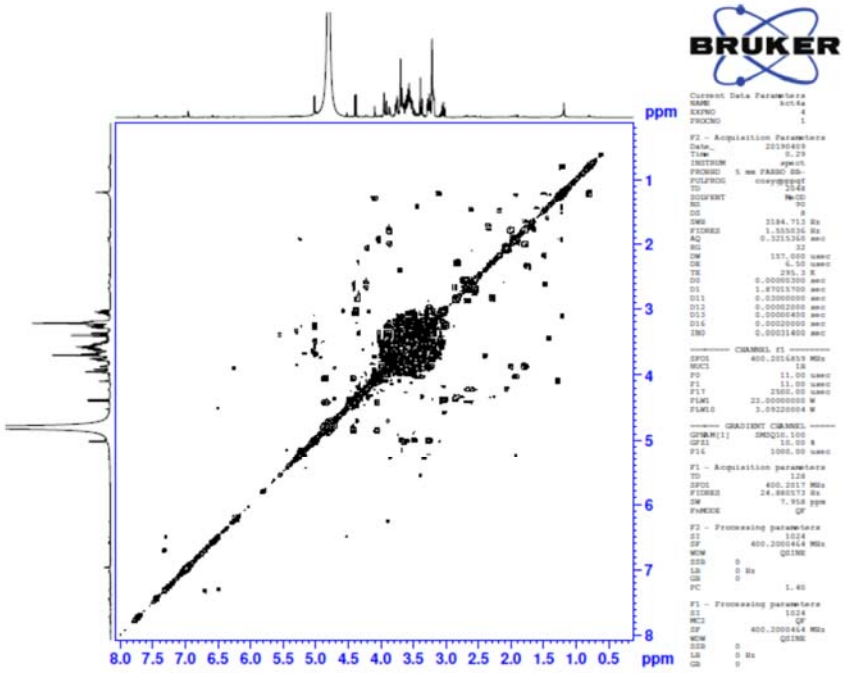

Figure E2.2: COSY Spectrum of KTC34

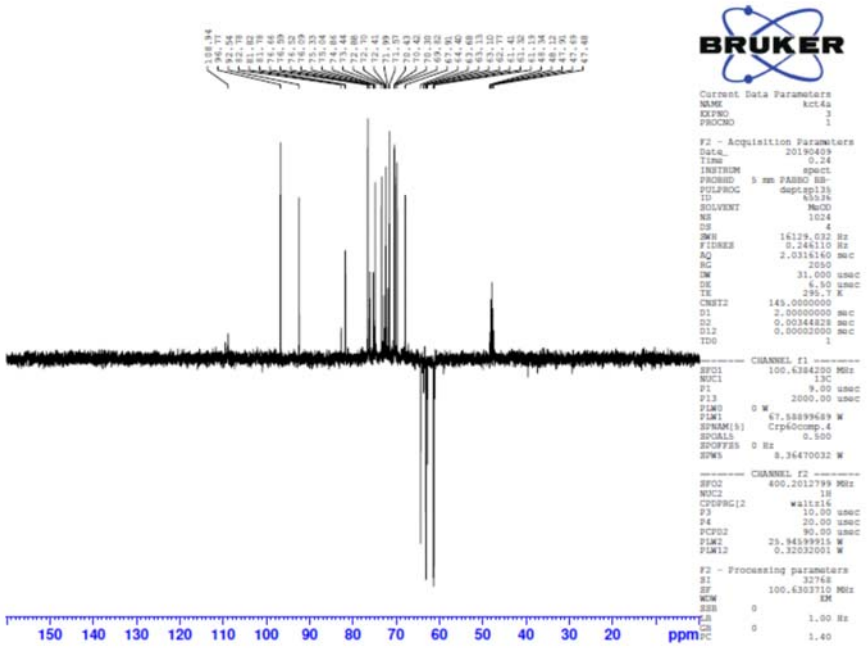

Figure I2.3: DEPT135 Spectrum of KTC4

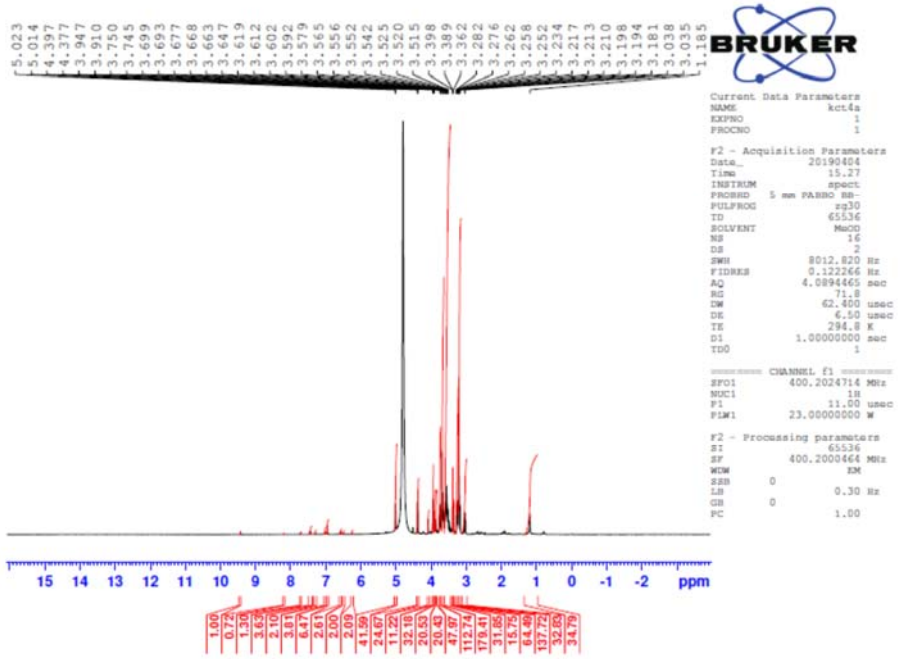

Figure I2.4: Proton Spectrum of KTC4

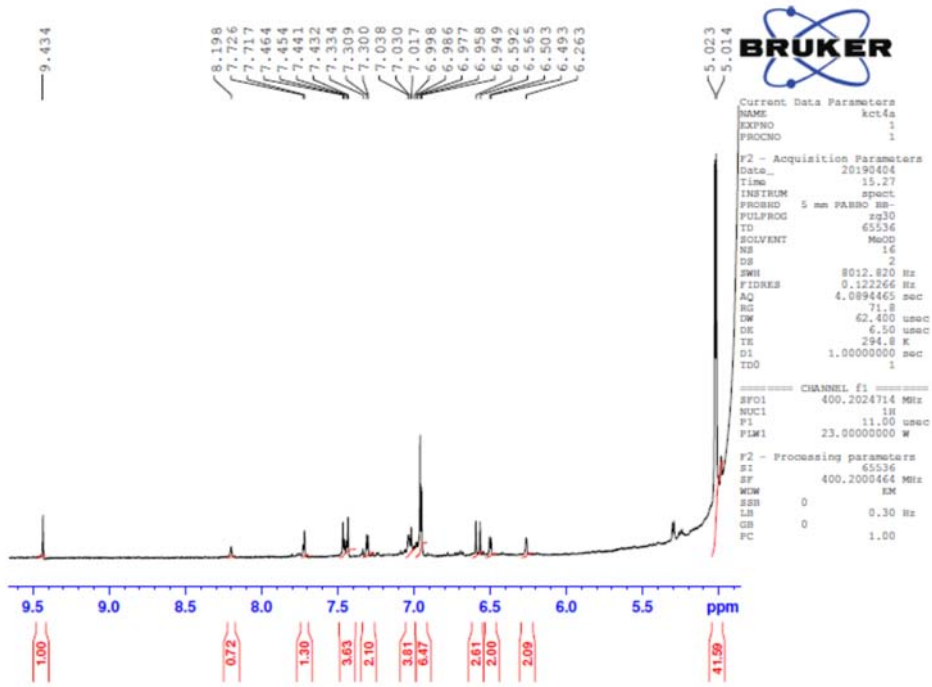

Figure I2.5: Proton NMR Spectrum of KTC4 (Expansion)

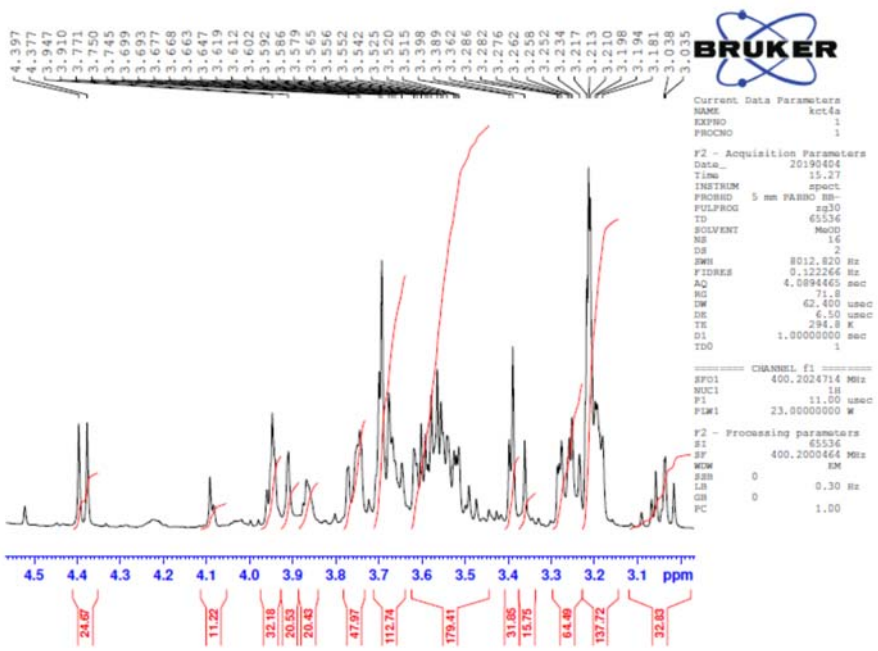

Figure I2.6: Proton NMR Spectrum of KTC4 (Expansion1)

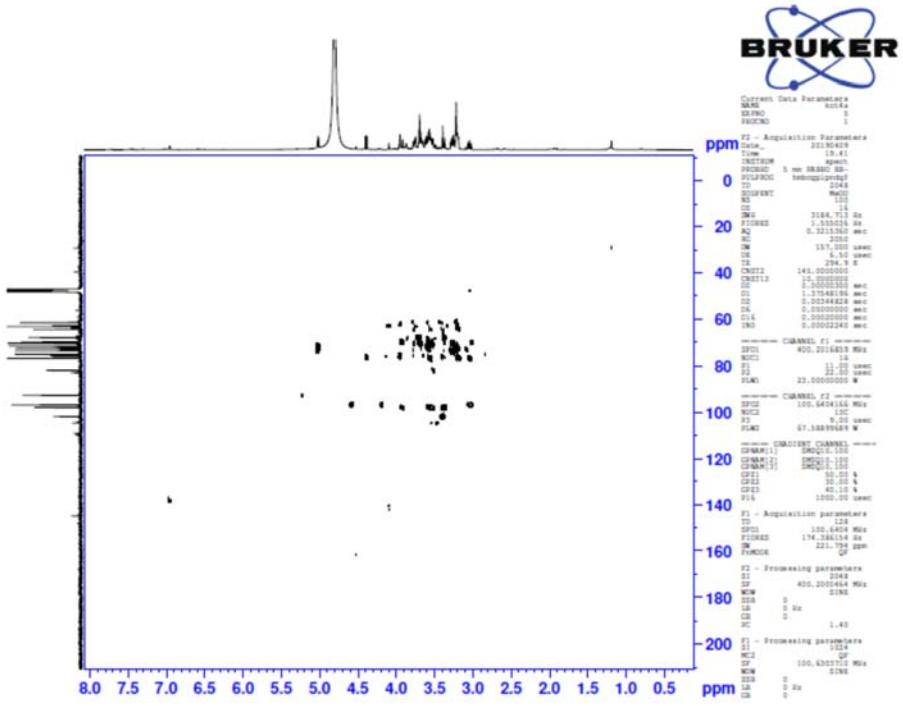

Figure I2.7: HMBC Spectrum of KTC4

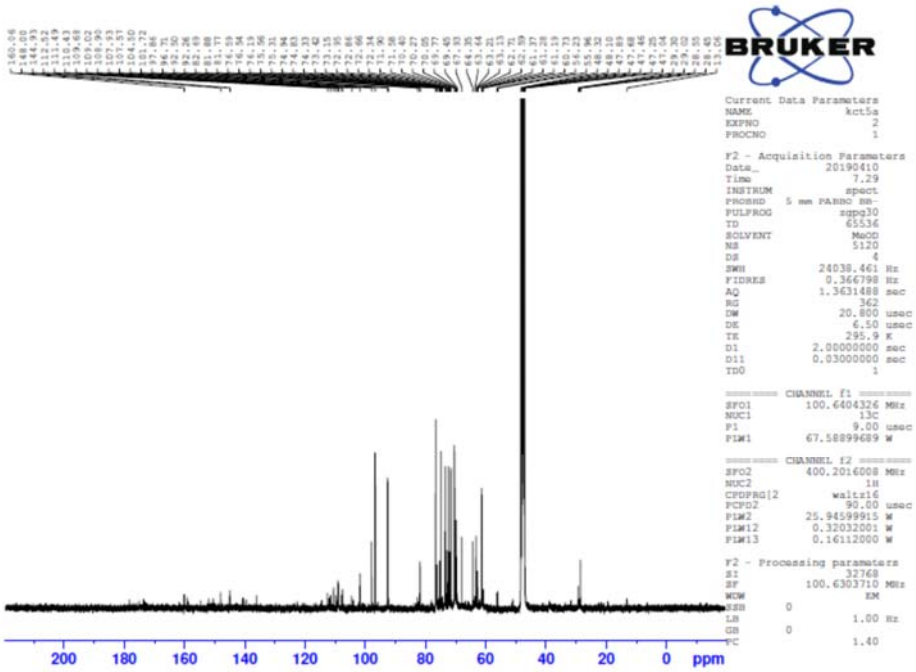

Figure I3.1: ^{13}C Spectrum of KTC5

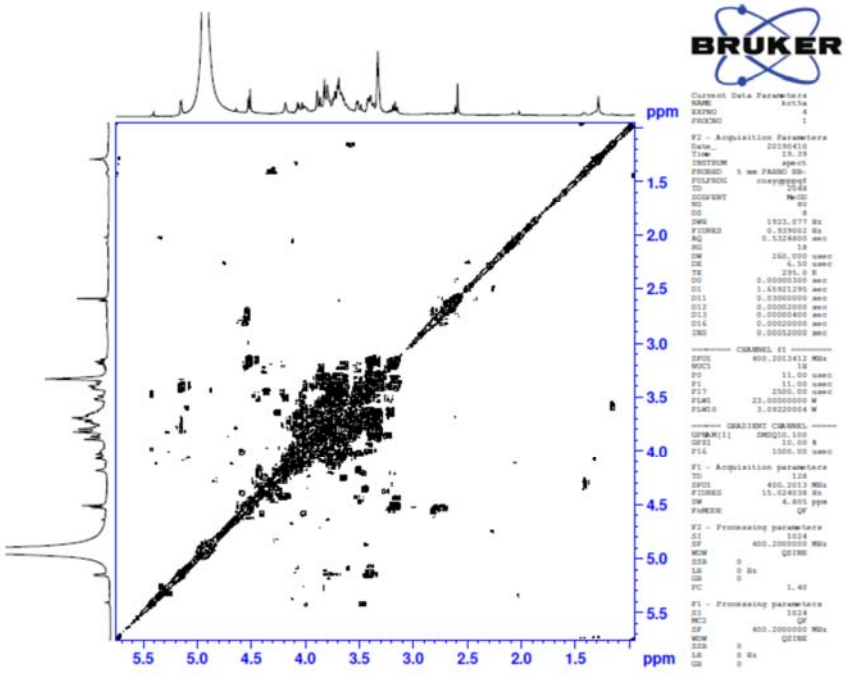

Figure I3.2: COSY Spectrum of KTC5

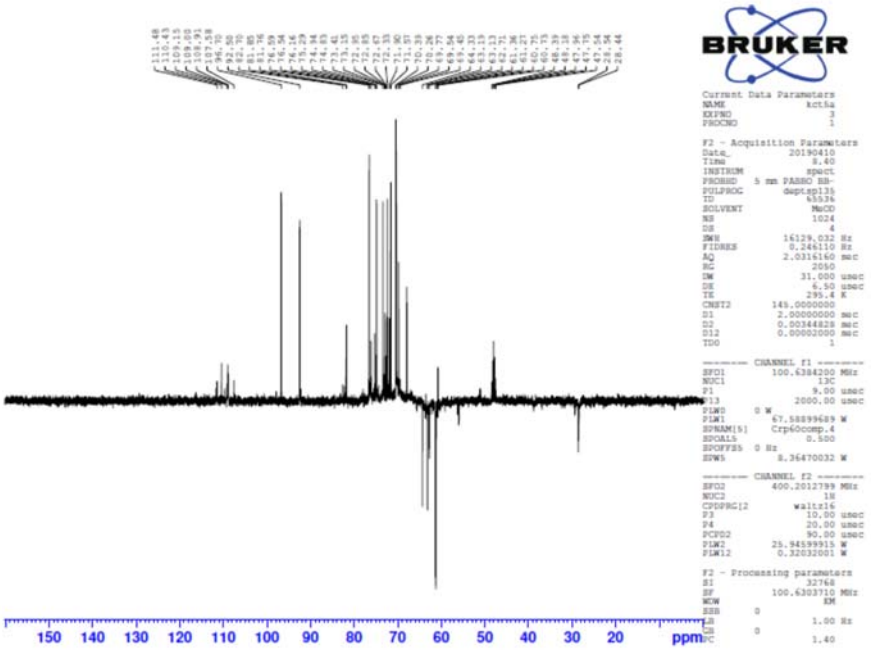

Figure I3.3: DEPT135 Spectrum of KTC5

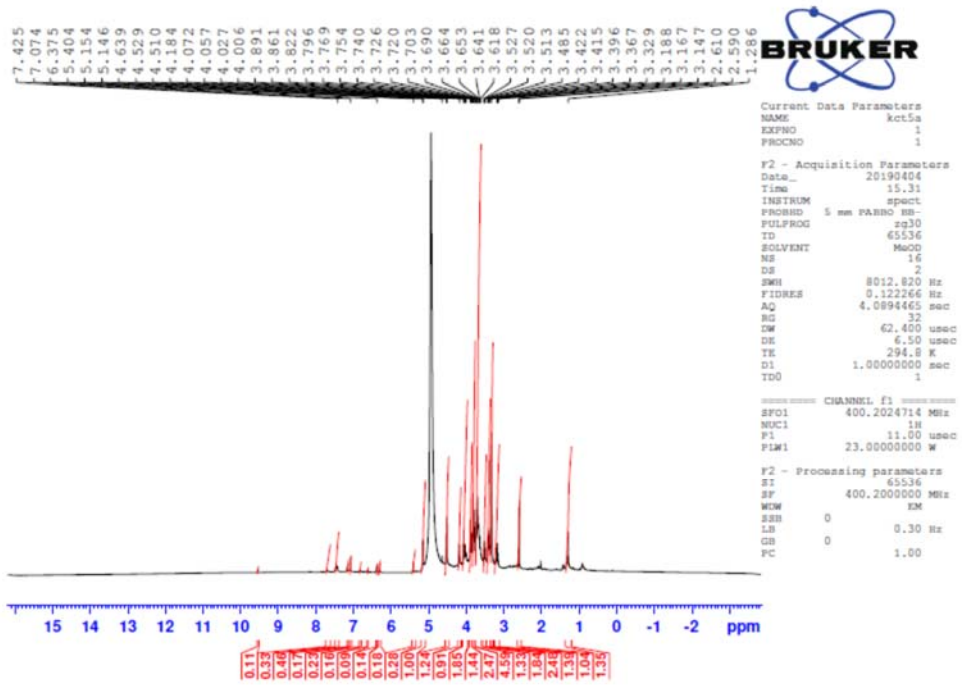

Figure I3.4: Proton Spectrum of KTC5

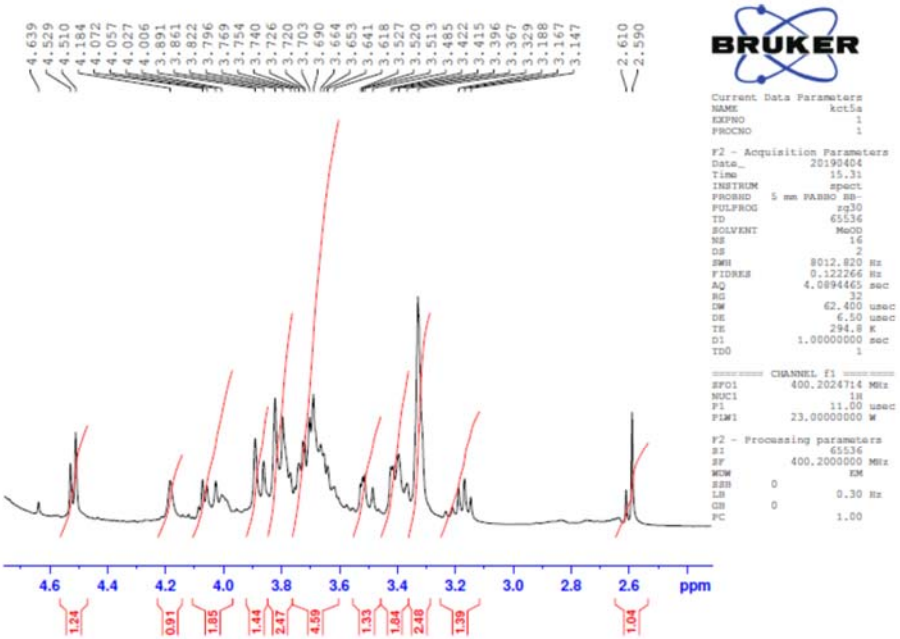

Figure I3.5: Proton NMR Spectrum of KTC5 (Expansion)

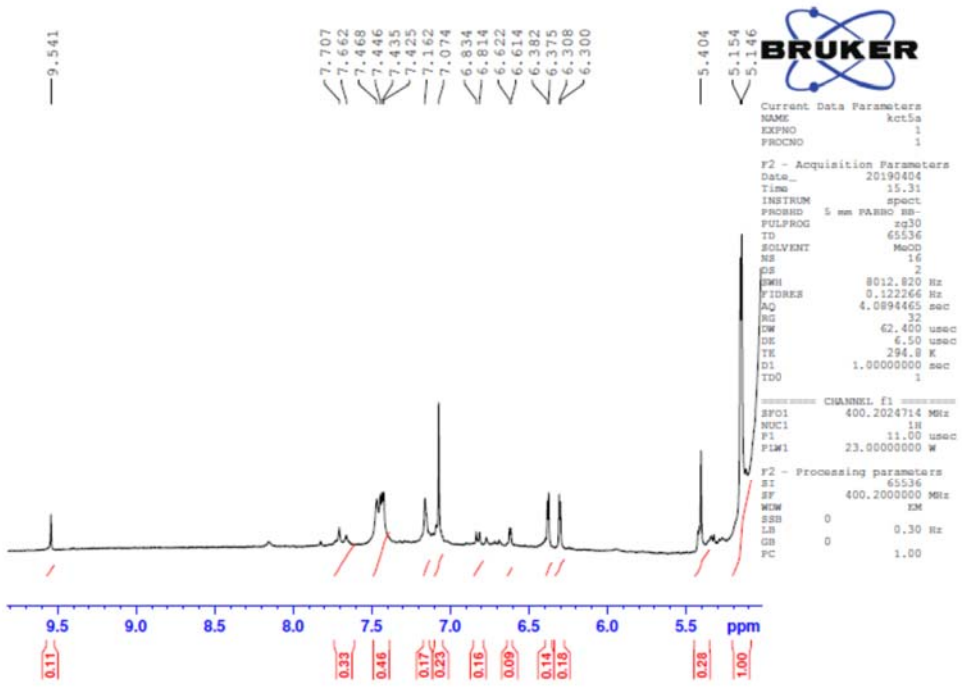

Figure I3.6: Proton NMR Spectrum of KTC4 (Expansion1)

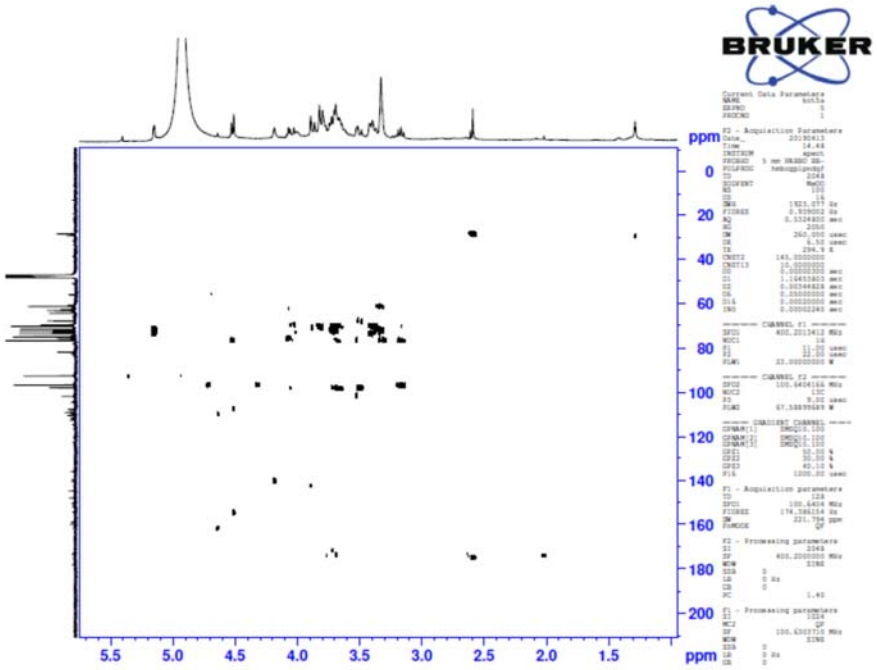

Figure I3.7: HMBC Spectrum of KTC5

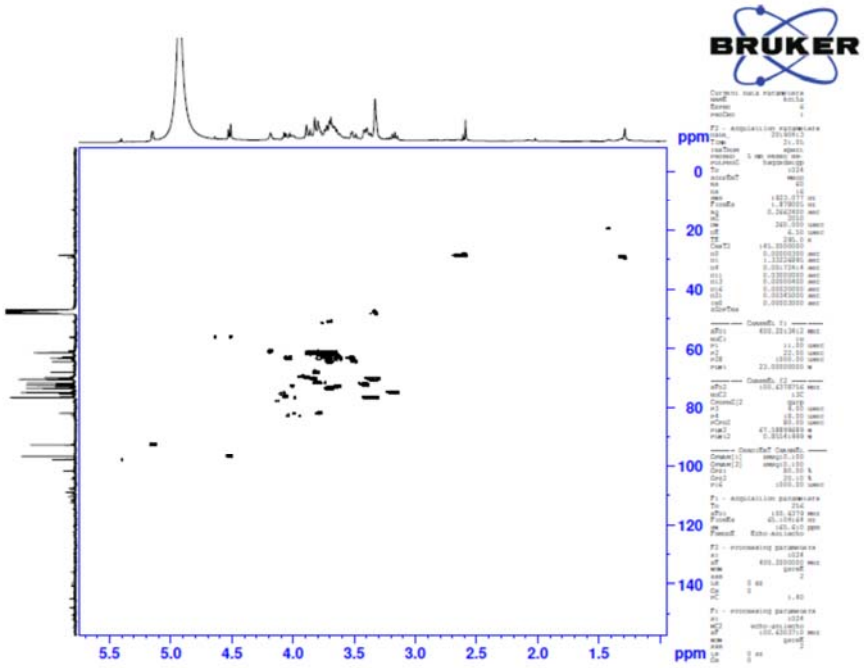

Figure I3.8: HMBC Spectrum of KTC5