A contribution to the phytochemical and antibacterial characteristics of *Crinum macowanii* bulbs extracts

By

Tendani Edith Sebola

Student number: 200905353

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SUPERVISOR: DR. DEREK NDINTEH

CO-SUPERVISOR: DR. VUYO MAVUMENGWANA

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EXECUTIVE SUMMARY

The World Health Organization (WHO) in 2014 indicated that about 80% of the world's population depend on medicinal plants for their primary health care, due to accessibility, affordability and cultural significance. However, issues such as extinction due to cultivation and lack of scientific validation of the therapeutic potential of medicinal plants through phytochemical and pharmacological screening hinder the sustainability and conversion of medicinal plants into a commodity of high value.

Crinum macowanii Baker is a plant belonging to the *Amaryllidaceae* family under the genus Crinum. The species from the genus occurr world-wide in America, Africa, and southern Asia to Australia, with Africa having the most species. *Crinum macowanii* Baker grows in many habitats like grasslands, beside rivers and along the coast and in various types of soil. The plant has a deciduous bulb, with fleshy roots and bright green to bluish green leaves and large white lilies with dark pink stripes. The flower produces about 20 to 80 small seeds that appear as smooth, pale green to silvery and fleshy. The bulbs are 6-25 cm in diameter. The flowers have a heavy scent and are normally 4-20 cm and with pedicels up to 6.5 cm long.

Traditionally, *C. macowanii* has been used for different applications in humans and animals since its known to possess medicinal properties. *Crinum macowanii* is used traditionally as a remedy for the treatment of boils, diarrhoea, fever, inflammation, respiratory system problems, skin rashes, tuberculosis, wounds and urinary tract problems. The bulbs are also used to increase lactation in women and cows. such indicates that the plant has phytochemicals which explains its continued used. The plant has shown to have antibacterial, antifungal, antiviral and anti-inflammatory properties. A number of alkaloids have been isolated from the plant which cannot all account for the biological uses of the plant and therefore this study was done to investigate the phytochemicals present in the plant and its antibacterial activity.

Crinum macowanii bulbs were purchased from Faraday Muti Market in Johannesburg South Africa. The bulbs were subjected to solvent extraction and pressurized hot water extraction. The solvent extraction produced the highest yield as compared to the pressurized hot water extracts.

From the phytochemical screening done, compound classes such as alkaloids, saponins, tannins, flavonoids, steroids and cardiac glycosides were detected in the bulbs. Concentrations of each compound could not be determined as quantitative tests were performed. The compounds detected have been reported to have biological uses such as antimicrobial, antidiarrheal and anticancer. Phytochemical analysis of both extracts was performed by two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GCTOF-MS), where this chromatographic technique was the first to be done on *C. macowanii* bulbs. Phytochemicals from compound groups such as fatty acid, sterol, volatile oil, alkaloid, cyclic ether, phenolic aldehyde and flavonoid were identified from both crude extracts (solvent extracts and pressurized hot water extracts). The isolated phytochemicals are known to have biological applications such as anti-inflammatory, anti-diabetic, anti-inflammatory, anti-cancer, anti-bacterial and anti-microbial. This can support and justify the traditional use for the plant for treatment of heart disease, kidney and bladder diseases, treatment of tuberculosis, for back pain and remedy for skin problems such as boils, sores and acne. Alkaloids were the most abundant from both crude extracts (organic solvent extract and pressurized hot water extract) and this supports literature since the plant is known to be rich in alkaloids.

Antibacterial activities of *C. macowanii* bulbs were determined by disc diffusion method and minimum inhibitory concentration (microdilution method). the tests were done on both crude extracts (organic solvent extract and pressurized hot water extract). A good antibacterial activity was demonstrated against *Bacillus cereus*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This provides justification for the traditional uses of the plant in the treatment of skin, stomach, respiratory, kidney and bladder infections. The pressurized hot water extracts (PHWE) were less active to most strains that were inhibited by the solvent extracts. The activity was exhibited against gram positive strains in both the test methods used and by both extracts. Most gram negative bacterial species were not inhibited by both extracts and both test methods used.

The metals analysis performed were analyzed by ICP-OES and metals such as calcium, cadmium, chromium, copper, iron, mercury, potassium, manganese, sodium, nickel, lead, strontium and zinc were detected. All metals with permissible limits were below those prescribed by WHO except for

mercury which was 0.544 mg/kg with the permissible limit being 0.20 mg/kg. Metals such as iron, zinc and chromium are known to have pharmacological effects such as resistance to infections, wound healing and prevention of atherosclerosis, this also supports the traditional uses of the plant for the treatment of heart disease and as a remedy for skin problems such as boils, sores and acne.

Keywords: *Crinum macowanii*, Antibacterial, Pressurized Hot Water Extracts, Metal analysis, GC×GC-TOFMS

DECLARATION

I hereby declare that this dissertation, which I herewith submit for the research qualification

MASTER OF TECHNOLOGY (MTech) IN BIOTECHNOLOGY

to the University of Johannesburg, Department of Biotechnology and Food technology, is, apart from the recognised assistance of my supervisors, my own work and has not previously been submitted by me to another institution to obtain a research diploma or degree.

_____ on this ____ day of _____

(**Candidate**)

_____ on this ____ day of _____

(Supervisor)

_____ on this ____ day of _____

(Co-supervisor)

DEDICATION

I dedicate this work to the Lord God Almighty who through Him all things are possible. My dad Masia Sebola, my mom Mukondeleli Sebola, my sister Unarine and my brother Mpho for their love, support and prayers.

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RESEARCH OUTPUTS

- T.E. Sebola, V. Mavumengwana, N. Niemann, and D.T. Ndinteh. Metal analysis, Phytochemical screening and antibacterial investigation of *Crinum macowanii* bulb. **Oral presentation** at the International Conference on Advances in Science, Engineering, Technology and Natural Resources (ICASETNR-16), to be held in November 24-25, 2016 in Parys, South Africa. http://doi.org/10.15242/IAE.IAE1116435.

- T.E. Sebola, V. Mavumengwana, D.T. Ndinteh, and N. Niemann. Phytochemical and antibacterial investigation of the medicinal plant *Crinum macowanii* bulbs. **Poster presentation** at the 2016 Autumn International Scientific Conference on Food Safety and Security, May 15-18, 2016, Johannesburg, South Africa, 2016. Won the best poster presentation award.

- T.E. Sebola, D.T Ndinteh, N. Niemann, and V. Mavumengwana. A Contribution to the Chemical Composition and Biological Activity of the Plant *Strelitzia reginae*. **Poster presentation** at the 2015 Women in Science Technology Engineering and Mathematics (STEM) conference, October 26-28, 2015, Johannesburg, South Africa.

- T.E. Sebola, D.T. Ndinteh, V. Mavumengwana, and N. Niemann. A Contribution to the Chemical Composition and Biological Activity of the Plant *Strelitzia reginae*. **Poster presentation** at the University of Johannesburg Cross Faculty Symposium, October 13, 2015, Johannesburg, South Africa.

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LIST OF ACRONYMS

AChE	Acetylcholinesterase
ATCC	American Type Culture Collection
Ca	Calcium
Cd	Cadmium
Cr	Chromium
Cu	Copper
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
Fe	Iron
Ga	Gallium
GC×GC-TOFMS	Comprehensive Two dimensional gas chromatography coupled to time of flight Mass Spectrometry
HCW	Hot compressed water
Hg	Mercury
H_2O	Water
In	Indium
K	Potassium
MIC	Minimum inhibitory concentration
Mn	Manganese
Na	Sodium
NCW	Near-critical water
Ni	Nickel
Pb	Lead
PPM	Parts per million
PHW	Pressurized hot water
PHWE	Pressurized Hot Water Extraction
PWE	Pressurized Water Extract
R_f	Retention factor
Rh	Rhodium

RNA	Ribonucleic acid
sdH_2O	Sterile distilled water
SCW	Subcritical water
Sr	Strontium
TLC	Thin Layer Chromatography
UV	Ultra violet light
WHO	World Health Organization
Zn	Zinc
1D GC	One dimensional gas chromatography

CHAPTER 1
GENERAL INTRODUCTION

1.0 BACKGROUND

Since the existence of mankind, nature (particularly plants) has provided food, shelter, tools, and medicine to various civilizations across the globe. This was found to be in form of wildflowers, edible bulbs, and carefully groomed grasslands (Reid and van Wishingrad,2009). People have engaged in a relationship with medicinal, edible, and otherwise useful native plants, which most occur naturally. Plants have been used as a source of medicinal compounds due to the presence of certain bioactive scaffolds within them. These medicinal compounds are believed to be useful in the treatment of certain disorders (Patel, 2015).

Given that health has been an important aspect to people, this has led to finding ways of achieving and maintaining a state of well-being that is free of disease and infirmity (Rajbhandary, 2014). Gupta *et al.,* (2014) stated that different therapeutic methods have been developed to combat diseases; these methods vary from cultural beliefs and communities hence Chinese traditional medicine and African traditional medicine, just to name a few of these healing methods used (Ndhlala *et al.*, 2011). With the increasing usage of traditional medicine, Payyappallimana (2009) stated that the World Health Organization (WHO) refers to traditional medicine as "health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being", which is based on practical experience and passed on from generation to generation through observation whether verbally or in writing (Adefolaju, 2011).

Despite civilization in most developing countries, the WHO reported that an estimated 80% of the world's population depends on traditional medicine for their primary health due to its easy access, its affordability and cultural significance (Brusotti *et al.,* 2014;Fennell *et al.,* 2004). Traditional knowledge and practices, customs and habits, constant processes of trial and error have influenced

and shaped traditional medicine to date (Rajbhandary, 2014). The utilization of traditional medicine is still widely spread across the world as seen in Figure 1.1.

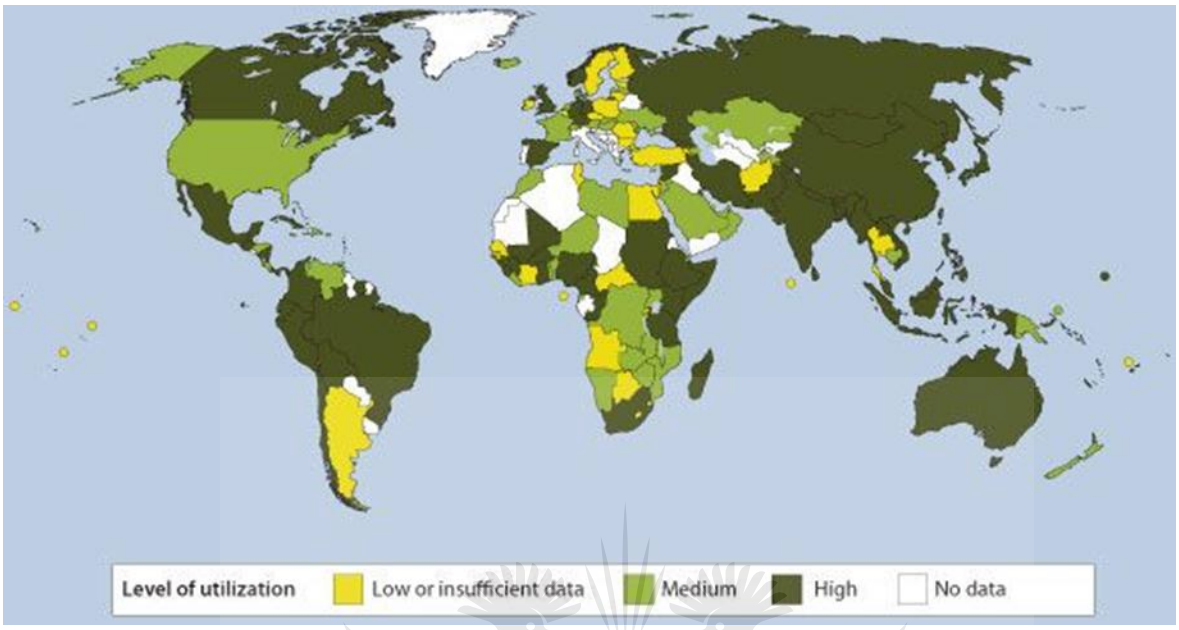

Figure 1.1: Map of the world showing the utilization of traditional medicine across the world (Alexander, 2015)

Africa, the cradle of humankind as it's known for its oldest medicinal system (van Vuuren, 2008), has a rich diversity of plants, with 25% of higher plants in the world being found in Africa (van Wyk, 2008). Arnold *et al.,* (2002) stated that traditional medicinal plants of Southern Africa amount to a total of 3481 plant taxa, of which 2942 are administered to people only as reported by van Wyk (2008). Pal and Shukla (2003) reported that herbal drugs are deduced as free from side effects and are cheap and locally available, hence their increasing interest and use. In South Africa only 38 of the estimated 3000 medicinal plant species that are used in traditional medicine have been processed into commodities of high value (including but not limited to *Hypoxis hemerocallidea*, *Gunnera perpensa* L. and *Aloe ferox* Mill. (van Wyk, 2008). This will support the view by Scott (1993) that indeed plants still remain the ''sleeping giant of drug development'' as also noted by Fennell *et al.,* (2004).

South Africa is a diverse country with different cultural groups and tribes, and therefore different applications of medicinal plants apply. It is also home to about 25 000 traditional healers, with the majority residing in provinces such as Mpumalanga, Limpopo, KwaZulu-Natal and the North West province (Campaign and Richter, 2003). In South Africa, traditional healers are classified as

diviners (Izangoma/Amagqirha), herbalists (Izinyanga/amaxhwele), prophets/faith healers (abaprofeti/abathandazeli), traditional surgeons (iingcibi), traditional birth attendants (ababelethisi /abazalisi) as stated by Mander *et al.,* (2007).

These traditional healers administer "muti" or medine which is the plant material to their patients to aid with the health process (Ndhlala *et al.*,2011). Urbanization was feared to be a threat to the utilization of traditional medicine but Ndhlala *et al.*, (2011) reaffirmed Marsland (2007) view that African Traditional Medicine (ATM) utilization is increasing in both urban and rural black communities. Ndhlala *et al.,* (2011) reported that for the past decade, an estimated 4 to 6% of annual incomes in South Africa have been spent on traditional medicine and related services.

1.1 Medicinal plants

Plants, especially medicinal plants have been proven to treat ailments and therefore are a valuable resource (Louw *et al.* 2002). Neuwinger (2000) reported that about 5400 medicinal plant taxa have been listed as medicinal plants in the African continent with over 16,300 medicinal uses assigned to them (van Wyk, 2008). van Wyk (2008) also stated that about 3000 medicinal plants are regularly used in Southern Africa for a number of different reasons as predicted by van Wyk and Gericke (2000). The interest in medicinal plants is increasing at accelerated rate, thus while resulting in greater market value, also threatens biodiversity. Mander (1998) estimated that more than 20000 tons of medicinal products with a turnover of approximately US$ 60 million per year are traded annually in South Africa. Most of these medicinal plants are sold in informal markets and therefore there is a great demand to develop and brand them so as to present those consumer products sold in formal market as over-the-counter medicines and herbal supplements (van Wyk, 2011).

With the demand of medicinal plants increasing, factors such as over-harvesting, overexploitation and extinction threaten this beautiful resource of South Africa. Plants such as African cherry (*Prunus africana*), devil's claw (*Harpagophytum spp*), aloe species and *Warburgia salutaris* (pepper bark) are South African plant species threatened by over harvesting and over-exploitation (Monakisi, 2007). Strategies such as large-scale cultivation, the use of alternate plants and plant part substitution have been put in place to help with the sustainability of the different plant species

(Monakisi, 2007). More scientific studies have to be done to assess the cultivation of threatened species, optimal harvesting systems and the regeneration of alternative potential species (Monakisi, 2007).

The "doctrine of signatures" has been used to state certain herbs that resemble various body parts and thus used to treat ailments of those body parts, where red-coloured herbs were used to treat blood diseases and goldenrod with a yellow hue was used to cure jaundice just to name a few (Salim *et al.*,2008). In this way, medicinal plants and their uses would be known. It has been documented in modern pharmacopoeia that 25% of therapeutic drugs are derived from plants and many other are built on template compounds isolated from plants (Scheffer,1991). Salim *et al.*, (2008) noted that morphine was the first pharmacologically active compound to be isolated in 1805 in pure form from a plant and its structure was ultimately elucidated in 1923. A number of alkaloids were isolated from various plants species in the 19th century, which included atropine from *Atropa belladonna*, caffeine from *Coffea arabica*, cocaine from *Erythroxylum coca* and morphine and codeine from *Papaver somniferum* (Table 1.1). Some of these compounds are still used as single agents or combinations or formulations in prescribed drugs (Salim *et al.*,2008). These discoveries lead to bioactive secondary metabolites from plants being used in medicines in their original state or in a modified form (Salim *et al.*, 2008), and thus the use of herbal medicines for healing purposes and production of herbal drugs gave birth to modern medicine (Pal and Shukla, 2003).

Table 1.1: Examples of pharmaceutical drugs developed from plants.

Pharmaceutical drugs	Plant	Drug Use
Cocaine	*Erythroxylum coca*	Stimulant
Atropine	*Atropa belladonna*	Painkiller
Caffenine	*Coffea arabica*	Stimulant
Morphine	*Papaver somniferum*	Analgesic

Adapted from Salim *et al.*, (2008)

For the past hundred years, most drugs known to effectively treat different ailments, originated from plants such as aspirin from willow bark used for the treatment of pain, quinine from cinchona bark used to treat malaria and digoxin from foxglove used to treat heart conditions (Vickers and

Zollman, 1999). The production of herbal products stopped in the mid-20th century due to impractical production costs as compared to synthetic drugs. Despite the latter being effective, in the 1960s issues such as side effects (associated with synthetic counterparts) began to arise. As such, natural products are reemerging as better alternatives contributing to their increased and preferred usage by some sectors of the population in developing countries (Pal and Shukla, 2003) Despite the discovery of plant derived drugs, diseases such as human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), cancer, tuberculosis and malaria still contribute to the high morbidity and mortality worldwide. Factors such as secondary infections and resistance of microorganisms to antibiotics are still concerns with regard to synthetic drugs, contributing to the high levels of recurring infections. This therefore, presents a threat to public health (Mahady, 2005). Therefore, scientific research of medicinal plants has to be thoroughly researched to find alternative effective drugs to treat and if possible cure this disease.

Medicinal plants have been used in the past for medicinal purposes, but their safety and efficacy is still of great concern, therefore toxicological tests have to be carried out to check for toxicity levels over a period of time (Ndhlala *et al.*, 2013). External factors such as climate, geographical distribution, growth conditions and season have been observed to have a great effect on the make-up of the plants and thus the chemical composition of the compounds (Patel, 2015).

1.3 Justification of study

South Africa is known for its botanical wealth with medicinal plants being the pride of the country. These plants have been used to sustain a health state for basic primary health care. Despite the popularity and great use of medicinal plants, there is a lack of scientific proof regarding the effectiveness, safety and toxicology of most plants. Therefore, scientific validation has to be acquired to allow traditional medicine to be used and sold in formal markets and also to discover new drugs and improve already existing ones. Most information regarding traditional medicine is passed on verbally from one generation to the next, with no formal documentation. This results in loss of valuable information so documentation of enthomedical data of medicinal plants has to be carried out. A thorough study of medicinal plants requires a multidisciplinary approach consisting of different fields such as botany (particularly taxonomy), chemistry, pharmacology,

microbiology, toxicology and horticulture. Taxonomic studies are important to find related species in order to fully understand the genetic make-up and metabolic diversity of the species. Ethnobotanical studies are normally used to document and explore social and cultural uses of the medicinal plants. Whilst chemical studies are necessary in identifying their phytochemicals, pharmacological studies are carried out to determine the mode of action of these medicinal plants. For traditional medicine to be scientifically validated, the identification of bioactive phytochemicals, determination of their mode of action, recommended dosages and toxicity levels and the chemical make-up of the plant are normally undertaken (Lukhele *et al.*,2009).

Crinum macowanii Baker from the *Amaryllidaceae* family is a medicinal plant indigenous to Southern Africa and it is used traditionally for a number of applications (Watt and Breyer-Brandwijk, 1962). The bulb is used traditionally to treat itchy rashes, boils, acne, backache and venereal disease and is also used to increase lactation in women and cows (Nair *et al.*,2000). It has been reported to have antibacterial and antifungal activity and this could support its traditional use (Elgorashi *et al.*, 2003; Fennell and van Staden, 2001). The C*rinum* genus possesses chemical compounds such as flavonoids, coumarins, alkaloids and terpenoids, which make them true representatives of the *Amaryllidaceae* family (Asmawi *et al.*,2011). Thus, this study is aimed at investigating the phytochemistry, antibacterial activity and the metal makeup of the plant's bulb. Results from the study could add value to the information already available with regards to the phytochemistry, biological activity and scientific validation of the traditional uses of *C. macowanii* and hopefully contribute to the continued search for more bioactive compounds from this species.

1.4 Objectives of study

The overall intention of the study is to provide a scientific basis for the use of *C. macowanii* in traditional medicine. Within this framework the following objectives were proposed:

- To extract the chemical components from *Crinum macowanii* bulbs by solvent extraction and pressurized hot water extraction.
- To perform phytochemical screening of *Crinum macowanii* and to check for different classes of compounds present in the plant.

- To perform phytochemical analysis on the crude extracts (solvent extracts and Pressurized Hot Water Extracts) using two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-TOFMS).
- To screen the crude extracts (solvent extracts and Pressurized Hot Water Extracts) against various bacterial strains using disc diffusion and microdilution methods.
- To analyze for metals using inductively coupled plasma optical emission spectrometry (ICP-OES) present in *Crinum macowanii* bulbs.

1.5 Thesis outline

In addition to the general introduction presented in this chapter, the thesis comprises of six more chapters. A brief description of what is discussed in each chapter is outlined:

Chapter 2 (Literature Review)

This chapter presents a thorough literature survey discussing some major groups of naturally occurring plant chemicals (phytochemicals), the botanical description of the plant under study and its related plant species and techniques used in this study.

Chapter 3 (*Crinum macowanii* bulbs phytochemical constituents and their GC×GC-TOFMS screening)

In this chapter, phytochemical analysis of the plant was studied. Different analytical techniques were used for the identification and isolation of phytochemicals. Although the chemical profile of the plant is known, the interest was to explore the screening of plant's phytochemicals using GC×GC-TOFMS.

Chapter 4 (Antibacterial activity of *Crinum macowanii* bulbs)

In this chapter the biological activity of the plant was studied. Antibacterial tests were carried out to investigate the activity of the different plant extracts. The rationale of the traditional uses of the plant with relevance to its biological activity was established.

Chapter 5 (Pressurized hot water extraction of *Crinum macowanii* bulbs)

In this chapter, the use of a pressurized hot water extraction system was investigated and the hot water extracts tested for possible antibacterial properties and compared to the organic solvent extracts. The extracts were also screened with GC×GC-TOFMS.

Chapter 6 (Inductively Coupled Plasma Optical Emission Spectrometry metal analysis of *Crinum macowanii* bulbs)

In this chapter, metal analyses of the plant were carried out by ICP-OES so as to ascertain the presence of heavy metals and possible permissible limits.

Chapter 7 (Conclusions and Recommendations)

This chapter is a summation of the conclusions and recommendations drawn from the outcomes of the study.

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CHAPTER 2

LITERATURE REVIEW

2.0 BRIEF

This chapter deals with the literature survey of *Crinum macowanii,* its phytochemicals (natural plant products) and their pharmacological activities and those of its closely related species, botanical description of the plant and its traditional medicinal uses and also the different techniques used in this study.

2.1 Natural plant products (Phytochemicals)

Plants naturally produce chemical compounds which are biologically active and known as phytochemicals (Saxena *et al.,*2013). Phytochemicals contribute to a plant's colour, aroma and flavour. They also protect plants from disease, damage and environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack (Saxena *et al.,*2013). Jayaprasad and Sharavanan (2015) reported that phytochemicals have been classified according to protective function, physical characteristics and chemical characteristics and over 4 000 phytochemicals have been listed with almost 150 having been studied in detail. Phytochemicals are found in different plant parts such as the roots, stems, leaves, flowers, fruits or seeds and bulbs (Saxena *et al.,* 2013). The accumulation levels of phytochemicals depend on factors such as processing, cooking, storing and growing conditions (Saxena *et al.,* 2013).

Natural plant products can be divided into two groups depending on the role they play in plants and the function they confer to the plant, these are known as primary and secondary metabolites (Hoffmann,1951). Primary metabolites are compounds responsible for the well-being and are essential for the survival of the plant. These compounds are responsible for processes like respiration, photosynthesis, growth and development. Examples of primary metabolites include, amino acids, nucleotides, common fatty acids and sugars (Hoffmann,1951). Secondary metabolites on the other hand have no distinct function and therefore are not involved directly in the reproduction, development and growth of plants but deal more with function of the plant in relation to its ecology. These functions include attracting pollinators or serve as chemical defenses against

insects or any other predator, examples include tannins, alkaloids, flavonoids, steroids, saponins and cardiac glycosides (Hoffmann,1951).

2.1.1 Common primary metabolites

2.1.1.1 Amino acids

Amino acids are described as any molecule containing both a carboxylic acid (-COOH) and an amino ($-NH_2$) functional group attached to the same tetrahedral carbon atom. They appear as colourless crystals which are soluble in water and insoluble in ether and other organics (Wade, 2010). Amino acids are chemical units or building blocks which when joined together form proteins (Hounsome *et al.,* 2008). There are twenty amino acids that are found as building blocks of plant proteins and also about several hundred non protein amino acids which are also present in plant species (Dunlop *et al.,* 2014). Non-protein amino acids are defined as compounds isolated from plants as secondary metabolites, which have a similar structure to the commonly used amino acids as the building blocks for proteins. Some roles played by non-protein amino acids include functioning as intermediates in the biosynthesis of the plant signaling molecules, vitamins, and other constituents (Velíšek *et al.,* 2006), examples include ornithine, canavanine and γ-aminobutanoic acid. Primary metabolites such as sugars and fatty acids are distinguished chemically from amino acids, in that amino acids have about 16 percent nitrogen whereas the others do not contain nitrogen (Velíšek *et al.,* 2006). Amino acids have biological functions such as acting as chemical messengers in a variety of biochemical pathways (as in the case of neurotransmitters and hormones), being precursors for a variety of complex nitrogen-containing molecules such as nucleotides and chlorophyll and also act as metabolic intermediates (McKee and McKee, 2008). Amino acids such as glycine, lysine, threonine and glutamate (Figure 2.1) which help to maintain intestinal integrity and health have also been detected from a variety of plants (Moran-palacio *et al.,* 2014).

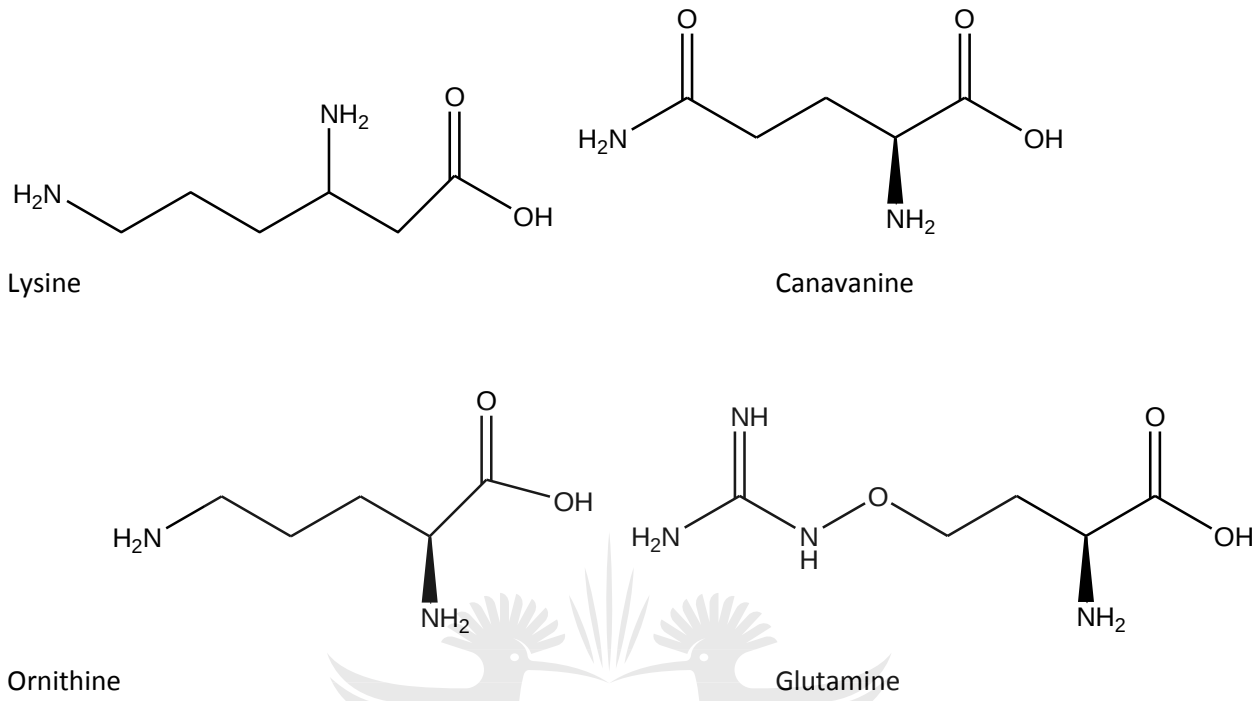
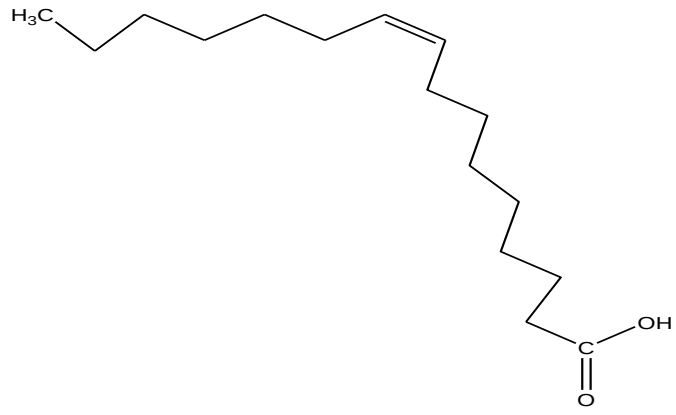


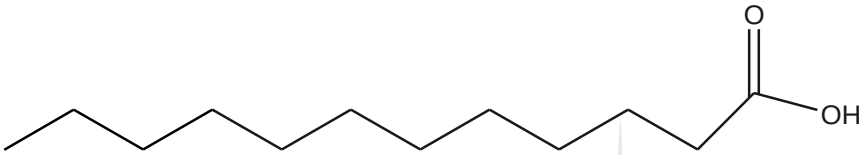
Figure 2.1: Structures of common amino acid

2.1.1.2 Fatty acids

Fatty acids are carbon chains with a methyl group at one end of the molecule and a carboxyl group at the other end (Rustan and Drevon, 2005). Fatty acids play important roles such as the storage and transport of energy, also are essential components of all membranes and function as gene regulators (Rustan and Drevon, 2005). Fatty acids are used to reduce inflammation among patients with rheumatoid arthritis and further used in cosmetics, detergents, fat emulsions and liposomes (Simopoulos, 2004). There are two types of fatty acids (saturated and unsaturated). The former are saturated with hydrogen and therefore have no double bonds. They contain an even number of carbon atoms ranging from 12 to about 22 carbon atoms. Examples include palmitic acid, lauric acid and capric acid (Figure 2.2). Unsaturated fatty acids have one or more double bonds between carbon atoms, occurring in different positions. The chain length varies between 16 to 22 carbon atoms possessing a cis or trans configuration. Examples include palmitoleic acid, oleic acid, vaccenic acid and linoleic acid (Millar *et al.,* 2000).



Palmitoleic acid

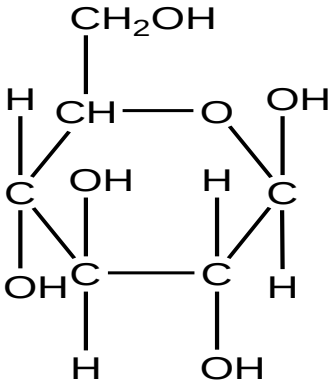


Lauric acid

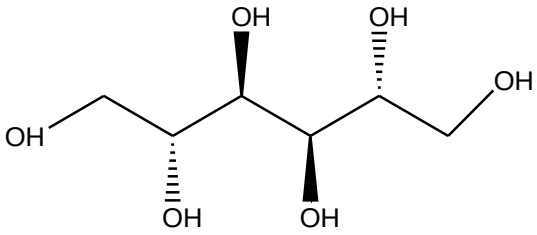
Figure 2.2: Structures of fatty acids

2.1.1.3 Carbohydrates

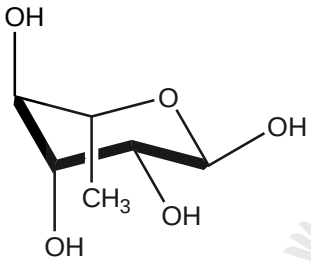
Carbohydrates are biological molecule consisting of carbon (C), hydrogen (H) and oxygen (O) atoms and occur in living organisms (Ahmad, 2007). They are referred to as the first complex organic compounds formed in the plants as a result of photosynthesis. Carbohydrates perform different roles in living organisms, in plants they provide building blocks for plant structural components, such as cellulose which is important in building cell walls and they also deliver energy for plant growth (Roberts and Caserio, 1977). Carbohydrates are divided into three groups according to their degree of polymerization, the groups include simple (i) sugars, (ii) oligosaccharides and (iii) polysaccharides (Ahmad 2007). Other roles played by carbohydrates include functioning as storage for energy while monosaccharide such as ribose and deoxyribose are the backbone of the genetic molecule known as ribonucleic acid (RNA) and Deoxyribonucleic acid (DNA) respectively. They also play vital roles in the immune system, fertilization and blood clotting (Khowala *et al.,* 2008). Examples of the different carbohydrates groups are shown in Figure 2.3 (Hounsome *et al.,* 2008).



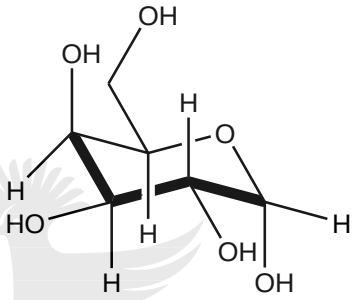
Glucose



Mannitol



Alpha-L-Rhamnose



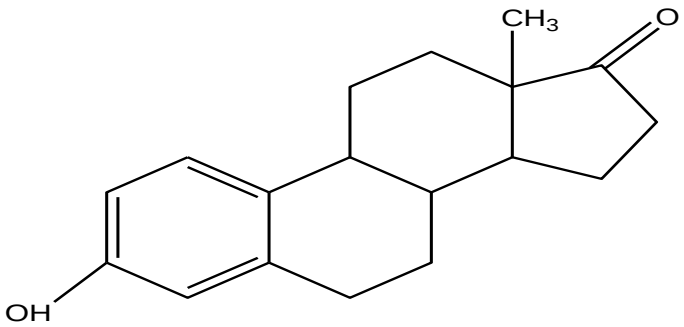
D (+) Galactose

Figure 2.3 Structures of carbohydrates

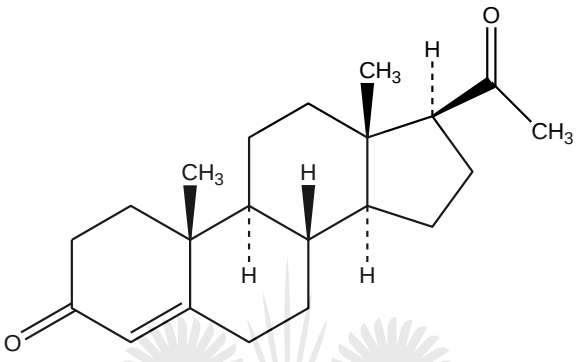
2.1.2 Common secondary metabolites

2.1.2.1 Steroids

Steroids are organic compounds classified as terpenoid lipids and contain a steroid nucleus (a carbon skeleton with four fused rings). Functional groups attached to the rings and oxidation state of the rings differentiate different classes of steroids. Different biological actions of steroids are determined by various groups attached to the common nucleus. When the nucleus contains alcohol and ketone groups, the steroids are called sterols and sterones respectively (Bhawani *et al.,* 2010). Many steroids include hormones, alkaloids and vitamins. Steroids such as androgens, estrogens and progestogens (Figure 2.4) function as hormones for controlling metabolism, for developing and appropriate functioning of sexual organs (Bhawani *et al.,* 2010). Progesterone regulates female reproductive functions such as induction of ovulation, facilitation of implantation, maintenance of early pregnancy and lobular-alveolar development in preparation for milk secretion (Al-asmakh, 2007).



Estrogens

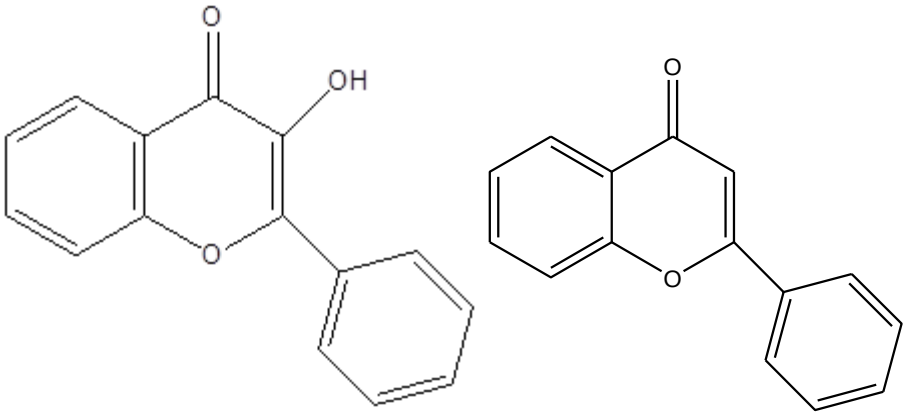


Progesterone

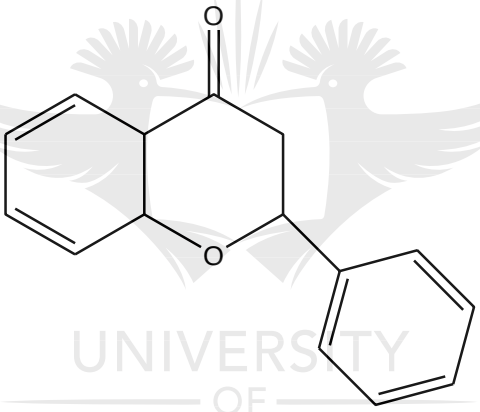
Figure 2.4: Structures of steriods

2.1.2.2 Flavonoids

Flavonoids are secondary metabolites obtained from plants and fungi and consist of a large group of polyphenolic compounds containing a benzo-γ-pyrone structure (Kumar and Pandey, 2013). A general structure of a flavonoid consists of a 15-carbon skeleton, which consists of two phenyl rings and a heterocyclic ring and can be abbreviated as C_6-C_3-C_6 (López-lázaro 2009). Flavonoids perform different functions in plants such as plant pigmentation for pollen attraction and act as chemical messengers and physiological regulators (Kumar and Pandey 2013). Tiwari *et al.,* (2011) reported that flavonoids have been noted to have bactericidal effects on several strains (for instance, strains such as *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aerginosa*) of bacteria by complexing with the cell wall and binding to adhesins (cell wall surface components involved in influencing adherence of bacteria with each other) (Griff *et al.,* 2013; Tereschuk *et al.,* 1997). Flavonoids may be divided into six groups that include flavanone, anthocyanin, flavonol, flavanol, isoflavone and flavone (Figure 2.5) depending on the linkage of the aromatic ring to the benzopyrano and the different groups (Kumar and Pandey, 2013; Ververidis *et al.,* 2007).



Flavon Flavone



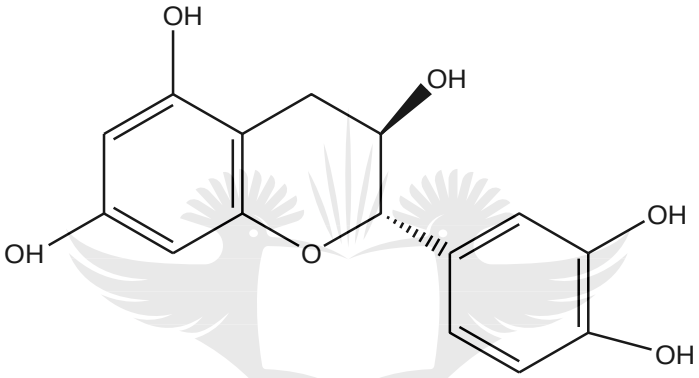
Flavanone

Figure 2.5: Structures of flavonoids

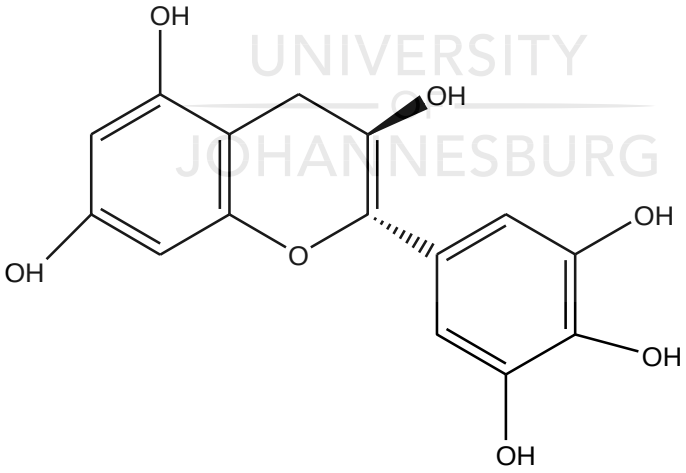
2.1.2.3 Tannins

Tannins are polyphenolic compounds containing two or three phenolic hydroxyl groups on a phenyl ring, in a molecule of moderately large size (Okuda and Ito, 2011). Tannins are found distributed in many plants and in different plant parts and are known to either bind and precipitate proteins. Upon consuming unripe fruits or red wine, the mouth experiences a dry sour feeling which is caused by the astringency from the tannins. They function in plant defensive mechanisms against predators and also play a role in plant growth regulation (Wina, 2010; Ashok and Upadhyaya, 2012). Saxena *et al.*, (2013) reported that traditionally, medicinal plants containing tannins are used as astringents, against diarrhea since they tighten and contract human tissue.

Tannins (Figure 2.6) are also known to form a protective layer over exposed tissues so as to prevents further infection of the wound facilitating wound healing internally (Ashok and Upadhyaya, 2012). These polyphenolic biomolecules can be assembled into three groups' namely hydrolysable tannins, non-hydrolysable tannins and pseudo-tannins. Upon heating with hydrochloric or sulfuric acid, hydrolysable tannins yields gallic or ellagic acids, whereas non-hydrolysable tannins yield phlobaphenes like phloroglucinol and pseudo-tannins (upon treatment with the same acids) which are low molecular weight compounds (Okuda and Ito, 2011).



Catechin



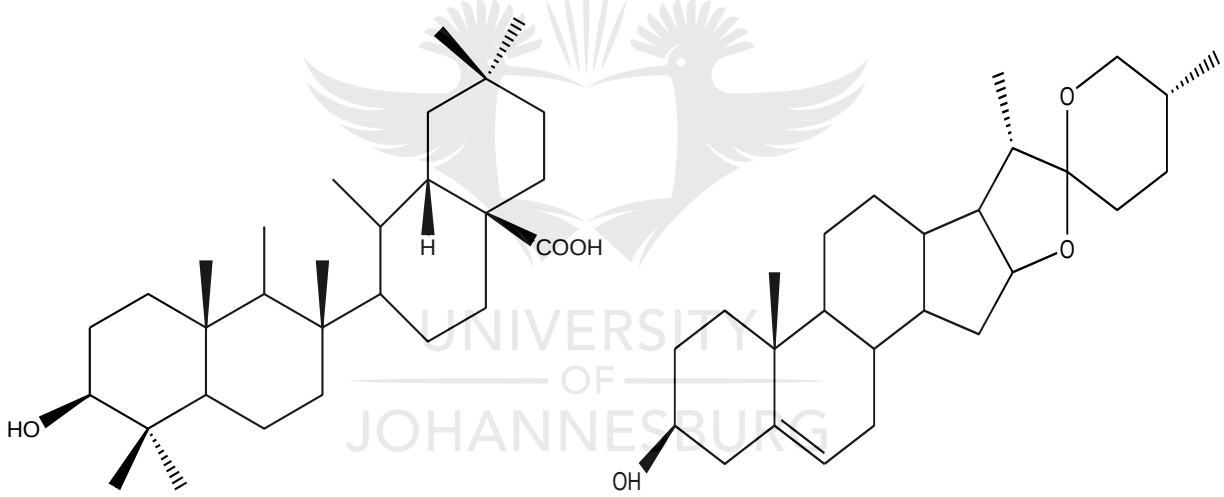
Gallocatechin

Figure 2.6: Structures of tannins

2.1.2.4 Saponins

Saponins contain both polar and nonpolar domains and are therefore called amphipathic glycosides where the aglycon (glycoside-free) is called sapogenin and is nonpolar (Negi *et al.,* 2013).

Saponins (Figure 2.7) are usually obtained from plants whilst some have been isolated from marine organisms and insects (Thakur *et al.,* 2011). The ability of saponins to reduce the surface tension of a liquid in which they are dissolved in distinguish these compounds from other glycosides. When saponins dissolve in water, they form a colloidal solution that foams upon shaking (Sparg *et al.,* 2004). They have a bitter taste and some are toxic (sapotoxin) (Tafadzwa, 2012). The anti-microbial activities of saponins have been observed with *Staphylococcus aureus* and *Enterococcus faecalis* where their inhibition by diosgenyl 2-amino-2-deoxy-beta-D-glucopyranoside was effectively demonstrated using a wound healing model (Thakur *et al.,* 2011). These amphipathic glycosides can be gathered into two groups (steroidal saponins and triterpenoid saponins) based on their aglycone skeleton (Sparg *et al.,* 2004). Examples of saponins include diosgenin and oleanolic acid.



Oleanolic acid Diosgenin acid

Figure 2.7: Structures of saponins

2.1.2.5 Alkaloids

Alkaloids are chemical compounds containing heterocyclic nitrogen atoms but are not amino acids, they are basic due to the inherent nitrogen and exist in the plant as a salt (Saxena *et al.,* 2013). Diaz *et al.,* (2015) stated that alkaloids occur naturally in plants even though some have been isolated from algae, insects, marine and land animals, microorganisms and fungi. Most alkaloids appear as colourless, crystalline material and have a bitter taste (Saxena *et al.,* 2013). Doughari (2012) noted

that alkaloids have different functions such as protecting plants against herbivores and pathogens and are used as pharmaceuticals and poisons due to their effective biological activities. The alkaloid quinoline and its derivatives have been reported to show antibacterial activity against *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* which cause conditions such as wound, skin infections and boils (Archer 2006; Davies and Davies 2010; Kumar *et al.*, 2009). Souto *et al.,* (2011) reported that about 40 alkaloids, especially isoquinolines have been found to have anti-inflammatory properties. Alkaloids can be grouped into three types which are true alkaloids, proto-alkaloid and pseudo-alkaloids. True alkaloids have a heterocyclic ring with nitrogen whereas proto-alkaloids do not possess this functionality but both are derived from amino acids whereas, although pseudo-alkaloids contain a heterocyclic ring they are not generally derived from amino acids (Ngwenya, 2012). According to Saxena *et al.,* (2013), factors such as biological activity, chemical structure and biosynthetic pathways further contribute to the classification of alkaloids into distinct biochemical groups such as indole, isoquinoline, isoxazole, phenethylamine, purine, pyrrolidine and piperidine, quinolizidine and tropane (Figure 2.8).

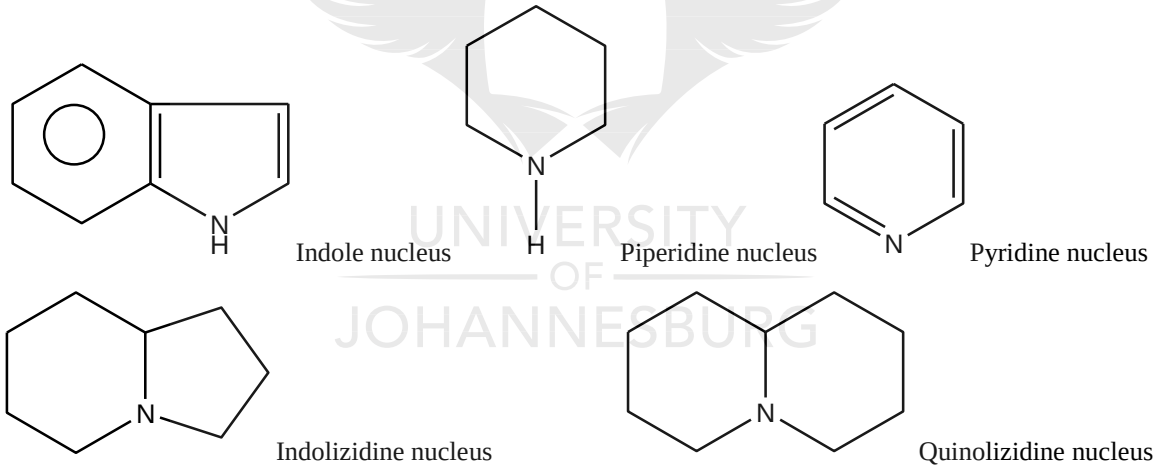


Figure 2.8: Structures of alkaloids

2.2 An overview of the *Amaryllidaceae* family

The plant under investigation is *Crinum macowanii* Baker which belongs to the family *Amaryllidaceae* or *Amaryllis* as is commonly known. The *Amaryllidaceae* or *Amaryllis* are a family of herbaceous monocotyledonous flowering plants mostly perennial, bulbous and not often rhizomatous (Nair and van Staden, 2013). The family consists of about 1000 species and has been divided into about 60 genera distributed throughout the world (Nair and van Staden, 2013) with South America containing about 28 genera, South Africa 19, the Mediterranean region 8 and

Australia with only 3 genera (Iannello, 2014). The *Amaryllidaceae* are widely distributed due to their great adaptability to different environments and thus found in tropical and subtropical regions, but also in temperate areas (Guo, 2015). In general, *Amaryllidaceae* plants (Figure 2.9) have fleshly leaves with parallel veins showing different shapes of leaves, the flowers are bisexual, symmetrical and consist of six sepals which are similar in shape and size and form a floral tube. The ovaries may be superior or inferior. They also contain a dry or fleshy fruit (Iannello, 2014).

Figure 2.9: Different *Amaryllidaceae* plant species

Many species from the *Amaryllidaceae* plant family are known for their horticultural significance serving as ornamentals and medicinal plants due to their appeal and medicinal value (Nair *et al.*, 2000;Nair and van Staden 2013). Medicinally, the *Amaryllidaceae* have been used for a number of different applications such as poultices and decoctions for the treatment of sores and digestive disorders, for psychoactive effects and as protective charms (Nair and van Staden, 2013). In South Africa, various communities including the Zulu, Xhosa and Sotho societies, the bulbs of *Amaryllidaceae* plants have been traditionally used for illnesses such as colds, mental illnesses, kidney and liver conditions (Nair *et al.,* 2013). The different traditional medicinal uses of this family of plants are summarized in Table 2.1.

Table 2.1: Examples of some *Amaryllidaceae* plants with medicinal properties.

Plant name	Distribution	Isolated/known compounds	Ethnopharmacological usage
Amaryllis belladonna L.	South Africa	Lycorine Ambelline	Treatment of tumours and antispasmodic action (Abou-Donia *et al.,* 2006).

Crinum bulbispermum	Southern Africa	Bulbispermine, Cherylline, Crinine	Kidney and bladder infections (Louw *et al.,* 2002).
Boophone disticha	South Africa	Buphanidrine, Buphanisine, crinine and distichamine	Treatment of wounds, infections and inflammatory conditions (Nair and Staden, 2014).
Brunsvigia josephinae	Southern Africa	Alkaloids	Antiseptic dressings on fresh wounds and used for the treatment of coughs and colds (Louw *et al.,* 2002).
Ammocharis coranica	Southern Africa	Alkaloids and triterpenoids	Used as enemas for blood cleansing or applied topically to open wounds or boils (Louw *et al.,* 2002)
Clivia miniata (Lindl.) Regel	South Africa	alkaloids	Used to augment or induce labour and against infertility (Louw *et al.,* 2002)

2.2.1 Phytochemicals from Amaryllidaceae plants

Chemical compounds such as alkaloids, flavonoids, coumarins and terpenoids are present in *Amaryllidaceae* plants and are used as chemical traits for this plant family and thus genera containing these traits are true representatives of the *Amaryllidaceae* family. These phytochemicals create a platform for drug discovery since they are responsible for many biological activities (Asmawi *et al.,* 2011; Nair *et al.,* 2013). These plants are known for their unique isoquinoline alkaloidal structures which are not known to occur in any other family of plants and over 500 of such alkaloids have been described from various species of this family (Iannello, 2014; Bastida *et al.,* 2006). The alkaloids from the *Amaryllidaceae* family are categorised into nine groups which include the norbelladine, lycorine, homolycorine, crinine, haemanthamine, narciclasine, tazettine, montanine and galanthamine (Bastida *et al.,* 2006). The alkaloid, galanthamine (Figure 2.10) isolated from Galanthus woronowii (another plant from the

Amaryllidaceae family) has been used as a prescription drug in the treatment of Alzheimer's disease, (Nair *et al.*, 2013; Rønsted *et al.*, 2012). Pancratistatin (Figure 2.10) isolated from the spider lily (a common name for a number of different plant species within the *Amaryllidaceae* family and belonging to the *Crinum*, *Hymenocallis* and *Lycoris* genera) has shown potential as an anticancer agent since it has antitumor activity in animal and human cell lines (Takos and Rook, 2013; Bastida *et al.*, 2006). Antimicrobial and anti-inflammatory activities have also been reported from *Amaryllidaceae* (Nair and van Staden, 2013).

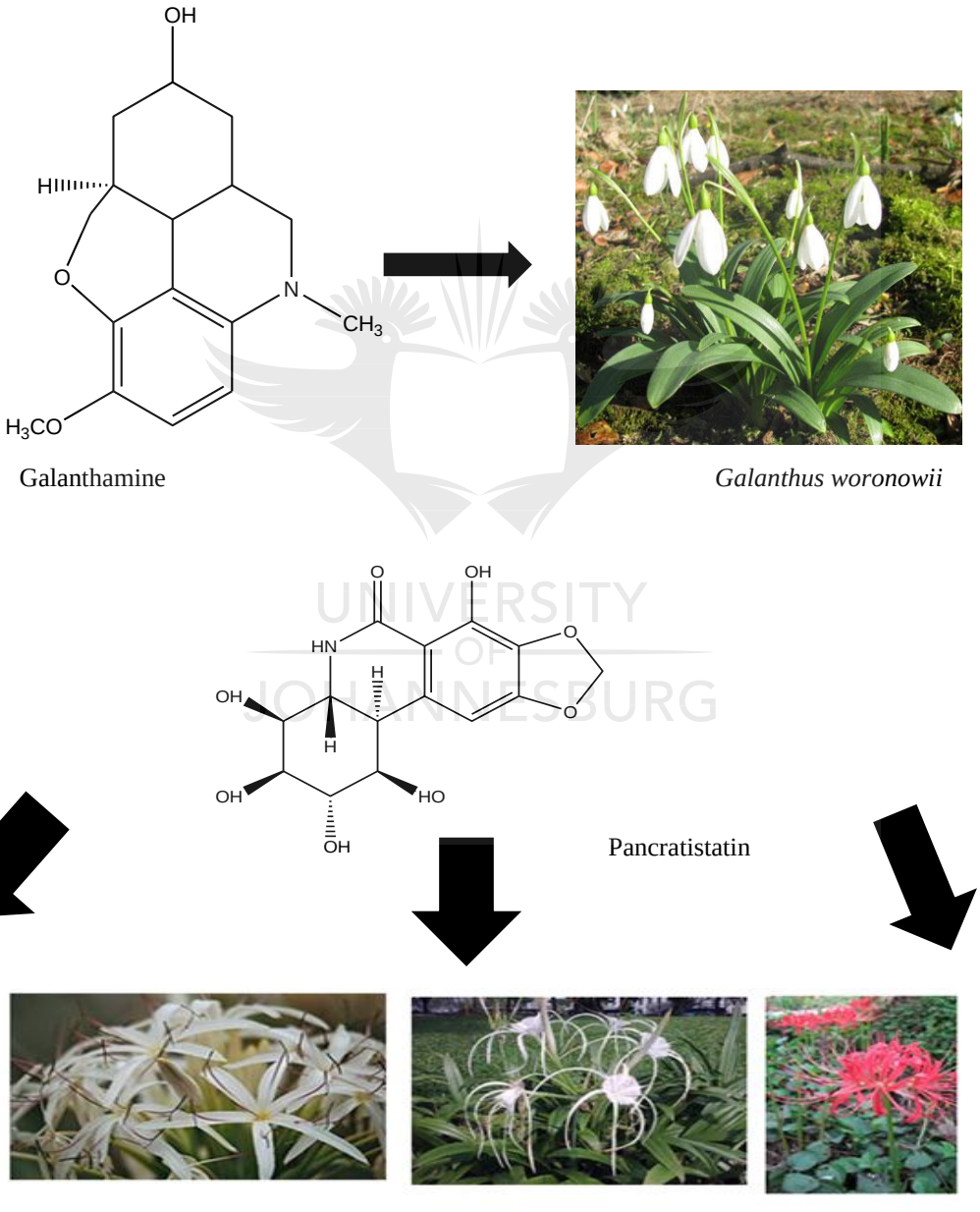


Figure 2.10: Different *Amaryllidaceae* plant species with isolated phytochemicals

2.3 *Crinum macowanii* Baker

The genus *Crinum* L., derives its name from the Greek Krinon, which means "white lily" since most species have white or whitish flowers. The genus contains roughly 65 species occurring world-wide in America, Africa, and southern Asia to Australia, with Africa having the most species (Kwembeya *et al.,* 2007). About 23 species of Crinum are recognized in Southern Africa (Meerow *et al.,* 2003). Some species from this genus include *C. americanum* L., *C. asiaticum* L., *C. bulbispermum*, *C. latifolium* L. and *C. moorei* Hook.f.

Crinum macowanii Baker from the *Amaryllidaceae* family is native to Southern Africa and it grows widely over the subcontinent (Watt and Breyer-Brandwijk, 1962). The plant is generally found in Angola, Malawi, Mozambique, Zambia, Zimbabwe, South Africa, Botswana, Namibia, Swaziland, Kenya, Uganda, Tanzania and Congo. In South Africa, it is commonly known as the cape coast lily or cape lily (English), riverlelie (Afrikaans), intelezi (Xhosa), umduze (Zulu), whilst in Namibia and Kenya it is respectively reffered to as Grosse Omurambalillie (German) and gûtûngûrû kla ngoma (Kikuyu) (Notten, 2013).

2.3.1 Plant Description

Crinum macowanii Baker (Figure 2.11) grows in many habitats like grasslands, beside rivers and along the coast and in various soils (Watt and Breyer-Brandwijk, 1962). This plant develops a deciduous bulb, with fleshy roots and bright green to bluish green leaves and large white lilies with dark pink stripes. The flower produces about 20 to 80 small seeds that appear as smooth, pale green to silvery and fleshy (Notten, 2013). The bulbs are normally 6-25 cm diameter. The leaves are 80 cm long, or longer and 2-16 cm broad. The flowers have a heavy scent and are normally 4-20 cm and with a pedicel up to 6.5 cm long. *C. macowanii* can be distinguished from other crinum species such as *Crinum moorei* and *Crinum bulbispermum* by the appearance of its large bell-shaped flowers with black anthers while *Crinum moorei* and *Crinum bulbispermum* have light grey and pale brown anthers respectively (Elgorashi, 2000). The fruits appear as green to a fading dull yellow colour. The fruits and seeds appear as non-spherical balls with about 3-6 cm and 2 cm diameter respectively.

Figure 2.11: Morphology of *C. macowanii*, flowers, leaves and seeds (Elgorashi, 2000)

2.3.2 Plant Distribution

C. macowanii is found in areas with large seasonal variation in water supply especially grassland on heavy black soil and disturbed places such as roadsides and abandoned cultivations. The species has been recorded in most provinces of South Africa but is absent in the south -western Cape (Elgorashi, 2000). Figure 2.12 shows the distribution of the plant in Southern Africa.

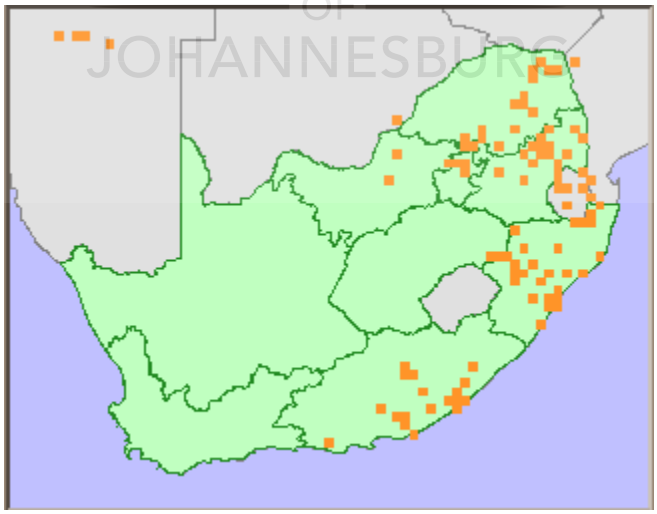


Figure 2.12: Map of Southern Africa showing the natural distribution of *C. macowanii*. Distribution map obtained from (Maroyi, 2016)

2.3.3 Ethnobontany of *C. macowanii*

The plant is extensively used across the African continent where the fibre from the bulb is utilised as bandages. Socially, the plant finds applications in ritual ceremonies and wizardry, where in Lesotho the leaves and bulb are used to make protective charms. The bulbs are also used to increase lactation in women and cows (Elgorashi *et al.,;* 2003;Nair *et al.,* 2000).

Table 2.2: Ethnomedicinal uses of *C. macowanii*

Conditions to be treated	Traditional applications
Circulatory System Disorders	Decoctions of the bulbs is used by the Zulu people for the treatment of heart disease. The bulbs and leaves are used as remedy for rheumatic fever by Zulu people (Nair *et al.*, 2000).
Digestive System Disorders	An infusion of the bulbs is used as emetic for humans and the plant is known to treat stomach diseases (Elgorashi *et al.*, 2003).
Genitourinary System Disorders	The Zulu people ingest the decoctions of the bulb orally for the treatment of kidney and bladder disease. The leaves are used as remedy for the bladder and kidneys. The overall plant is known to be used as an aphrodisiac (Elgorashi *et al.*, 2003).
Infections	The Zulu people ingest the decoctions of the bulb orally for the treatment of tuberculosis. In Zimbabwe, powdered bulb is taken in porridge for the treatment of venereal diseases. The bulbs and leaves are used as remedy for fever and scrofula (Nair *et al.*, 2000).
Pain	The boiled bulb is used in Zimbabwe as a compress for back pain (Elgorashi *et al.*, 2003)
Sensory System Disorders	The plant is known to be used for the treatment of eye diseases (Nair *et al.,* 2000).
Skin/Subcutaneous Disorders	The bulbs and leaves are used as a remedy for skin problems such as boils, sores and acne. The plant is used for the treatment of itchy rashes and swelling of the body (Elgorashi *et al.*, 2003).

(Elgorashi *et al.*, 2003; Nair *et al.*, 2000)

2.3.4 Phytochemicals isolated from *C. macowanii* and their pharmacological activity

As stated earlier, *C. macowanii* is known to contain isoquinoline alkaloids since the *Amaryllidaceae* family is unique to such. Chemical compounds such as flavonoids, coumarins, alkaloids and terpenoids have been isolated from *Crinum* species (Asmawi *et al.,* 2011). Alkaloids such as galanthamine, lycorine, crinine, homolycorine, tazettine and monotamine have been extracted from different plant parts such as the bulbs, roots, leaves and flowering stalks of three closely related *Crinum* species namely *C. bulbispermum*, *C. macowanii*, and *C. moorei* (Machocho *et al.,* 2004). According to Elgorashi *et al.,* (2003) crinine, powelline, undulatine, epideacetylbowdensine, crinamidine, and 3-O-acetylhamayne have been extracted and identified from *C. macowanii*. Compounds such as oleic acid, lycorine, flexinine, crinan-3-ol,1,2-didehydro-(3-alpha), buphanisine, criwelline, tazettine, powelline, heptacosane and stigmasterol have been reported before as being contained in crinum species on either the bulb, leaves or the whole plant (Elgorashi *et al.,* 2003; Nair *et al.,* 2000; Refaat *et al.*, 2012; Refaat *et al.,* 2013). Although the bioactivity of the plant has been documented particularly with respect to crude extracts, some bioactivities have been successfully linked to pure compounds from within the plant. For instance, crinamine and lycorine possess anti-bacterial activity whilst 6-hydroxycrinamine and undulatine display anti-cancer activity (Elgorashi *et al.,* 2003). Vittatine and crinamine have been shown to be active against *Bacillus subtilis* and *Staphylococcus aureus* (Fennell and van Staden, 2001). *S. aureus* and *Candida albicans* have been shown to be susceptible to vittatine at minimum inhibitory concentrations (MIC) of 63 mg/mL and 31 mg/mL respectively (Fennell and van Staden, 2001). While extensive research has been done on most *crinum* species resulting in the appreciation of its alkaloidal contents where most belong to crinine-type alkaloids, other compounds such as flavonoids, coumarins have thus not been thoroughly investigated (Refaat *et al.,* 2013). The wide range of traditional medicinal uses of the plant suggest good antimicrobial activity while the limited knowledge available on the phytochemistry and pharmacological activity of *C. macowanii* is still very incomplete particularly with regards to classes of other compounds besides alkaloids. Such lack of information hinders the conservation and transformation of the medicinal plant into a commodity of high value (Fennell and van Staden, 2001).

2.4 Extraction of phytochemicals

The following section present some techniques used in natural product research giving insight into the extraction techniques, chromatographic and spectroscopic techniques with special emphasis on those used in this study.

2.4.1 Extraction technique

Extraction of plant based bioactive constituents is defined as the separation of medicinally active portions of plant tissues using selective solvents and standard procedures (Tiwari *et al.*, 2011). The plant material of interest is either dried or used fresh, it is blended and soaked in a solvent of interest, this is done in order to obtain the desired therapeutic portions and to eliminate unwanted material (insoluble cellular matrix, chlorophyll, cellulose, salts and glycoproteins) (Tiwari *et al.*, 2011).

2.4.1.1 Solvent extraction

Different techniques are used in the extraction of medicinal plants bioactive compounds. The bioactive compounds of interest determine the selection of the solvent system and for the purpose of this study, a mixture of dichloromethane/methanol in a ratio of 1:1 was used as this is known to extract more lipophilic compounds (Sasidharan *et al.*, 2011). In the extraction process, certain aspects have to be taken into consideration and these include; the:- (1) Nature of solvent used since it affects the composition of the crude extract due to the fact that the chemical makeup of the plant is usually unknown, (2) Effect of the choice of solvent on other procedures such as antimicrobial bioassays since harsh organic solvents are known to be detrimental to microbial and mammalian cells, (3) Temperature, since it has been observed to affect the stability of phytochemicals. As such, mild extraction temperatures are usually recommended and the crude extract is generally stored at 4 °C in the dark since light can also affect the compound's stability (Sasidharan *et al.*, 2011). Buwa and Afolayan (2009) noted that even though water is the commonly used solvent by traditional healers, solvents such as dichloromethane and ethanol have been reported to have extracted more bioactive compounds. Knowledge of the cellular and subcellular localization of plant metabolites is an essential consideration for such studies in order to facilitate and enhance

the extraction processes, since plant cells contain vacuoles and other organelles in the cytoplasm surrounded by a rigid wall consisting of a framework of cellulose and micro fibrils embedded in a hydrophilic matrix of pectin and hemicelluloses (Tiwari *et al.,* 2011). As such, primary plant metabolites such as lipids are found in intracellular vacuoles (spherosomes) and proteins are located in the storage vacuoles of differentiated cells of the embryo and endosperm while secondary metabolites such as phenolic are accumulated in cell vacuoles whereas essential oils are mostly present in special glands called trichomes. The metabolites of interest determine the extraction method and solvent used, extraction steps include:- (i) penetration of the solvent into the solid, (ii) solubilisation–desorption of the solute from the solid matrix and/or hydrolysis, (iii) diffusion of the components of interest to the surface, and (iv) external transfer into the bulk solution. The disruption of the plant cells makes the protoplasm more permeable while grinding destroys cell integrity allowing the access of the solvent to plant metabolites located in the cell walls or in the cytoplasm (Flórez *et al.,* 2014).

2.4.1.2 Subcritical water

Subcritical water (SCW) is a term used to refer to liquid water at temperatures between the atmospheric boiling point and the critical temperature (374°C) and pressures above 221 bar, terms such as pressurized hot water (PHW), hot compressed water (HCW), near-critical water (NCW) or superheated water are also used to describe subcritical water (Figure 2.13) (Gbashi *et al.,* 2016; Shams *et al.,* 2015). Subcritical water has been used for the extraction of contaminants from soils and also as a solvent for the conversion of biomass. Recently it has been used to extract bioactive compounds from plants. Pressures ranging from 16 bars at 200°C to 226 bars at 374° C are applied to the water to keep it in a liquid state. Extraction conditions, chemical structure of solute and the nature of the sample determine the efficiency of SCW (Flórez *et al.,* 2014). Advantages of using subcritical water for extraction include; higher temperatures than in other extraction techniques, improved solute–solvent contact due to lower viscosity and surface tension and hydrolytic conditions that break the cell walls facilitating the release of target compounds. All these lead to enhanced extraction rates and yields (Luong *et al.,* 2015; Plaza and Turner, 2015). Also the temperature and pressure used in SCW produce high diffusion rates that promote efficient extraction of the raw materials, the rates differ according to chemical structures of organic compounds. This then promotes selective and rapid extraction by SCW (Liang and Fan, 2013;

Petersson *et al.*, 2010). The removal of organic solvents after extraction procedures is expensive and time-consuming. Disadvantages of extraction of plant bioactive compounds using organic solvents and their mixtures include long extraction times, high solvent usage and thermal degradation of the phytochemicals (Mukhopadhyay and Palash, 2008 ; Nematollahi *et al.*, 2014) Teo *et al.*, (2010) reported that hot water extracted greater amounts of phytochemicals than the cold water extraction since the hot water extraction had more antibacterial activities and therefore which justifies the efficiency of boiled plant extract when used as herbal medicine.

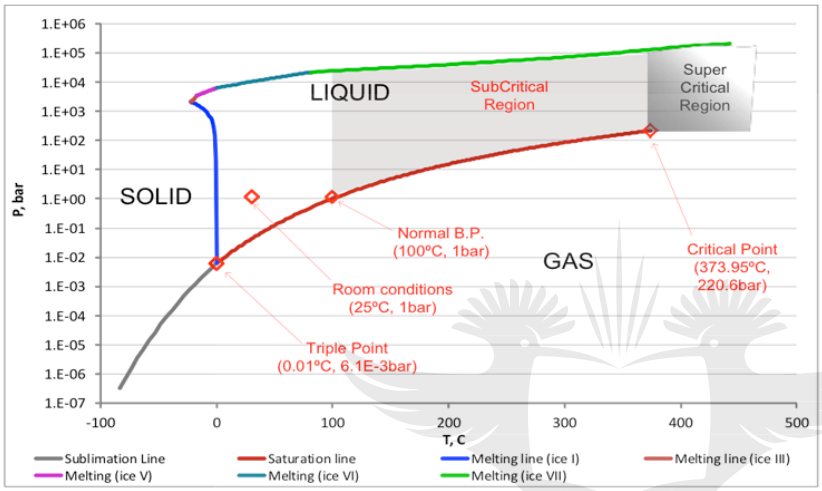


Figure 2.13: Different phases of water (Usha, 2007)

2.4.2 Chromatographic techniques

Chromatography is a technique used for the separation of components in a mixture of crude extracts under investigation (Faust, 1997). Since crude plant extracts contain mixtures of different phytochemicals with different polarities, procedures that deal with identification and characterization of bioactive compounds become a challenge since pure compounds are required (Sasidharan *et al.*, 2011). All chromatographic techniques usually contain a mobile phase which carries the mixture to be separated through a stationary phase. The various component of the mixture partition themselves in between the stationary phase, thereby effecting separation. Factors affecting separation are well documented and studies and involve adsorption, partition, ion exchange or molecular exclusion (Faust, 1997).

2.4.2.1 Two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-TOF-MS)

Two-dimensional gas chromatography coupled to time-of-flight mass spectrometry is a form of chromatography used for the separation and analysis of organic volatile compounds, where the stationary phase is fixed on a solid support and the mobile phase is a gas. This method was introduced to increase chromatographic resolution of traditional one-dimensional gas chromatography and together with time-of-flight mass spectrometry, allowing organic compounds to be identified and mass spectra determined (Naeher *et al.*, 2016). Samples such as biological and geological samples usually contain complex mixtures that vary in concentration and composition leading to complicated separation processes however, the high resolution separation in comprehensive two-dimensional gas chromatography (GC×GC) has emerged as a solution to this problem (Eiserbeck *et al.*, 2008; Eiserbeck *et al.*, 2012). In this study, GC×GC-TOFMS was used for the first time in the identification of compounds from the extracts of *Crinum macowanii* bulbs. The sample is subjected to separate in two columns occurring in a single run. The analytes separate as they move along the columns on the basis of their boiling points and interaction with the stationary phase and the separated analyte signals are monitored by a detector (Machado *et al.*, 2011). The sample constituents are separated according to compound class or chemical structure which is useful in complex hydrocarbon mixtures so that compound identification and quantitation are not compromised (Gorst-Allman *et al.*, 1993). The peak capacity offered by GC×GC allows for complete separation of analytes (Gorst-Allman *et al.*, 1993). Machado *et al.*, (2011) observed that components having identical molecular masses and similar fragmentation patterns are difficult to separate since they co-elute at the same time when analysed by 1D GC while in GC×GC–TOFMS (Figure 2.14), aided by two columns, mass spectra are used for the identification and quantification purposes.

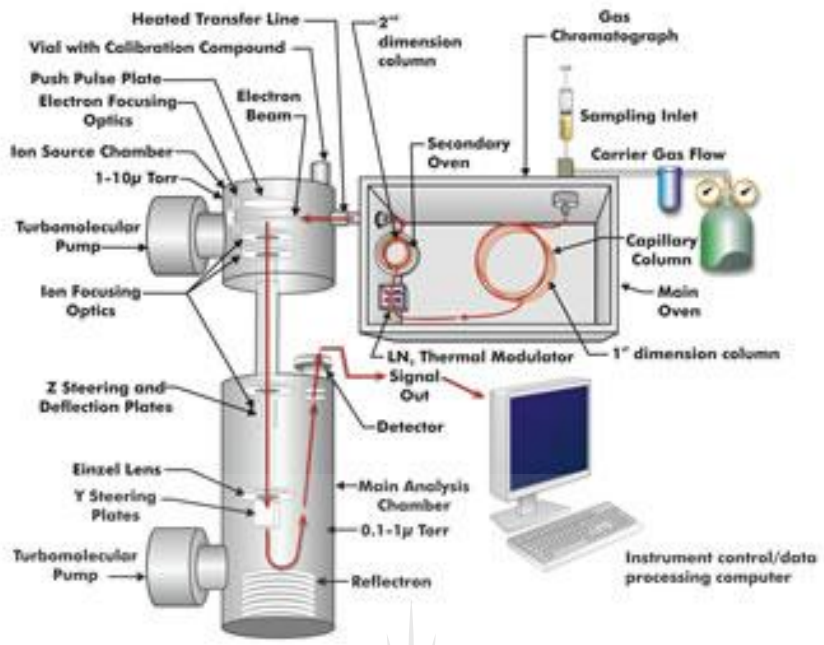


Figure 2.14: GC×GC-TOFMS instrumental unit (Heim *et al.,* 2015)

2.5 Using plants metabolites to eliminate ailments caused by bacteria

The World Health Organization (WHO) is planning to stop the spread of infectious disease by 2020 with reference to the Millennium Development Goal (United Nations, 2000). Most plant metabolites have been found to have antimicrobial activity against opportunistic pathogenic bacteria. Mahady (2005) stated that 25% of all prescription drugs dispensed in the United States of America have been derived from plants. Historically, plants have been a source of inspiration for novel drug compounds as plant derived medicines made large contributions to the human health and well-being. Many drugs currently used clinically are derived from medicinal plants. The WHO listed that 11% of 252 essential drugs were obtained from flowering plants. Some examples of drugs obtained from plants include quinine from *Cinchona* used for the treatment of malaria, reserpine from *Rauvolfia serpentine* used for the treatment of hypertension and taxol from *Taxus brevifolia* species used for the treatment of ovarian cancer (Mahady, 2005). The developments of drugs and the discovery of new ones help in combating multi-drug resistant bacteria and emerging new pathogens (Brusotti *et al.,* 2014).

Despite the broad use of plants for health related issues such as treatment of various ailments, the lack of scientific knowledge verifying their traditional use and their benefits hinder the regulation

and quality standards of plant based remedies and hence, most countries especially in Southern Africa do not have polices for such (Skalli *et al.,* 2008). Inadequate documentation is present on the antibacterial activity of extracts from most plant sources (Samie *et al.,* 2005), this is due to the fact that this information is orally passed on.

Buwa and Afolayan (2009) stated that further research on natural products from plants can increase knowledge about the link between the chemical structures of phytochemicals and their biological properties and it has been evident that higher plants represent a potential source of novel antibiotic prototypes due to the screening of plant products for antimicrobial activity (as seen in Table 2.3). Even though the efficiency of medicinal plants is known in most rural communities, their mode of action and safety still remains unknown, therefore pharmacological testing and research on natural products is a major strategy for discovering and developing new drugs (Fadipe *et al.*, 2015).

Fortunately, the South African government has introduced traditional medicine into the mainstream health care system through the national health policy. This is further supported by Nair and van Staden (2013) that this will help in the regulation of sustainable use of *Amaryllidaceae* plants which many of them are threatened in the wild. In so doing the gaps in indigenous knowledge with relation to medicinal plants will be filled and allow unrestricted use, distribution, and reproduction in any form (Maroyi, 2013).

Most people in developing countries tend to use natural medicine as compared to modern synthetic drugs since they are believed to be more tolerable by the human body. Given that morbidity and motility rates in some developing countries continue to increase alarmingly due to the spread of infectious disease (Ncc and Fernandes, 2010) most probably as a consequence of failing health care systems, the emergence of antibiotics has been a vital aspect in therapeutic discoveries since this meant treatment for bacterial infections was possible, however, this is proving to be not the case anymore. Factors such as random and continuous use of these synthetic products has led to only one third of known infectious diseases being successfully treated and in a number of instances resulting in pathogens developing drug resistance. Efforts are currently underway for a natural way of treating such infections (Sen and Batra, 2012), and medicinal plants are regarded a natural and renewable target for this (Selvamohan *et al.,* 2012).

Table 2.3: Mode of action of phytochemicals

Phytochemicals	Activity	Mechanism of action
Flavonoids	Antimicrobial	Complex with cell wall, binds to adhesins
	Antidiarrhoeal	
Tannins	Antimicrobial	Binds to adhesins, enzyme inhibition, substrate deprivation, complex with cell wall, membrane disruption, metal ion complexation
	Antidiarrhoeal	
	Anthelmintic	Makes intestinal mucosa more resistant and reduces secretions, stimulates normalization of abnormal water transport across the mucosal cells and reduction of the intestinal transit.
Terpenoids essential oils	Antimicrobial	Membrane disruption
	Antidiarrhoeal	
Alkaloids	Antimicrobial	Intercalate into cell wall and DNA of parasites
	Antidiarrhoeal	Inhibits release of autocoids and prostaglandins
	Anthelmintic	Possess anti-oxidating effects, thus reduces nitrate generation which is useful for protein synthesis, suppresses transfer of sucrose from stomach to small intestine, diminishing the support of glucose to the helminthes, acts on Central Nervous System causing paralysis
Glycosides	Antidiarrhoeal	Inhibits release of autocoids and prostaglandins
Saponins	Antidiarrhoeal	Possesses membrane permeabilizing properties

	Anticancer	
	Anthelmintic	
Steroids	Antidiarrhoeal	Enhance intestinal absorption of Na^+ and water

Adapted from Tiwari *et al.*, (2011).

2.5.1 Common human pathogenic bacteria relevant in the study

2.5.1.1 *Staphylococcus aureus*

The bacterium belongs to the *Micrococcus* family. A gram-positive facultative anaerobe bacterium, which is salt tolerant, non-motile and non-spore forming. It is able to grow in various of media and is known to withstand temperatures greater than 60 °C (Adekunle and Olatunji, 2011). This bacterium is commonly known to cause skin infections such as boils and has been reported to cause nosocomial infections and is now being regarded as a major community-acquired (CA) pathogen, and in some settings it is acknowledged to be drug-resistant (e.g. Methillicin Resistant *Staphylococcus aureus* or MRSA in short) (Davies and Davies, 2010).

2.5.1.2 *Escherichia coli*

This bacterium is negative, motile, petrichious, fimbriate, non-spore forming and is a member of the bacterial family of *Enterobactericeae*. The bacterium is a facultative anaerobe and mostly found in the intestinal flora and gastrointestinal of humans and warm-blooded animals and it released into the external environment through excretion in faeces (Allocati *et al.,* 2013). Some pathogenic strains of *E. coli* are known to cause urinary tract infection. It has been reported that *E. coli* is resistant to fluoroquinolones a broad-spectrum antibiotic (Levy and Marshall, 2004).

2.5.1.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, rod-shaped and non-sporing bacterium that can cause disease in plants, animals and humans. When the bacterium is grown on agar medium, it appears as large, flat spreading and irregular shape colonies that are grayish in colour. *P. aeruginosa* has been isolated from human and animal material, from food and environmental samples. This bacterium is known as an opportunistic pathogen of humans that can invade virtually any tissue (Rossolini and Mantengoli, 2005). Recent reports have listed *P. aeruginosa* as a leading cause of hospital-acquired (nosocomial) infections. Due to the high reports of infections by this bacteria, antibitiocs such as polymyxin, gentamicin and carbenicillin are effective against the bcateria even though it is now very resistant to other types of antibiotics (Rossolini and Mantengoli, 2005). The movement of bacterial species in different conditions such as environmental populations, clinical populations and commensals of other living species has allowed bacteria to evolve due to gene flux and thus acquire survival characteristics such as antibiotic resistance and virulence (Davies, 2006). The evolution of bacteria such as *P. aeruginosa*,

has led to the introduction of new antibiotic derivatives thus resulting in antibiotic resistance mechanisms from the evolved microorganisms (Davies and Davies 2010).

2.5.1.4 *Bacillus cereus*

Bacillus cereus is a Gram-positive, rod-shaped, aerobic, motile bacterium, which is able to lysis red cells in the media. The bacteria can produce protective endospores when conditions are not favorable. They can be found in soil and food and have virulence factors (cereolysin and phospholipase C) which contribute to their pathogenicity. Some strains are harmful to humans (e.g eye infection) and cause foodborne related illness, while others are beneficial as probiotics (Bottone, 2010).

2.5.1.5 *Mycobacterium smegmatis*

Mycobacterium smegmatis is a Gram-positive, bacillus shape aerobic bacteria which is 3.0 to 5.0 μm long and has an inner cell membrane and a thick cell wall. The bacterium is mostly found in the soil, water, and plants (Davies and Davies, 2010). When accessible nutrients are available, *M. smegmatis* has a waxy appearance because of the high amount of unique Gram-positive cell wall coated with mycolic acids. *M. smegmatis* is distinct from other Gram-positive bacteria since its cell wall contains mycolic acids, long, branched fatty acids that are normally present in acid-fast bacteria hence the acid-fast stain is used and not the Gram stain and also the cell wall is irregularly thick as compared to other gram-positive bacteria (Davies and Davies, 2010). It was not until the last 20 years that *M. smegmatis* has been classified as a human pathogen that causes infections such as skin or soft-tissue infections and pulmonary infections in immunocompetent individuals (Pierre-Audigier *et al.,* 1997).

2.5.1.6 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative, non-motile, facultative anaerobic encapsulated rod-shaped bacterium. *K. pneumoniae* is commonly found in the normal flora of the mouth, skin, and intestines. The bacterium is the common cause of community-acquired and nosocomial infections. Some of the nosocomial infections caused by *K. pneumoniae* include the urinary tract, wounds, lungs, abdominal cavity, intra-vascular devices, surgical sites, soft tissues and subsequent bacteremia (Iannello *et al.,* 2014). *K. pneumoniae* is the second common bacteria as compared to *E. coli* for causing urinary tract infections (UTIs) most especially with indwelling urinary catheters. It is also the second common bacteria for causing bacteremia at (10%) with

Staphylococcus aureus being the first at (30%). Most biofilms created by *K. pneumoniae* cause infections that are resistant to available antibiotics. *K. pneumoniae* has been reported as the most common bacteria causing wound infections in hospitalized adults (especially for burn related injuries) (Vuotto *et al.,* 2014).

2.5.1.7 *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive, facultative anaerobe, motile, non-spore forming cocci shaped bacterium which appear as single cocci or in chains. *Enterococcus faecalis* is a commensal microbiota living in the gastrointestinal tracts of humans and other mammals. *E. faecalis* is ranked the widest spread multidrug resistant hospital pathogens worldwide and it is capable of causing a number of infections such as endocarditis, sepsis, surgical wound infections, and urinary tract infections (Tyne *et al.,* 2013). This bacterium is known as lactic acid bacteria (LAB), which produces lactic acid as the major metabolic end product of carbohydrate fermentation (Fisher and Phillips 2009). The pathogenicity of *E. faecalis* is related to the high levels of antibiotic resistance, where the bacterium causes human infections in nosocomial (hospital) environment such as urinary tract infection and bacteremia (Fisher and Phillips, 2009).

2.5.1.8 *Bacillus subtilis*

Bacillus subtilis is a Gram-positive, catalase-positive facultative aerobe rod-shaped bacterium, which is able to form a tough protective endospore allowing it to adapt to various environmental conditions. The bacterium is found in soil and the gastrointestinal tract of ruminants and humans. This bacterium has been used for a number of different applications such as producing enzymes used as additives in laundry detergents. Bacterial cultures of *B. subtilis* were used in the 1900 as immune stimulatory agent to help in the treatment of gastrointestinal and urinary tract diseases (Olmos and Paniaua-Michel, 2014).

2.5.1.9 *Enterobacter aerogenes*

Enterobacter aerogenes is a Gram negative, oxidase negative, catalase positive, citrate positive, indole negative, non-spore forming, rod shaped bacterium. *E. aerogenes* is normally found in the human gastrointestinal tract and is not known to cause disease in healthy individuals (Davin-Regli and Pages, 2015). Reports have classified *E. aerogenes* as a nosocomial and pathogenic bacterium which causes opportunistic infections such as bacteremia, skin/soft tissue infections and lower respiratory tract and urinary tract infections mostly to patients on mechanical ventilation (Grimont

and Grimont, 2006). Even though most antibiotics are reactive to *E. aerogenes*, the bacterium is known to produce lactamase, which makes the bacterium more resistant to standard antibiotics used for treatment. This results in changing the recommended antibiotic to stop the presence of harmful bacteria and their toxins in the tissues of wound infections (Levy and Marshall, 2004).

2.5.1.10 *Enterobacter cloacae*

Enterobacter cloacae is also a Gram-negative, oxidase-negative, catalase-positive, facultative anaerobic, rod-shaped bacterium which uses flagella for locomotion (Grimont and Grimont , 2006). *E. cloacae* exist, as a commensal in water, sewage, soil, meat, hospital environments, the skin, and in the intestinal tracts of humans and animals. In a study conducted by van der Waaij *et al.,* (1977), this bacterium was the most widespread Gram-negative isolate found on the feces of leukemia patients. Grimont and Grimont (2006), reported that *E. cloacae* result in infections and bacteremia in both hospitalized and non-hospitalized patients, such infections include meningitis, urinary tract infections, pneumonia and arthritis.

2.5.1.11 *Klebsiella oxytoca*

Klebsiella oxytoca is a Gram-negative non-motile, indole positive, methyl red negative, citrate positive, urease positive rod-shaped bacterium. *K. oxytoca* is known to be a diazotroph, since it is able to occupy plant hosts and fix atmospheric nitrogen into a useful form which the plant can use. *K. oxytoca* is an opportunistic pathogen causing nosocomial infections especially to immunocompromised patients such as the elderly or very young, patients with burns or excessive wounds, those with HIV/AIDS infection or those requiring intensive care. Contamination of environmental reservoirs such as disinfectants, humidifiers and ventilators by *K. oxytoca* have been the leading cause of health care associated infection. Lowe *et al.,* (2012) indicated that handwashing sinks in high-intensity hospital care areas, might be a reservoir for *K. oxytoca* and such could lead to individuals being infected. This pathogen has recently been reported to cause serious infections such as destructive pneumonia (World Health Organization, 2004).

2.5.1.12 *Proteus mirabilis*

Proteus mirabilis is a Gram negative-negative, facultative anaerobic, motile, urease positive, catalase positive, rod-shaped bacterium. *P. mirabilis* is commonly found in the intestinal tracts of humans, soil and water. *Proteus mirabilis* is known as an opportunistic bacterial pathogen, which can cause wound infections, and urinary tract infections (UTIs). *P. mirabilis* strains have

developed resistance against antibiotics such as tetracycline, nitrofurantoin, cephalosporins and ampicillin (Różalski *et al.*, 2012). The pathogen is able to cause blood related infections and infections to the bile duct which further lead to infection of urethra, bladder, ureters and kidneys (Ro'zalski *et al.*, 2012).

2.5.1.13 *Proteus vulgaris*

Proteus vulgaris is a Gram-negative, catalase-positive, indole positive, motile by flagella rod-shaped bacterium. The bacteria live in the intestinal tracts of humans and animals and can be found in soil, water, and fecal matter (World Health Organization, 2004). *P. vulgaris* is known as an opportunistic pathogen and is known to cause wound infections and urinary tract infections and also produce severe abscesses (Ghaidaa *et al.*, 2013). *P. vulgaris* is known to be sensitive to antibiotics such as ciprofloxacin, netilmicin and ampicillin.

2.5.1.14 *Staphylococcus epidermidis*

Staphylococcus epidermidis is a Gram-positive, non-motile, coagulase negative, catalase-positive, facultative anaerobe, cocci shaped bacterium which is non-hemolytic on blood agar (Gara and Humphreys, 2016). *S. epidermidis* forms part of the normal human skin flora and mucosal flora. *S. epidermidis* is opportunistic pathogen causing nosocomial infection in immune-compromised patients. *S. epidermidis* in known to form biofilms on catheters and other surgical implants thus leading to infections and complicated therapy (Sharma *et al.*, 2014). Infections usually occur in catheters, medical prostheses, which further leads to endocarditis and sepsis in hospitalized patients. Antibiotics such as vancomycin, rifampin and aminoglycoside are known to be effective against the pathogen. Good hygienic practices such as the washing of hands helps lessen the spread of infections. Recent research has indicated that *S. epidermidis* is found inside affected acne vulgaris pores and where *Propionibacterium acnes* is the common cause of such as skin disease (Sharma *et al.*, 2014).

2.6 Metals in plants

A metal is defined as a material that is typically solid, cloudy, shiny, and it can conduct heat and electricity. A metal's shape can be changed by hammering or pressing it permanently without breaking or cracking it. Also they can be melted and stretched. In the periodic table of elements, about 91 of the 118 elements are metals while the others are nonmetals or metalloids and some

appear as both metallic and non-metallic forms (Nonresident training course, 1996). Chemical elements can be found in the earth crust, soils, seawater, the Sun and solar system (as seen in Table 2.4). Elements found on the earth's crust are measured in percentage or parts per million (ppm) in mass; 10,000 ppm = 1%. The appearance of chemical elements is reflected on the ecological and geochemical characteristic which are due to natural occurring processes (Alekseenko and Alekseenko, 2014).

Table 2.4: Abundances of some elements in different locations

Element	**Earth crust (kg/kg)**	**Soils (ppm)**	**Sea water (kg/L)**	**Sun**	**Solar system**
Calcium Ca	4.15×10^{-2}	4.15×10^{-2}	53800	4.12×10^{-4}	6.4×10^{-2}
Cadmium Cd	1.5×10^{-7}	0.9	1.1×10^{-10}	2.0×10^{-6}	1.6×10^{-6}
Chromium Cr	1.02×10^{-4}	80	3×10^{-10}	1.3×10^{-2}	1.3×10^{-2}
Copper Cu	6.0×10^{-5}	39	2.5×10^{-10}	4.5×10^{-4}	5.2×10^{-4}
Iron Fe	5.63×10^{-2}	22300	2×10^{-9}	9.0×10^{-1}	9.0×10^{-1}
Gallium Ga	1.9×10^{-5}	16.2	3×10^{-11}	2.1×10^{-5}	3.8×10^{-5}
Mercury Hg	8.5×10^{-8}	0.88	3×10^{-11}	3.4×10^{-7}	3.4×10^{-7}
Potassium K	$2.09x10^{-2}$	13400	$3.99x10^{-4}$	$3.7x10^{-3}$	$3.7x10^{-3}$
Manganese M	9.50×10^{-4}	729	2×10^{-10}	6.9×10^{-3}	9.5×10^{-3}
Sodium Na	2.36×10^{-2}	5800	1.08×10^{-2}	6.0×10^{-2}	5.7×10^{-2}
Nickel Ni	8.4×10^{-5}	33	5.6×10^{-10}	5.0×10^{-2}	5.0×10^{-2}
Lead Pb	1.4×10^{-5}	54.5	3×10^{-11}	2.0×10^{-6}	3.1×10^{-6}
Rhodium Rh	1×10^{-9}			4.0×10^{-7}	3.4×10^{-7}
Strontium Sr	3.70×10^{-4}	458	7.9×10^{-6}	2.2×10^{-5}	2.4×10^{-5}
Zinc Zn	7.0×10^{-5}	158	4.9×10^{-9}	1.1×10^{-3}	1.3×10^{-3}
Indium In	2.5×10^{-7}		2×10^{-8}	About $1.3x10^{-6}$	1.9×10^{-7}

Adapted from (Alekseenko and Alekseenko, 2014;Barth *et al.,* 2000).

2.6.1 Trace metals

Trace metals are element which appears in very low or small concentrations yet measurable and is mostly found in animal and plant cells and tissues. Sources of trace metals include environmental exposure, in animals through diet, in plants through the uptake of nutrients from the soil, from human vitamin pills and plant fertilizers (Rajan Prakash *et al.,* 2014). Exposure or ingestion of high amounts of trace metals can be toxic and fatal. Different applications of trace metals determine the definition such as; in biochemistry a trace element is defined as a dietary element that is needed in very small quantities for the proper growth, development, and physiology of the organism, in geochemistry a trace element is defined as an element is one whose concentration is less than 1000 ppm or 0.1% of a rock's composition and in analytical chemistry a trace element is defined as an element whose average concentration of fewer than 100 parts per million (ppm) measured in atomic count or less than 100 micrograms per gram (Blum *et al.,* 2009). Examples of trace metals include iron, magnesium, zinc, copper, nickel, cobalt, sodium, lead etc. The toxicity levels of trace metals in the environment are frequently difficult to determine due to factors such as location (water, soil or air), the source of the metals (mining or natural rock breakdown), the composition of the environment (acidic the environment) and composition of the metal (exists by itself or is part of larger chemical compounds) (Mtunzi *et al.,* 2015;Rajan- Prakash *et al.,* 2014; Wada, 2004).

The World Health Organization (Zhang, 1998) reported that extended and overdose ingestion of medicinal plants containing metals has led to a continuous buildup of different trace metals that cause various health problems. As reported by WHO, recommendations have been made that medicinal plants used as raw materials for medicinal purposes should be thoroughly checked for the presences of contaminants such as heavy/toxic metals, pesticides, fungi and microorganisms (World Health Organization, 2004; World Health Organization, 2013; Zhang, 1998; Zhang, 2007). The amounts of heavy metals present in medicinal plants have to be known as to provide a scientific database line for traditional practitioners as well as for pharmaceutical industries who will use the plants for medicinal purposes (Mtunzi *et al.,* 2012).

2.6.1.1 Antimony

Antimony (Sb) is a trace metal with an atomic number of fifty-one and is not essential to plants, even though plants can easily take it up if it is present in soluble forms in growth media. Safety

limits of Sb in agricultural crops are reported to range from <2 to 29 μg kg^{-1} (Blum *et al.*, 2009). Exposure to antimony can cause irritation of the eyes, skin and lungs. Antimony concentration of 9 mg/m^3 in the air causes health effects such as lung diseases, heart problems, diarrhea and stomach ulcers. Previously antimony was used as a medicine for parasite infections (Bosch, 2015).

2.6.1.2 Arsenic

Arsenic (As) is a non-essential element with an atomic number of thirty-three and is not required by the body. Since it is not required by the body, As is known to be toxic even at low and causes serious defects in the body such as being a carcinogen (Atinafu *et al.,* 2015). Arsenic is potent poison and disrupts ATP product. The safety limit of As is 1.0 ppm in herbal preparations as permitted by the WHO (Maobe *et al.,* 2012). In the past arsenic compounds were being used as pesticides in rice fields (Stanojkovic-sebic *et al.*, 2015).

2.6.1.3 Cadmium

Cadmium (Cd) is trace metals with an atomic number of 48 and not essential for either humans or plants. Since its not essential, it can easily cause toxic effects in humans even at low concentrations. The safety limit of Cd in edible plants is reported to be 1 μg kg^{-1} (Stanojkovic-sebic *et al.,* 2015; Ali, 2003). The metal is poisonous and known to cause causes birth defects and cancer when consumed or exposed in high amounts (Atinafu *et al.,* 2015).

2.6.1.4 Chromium

Chromium (Cr) is an essential micronutrient trace element with an atomic number of twenty-four important in human metabolic processes, even though high amounts are said to cause carcinogenic effects. Deficiency of Cr in the human body can cause disturbances in glucose, lipid and protein metabolisms (Pakade *et al.,* 2013; Stanojkovic-sebic *et al.,* 2015). Increased exposure to Cr can cause nasal mucosae injury, allergic and irritant contact dermatitis, upset stomach, lung cancer in the human body (Wada, 2004). Chromium has been used in the prevention and control of diabetes mellitus (Steenkamp *et al.,* 2006).

2.6.1.5 Cobalt

Cobalt (Co) is a trace element with an atomic number of twenty-seven and is needed by the body as a component of the vitamin B12 where the vitamin B12 is needed to ensure enough red blood cells are produced in the body (Fraga ,2005 ; Maobe *et al.,* 2012). Safety limits of Co range from 30 to 40 mg kg^{-1} Increased exospore of Co is said to be toxic and is known to be carcinogenic.

Effects of high exposure to cobalt dust include vomiting, diarrhea and skin irritations and rashes (Javed and Usmani, 2013; Steenkamp *et al.,* 2006).

2.6.1.6 Copper

Copper (Cu) is an element with an atomic number of twenty-nine and is essential for the proper functioning of the body and is needed in trace amounts to help enzymes transfer energy in cells, while it can become toxic at high concentrations (Fraga, 2005; Hunt, 2003). Copper helps in the proper functioning of enzymes, hemoglobin formation and vitamin synthesis in humans and have a safety limit of 4-20 μg/kg (Stanojkovic-sebic *et al.,* 2015). Copper is known to be a natural antibacterial. And it is used to prevent the spread of bacteria on brass doorknobs and handrails. When the concentration of Cu is higher than 20-100 mg kg^{-1} in dry plant material, it is known to be poisonous to plants (Korfali *et al.,* 2013).

2.6.1.7 Iron

Iron (Fe) is a trace metal which is essential for both the health of plants and the nutrient supply to humans and animals and contains a safety limit of 7-10 mg/day for humans (Fraga, 2005). The nutritional requirements of Fe concentrations vary with plants ranging from 50 to about 250 mg kg^{-1}, while the of grazing animals of 50-100 mg kg^{-1} (Fraga, 2005). Despite Fe being a trace essential element, excess amounts are stored in the liver, pancreas, pituitary, adrenals, heart, and skeletal muscles, this further moves into the blood and brain. While in the brain, Fe destroys neurons leading to neurodegenerative diseases and neurological dysfunction (Javed and Usmani, 2013). Iron concentrations are lower in boiled herbal medicines since boiling releases the iron as precipitate (Korfali *et al.,* 2013).

2.6.1.8 Lead

Lead (Pb) is an element with an atomic number of eighty-two, which is known to be a carcinogen and teratogenic (disturbs the development of an embryo or foetus) (Food Safety Authority of Ireland, 2009). The accumulation of lead has led to many diseases which include cardiovascular, kidney, blood, nervous, and bone diseases. In the human body, lead disrupt functions of vital organs and glands such as the brain, kidney and liver. Lead has a safety limit of 10 mg/kg and 10 ppm in herbal preparations (Maobe *et al.,* 2012). Continuous exposure to lead is known to decrease the performance of the nervous system and affects renal clearance and is a common cause of miscarriages in pregnant women. Therefore, with the dangers of lead and its exposure, herbal

preparations have to be checked for the presence of such a metal, since it's harmful to humans and also affect the intelligence of children (Stanojkovic-sebic *et al.,* 2015).

2.6.1.9 Manganese

Manganese (Mn) is an essential trace element of atomic number 25. Most enzymes such as the enzyme responsible for converting water molecules to oxygen during photosynthesis contains four atoms of manganese. When human bones lack manganese, they grow with holes inside and break easily (Fraga, 2005). The safety limit of manganese is 400 mg kg-1 of dry weight (Stanojkovic-Sebic *et al.,* 2015). Health effects related to manganese exposure include irritation of the lungs which could lead to pneumonia and inflammation of the kidneys and kidney stone formation (Maobe *et al.,* 2012 ; Nema *et al.,* 2015).

2.6.1.10 Molybdenum

Molybdenum (Mo) is an essential trace metal with an atomic number of forty-two and works together with some flavoprotein enzymes in plants and animals and has concentrations ranging between 0.2-5.0 mg kg^{-1} in plants (Pakade *et al.,* 2013). Plant species and soil types determine the variations of metal concentrations (Stanojkovic-sebic *et al.,* 2015). From previous research, Mo has been reported at low concentrations, since it is quickly removed from the body through the urine (European Medicines Agency, 2007).

2.6.1.11 Nickel

Nickel (Ni) is an essential element for healthy plant life and has an atomic number of twenty-eight. Nickel (Ni) requires an average daily intake of 0.3 mg / day, therefore, any concentration beyond 1 mg is said to be toxic according to the Environmental Protection Agency (EPA) (Steenkamp *et al.,* 2006). An excess amount of nickel is carcinogenic to the human body and excess exposure to nickel can cause lung cancer and fibrosis to the human body (Javed and Usmani, 2013 ; Mtunzi *et al.*,2012).

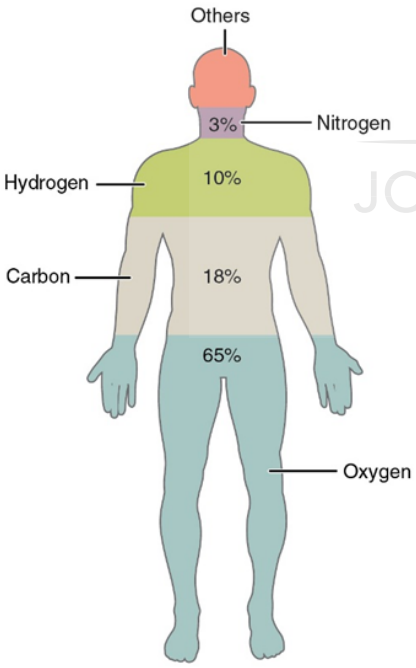
2.6.1.12 Zinc

Zinc (Zn) is an element with atomic number of thirty and is an essential trace element for plant growth and also plays an important role in various cell processes such as forming the active site in over 20 metalloenzymes (Hunt, 2003; Pakade *et al.*,2013) Increased accumulation of Zn is known to be toxic to both humans and animals. A total exposure of 60 mg/day of Zn is allowed for humans. Zinc is known to be carcinogenic if consumed in excess and if freshly formed zinc(II)

oxide is inhaled, a disorder called the ‘oxide shakes’ or ‘zinc chills’ can occur (Stanojkovic-sebic *et al.*, 2015; Fraga, 2005).

2.6.2. The effects of toxic metal chemicals on human health

Trace elements are needed in the human body for a number of applications such as they form part of enzymes, hormones and cells in the body. These metals can be obtained by eating a variety of foods from the different food sources (Nielsen and Hunt, 1989 ; Wada, 2004). Despite common knowledge that heavy metals such as mercury, lead, arsenic and cadmium are toxic metals and have mutagenic effects even at very low concentration and causes human disease, malfunction and malformation of organs, heavy metals are assumed to have therapeutic properties when introduced into herbal preparations (Nema *et al.*, 2015). Nema and colleagues, (2015) reported that before the introduction of penicillin, mercury was used for the treatment of syphilis and arsenic-derived compounds are still being used for the treatment of some forms of malignant tumours. It is then necessary that the body has trace “small” amounts of metals for normal body processes as indicated by Figure 2.15.



Element	Symbol	Percentage in Body
Oxygen	O	65.0
Carbon	C	18.5
Hydrogen	H	9.5
Nitrogen	N	3.2
Calcium	Ca	1.5
Phosphorus	P	1.0
Potassium	K	0.4
Sulfur	S	0.3
Sodium	Na	0.2
Chlorine	Cl	0.2
Magnesium	Mg	0.1
Trace elements include boron (B), chromium (Cr), cobalt (Co), copper (Cu), fluorine (F), iodine (I), iron (Fe), manganese (Mn), molybdenum (Mo), selenium (Se), silicon (Si), tin (Sn), vanadium (V), and zinc (Zn).		less than 1.0

Figure 2.15 The human body showing amounts of elements percentages within the body.

Adapted from (Dzomba *et al.*, 2012)

2.6.3 Common trace metals found mostly in the herbal preparations

The increasing use of traditional medicines is of special concern because they are not regulated and thus the focus of this study was to determine the amount of toxic heavy metals in *C. macowanii* bulbs. Contamination of traditional medicines by heavy metals is of major concern because of the toxicity, persistence and bioaccumulation of such metals. The effectiveness of medicinal plants for therapeutic purposes is often accounted for by their chemical constituents like flavonoids, alkaloids, terpenes, glycosides, essential oil, vitamins, etc. (Korfali *et al.,* 2013; Senila *et al.,* 2014). Additionally, minerals and trace metals are partially responsible for their medicinal and nutritional properties, as well as their toxic effects. These elements play an important role in plant metabolism and biosynthesis and as cofactors for enzymes (Gogoasa *et al.,* 2013 ;Korfali *et al.,* 2013).

The elemental composition of herbs is a reflection of the environment they grow in. The levels of essential elements in plants vary according to the geographical region, geochemical soil characteristics, and the ability of plants to selectively accumulate some of these elements. Generally, these elements are absorbed through the root systems and dispersed throughout the plant body (Korfali *et al.,*2013). Some metals are essential nutrients (zinc, iron, copper, and chromium). However, they become toxic at high concentrations, while others such as lead, mercury, arsenic and cadmium have no known beneficial properties and are toxic (Dzomba *et al.,* 2012 ; Korfali *et al.,* 2013). Elevated concentrations of essential elements (e.g., Fe, Mn, Zn, Cr, Cu) and low concentrations of non-essential elements (e.g., Cd, Ni, As) may present a potential hazard for human (Dzomba *et al.,* 2012).

Table 2.5: Trace elements and their pharmacological effects

Trace element	Pharmacological effects
Iron	Resistance to infections
Zinc	Wound healing
	Improved resistance to infections and immune functions
Chromium	Prevention of atherosclerosis
Selenium	Anti-cancer activity
	Prevention of ischemic heart disease
Iodine	Correction of latent iodine-deficient goiter

Adopted from (Wada, 2004)

2.6.4 Inductive coupled plasma-optical emission spectroscopy (ICP-OES)

With the desired need to determine the element content and concentration in the extract, a single extraction method had to be developed which uses less aggressive solutions and exchangeable metals, which are known to correlate better with plant uptake (Mtunzi *et al.,* 2015). The microwave-assisted sample digestion technique is a method adopted by the United States Environmental Protection Agency (EPA or sometimes USEPA) used to extract metals from sludge, soil or sediments and has been used for over thirty-six years (Mtunzi *et al.,* 2015). The method ensures a rapid, safe, and efficient digestion and prevents the loss of volatile metals and the results are analyzed by Inductive coupled plasma-optical emission spectroscopy (ICP-OES), which is an analytical technique for metal determination and has low detection limits, large dynamic range and high precision. ICP-OES is able to determine elements in liquids and solid samples (Senila *et al.,* 2014). The results obtained are presented and organized in tables in order to provide an easy overview of the method's performance (Pakade *et al.*,2013 ; Şenilă *et al.*,2011). Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) is used as a method for characterizing trace elements in medicinal plants (as seen in Figure 2.16) (Ebrahim *et al.*,2014). When compared to other methods used, ICP-OES has lower detection and quantification limits ranging between LoQ 50–1200 mg/kg, after digestion of e.g. 100 mg sample (Okem, 2014).

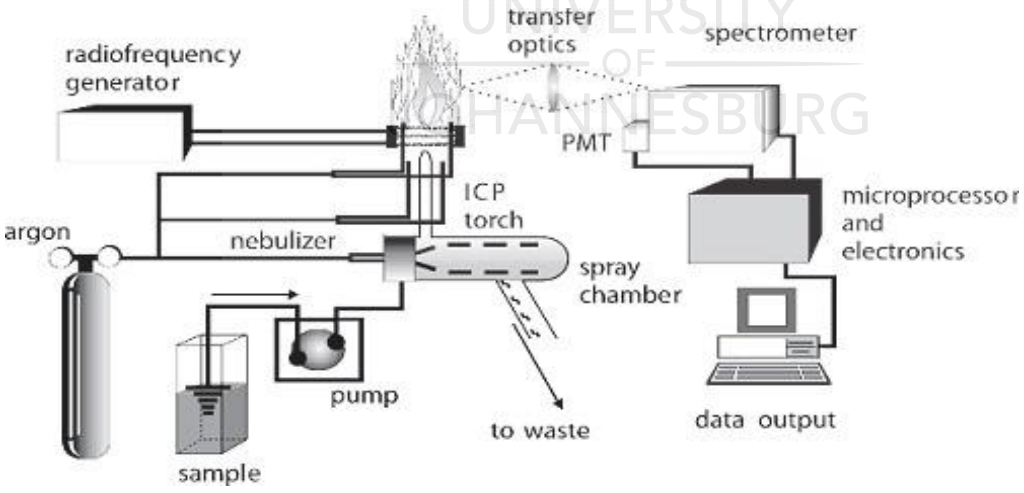


Figure 2.16 Schematic diagram showing major components and layout of a conventional ICP- OES instrument (Scientific, 2016)

2.7 Summary

From the literature reviewed so far, it is evident that *C. macowanii* is widely used for its medicinal purposes to treat a variety of ailments, and has the potential to contribute to health and wellbeing of those who use it since it possesses a wide array of chemical scaffolds (sometimes complex) known to efficacious bioactivities as far as in vitro experiments are concerned. Previous studies have demonstrated a need to study phytochemical composition with respect to antibacterial properties and metal composition of the plant in order to explore it as a valuable commodity. Moreover, this will assist in providing a scientific validation for the use of the plant in traditional medicine and also contribute to the safety and toxicology of the plant.

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CHAPTER 3

Crinum macowani bulbs phytochemical constituents and their GC×GC-TOFMS screening

Abstract

In this chapter, the phytochemical composition of *Crinum macowanii* bulbs solvent extracts were investigated. Qualitative phytochemical screening was undertaken to identify the possible compounds present in the bulbs where tannins, reducing sugars, flavonoids, steroids, alkaloids, saponins and cardiac gylcosides were identified. Furthermore, qualitative phytochemical probing of the same extracts was explored through the use of a comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry detection (GC×GC-TOFMS). To the best of our knowledge, this is the first report describing the use of GC×GC-TOFMS for the evaluation phytochemical constituents in *C. macowanii*. This method detects and identifies volatile compounds from the sample. The compounds detected included some alkaloids, fatty acids and sterols such as α-sitosterol, lycorine, xanthosine, oleic acid, dihydronormorphinone, 9,19-cycloergost-24-(28)-en-3-ol,4,14 dimethyl, acetate, (3á,4à,5à) and trisphaeridine just to name a few. These compounds have been associated with a number of biological applications such as anti-inflammatory, anti-microbial, anti-tumour, analgesia, lactation, anti-cancer and antidiabetic properties. This can justify the continued use of the plant for herbal preparations.

Keywords: GC×GC-TOFMS, phytochemical screening, *Crinum macowanii* and solvent extraction

3.1 Introduction

Medicinal plants are known to be rich in chemicals that are sources of novel drugs. These phytochemicals are also the main ingredients in traditional medicine and modern medicine where most notable active ingredients have their origins in phytochemicals (Saxena *et al.*, 2013; Tariq and Reyaz, 2013). Plants play a significant role in drug discovery, hence the WHO reported that up to 80% of the world's population depends on some form of traditional medicine for their primary healthcare needs (Brusotti *et al.,* 2014; Yadav and Agarwala, 2011). This is likely due to the affordability and availability of plants and their cultural significance to people since time immemorial (Fennell *et al.,* 2004). Natural products play a vital role in the field of new drugs research and development and therefor there is an urgent need to screen medicinal plants for the identification and isolation of bioactive compounds as drugs produced from plants that are biodegradable and considered safe (Tariq and Reyaz, 2013). Phytochemistry is defined as the study of compounds derived from plants, such studies are done to isolate small quantities of bioactive compounds from plants and techniques used in phytochemistry include extraction, isolation and structural elucidation of various plant secondary metabolites (Phillipson, 2001;Sasidharan *et al.,* 2011). GC×GC-TOFMS is used since plant samples are quite complex and characterizing possible compounds may prove difficult. GC×GC-TOFMS is sensitive even to small amounts and only little amounts are needed for the procedure. The use of gas chromatography (GC) for separation of metabolites and mass spectrometry (MS) for their quantification and identification makes this chromatographic technique advantageous over other techniques previously used (Adahchour *et al.,*2006).

3.2 Materials and Method

3.2.1 Plant material collection

For this study, *Crinum macowanii* bulbs were purchased at Faraday muthi market in Johannesburg South Africa in January 2015. A voucher specimen (no. BTNST01) is available at the UJ herbarium. Following the procedure of Yadav and Agarwala (2011), the bulbs were washed and chopped into smaller pieces and air-dried at room temperature. The dried plant material was blended into fine powder using a commercial blender.

3.2.2 Organic Solvent Extraction

Following the extraction procedures by Hasan *et al.,* (2009), 150 g of the prepared plant material was added into 2 L of a 50:50 methanol: dichloromethane (v:v) solution. This was allowed to shake for 3 days on a platform shaker. The solution was filtered through Whatman No. 1 filter paper and each day the filtrate was evaporated on a rotatory evaporator. The crude extract was allowed to air dry in a desiccator. Equation was used to determine extract recovery percentages.

$$\text{Extract recovery } \% = \frac{(\text{weight of extract+vial (g)}) - \text{weight of empyt vial (g)} X\ 100}{\text{weight of dry plant material used (g)}} \quad \ldots\ldots\ldots\ldots\ldots (1)$$

3.2.3. Phytochemical screening of Crinum macowanii

Phytochemical screening is used to check for possible phytochemical groups present in the crude extract. For this part of the study methods described by Tamilselvi *et al.,* (2012) and Yadav and Agarwala (2011) were followed with minor modifications.

Water extract preparation

10 g of dried crushed *Crinum macowanii* bulb was brought to boil with 200 mL distilled H_2O. The extract was filtered.

Tannins

2-3 drops of a 10% $FeCl_3$ solution was added to 2 mL of the water extract. A positive test resulted in the solution turning blackish-blue or blackish-green in colour.

Reducing sugars

2 mL of Benedicts's reagent was added to 10 mL of the water extract. The solution was placed in a boiling water bath and was allowed to heat. The following colour changes indicated the presence or absence of reducing sugars in the extracts: Bright blue solution signified the absence of reducing sugars. A green solution means there is a possibility of reducing sugars. Yellow solution – low presence of reducing sugars. Orange and red solutions signified definite presence of reducing sugars.

Alkaloids

5mL of a 1% HCl solution was added to 0.5 g of dried *C. macowanii* bulb powder. This was placed for 2-3 minutes in a boiling water bath. The solution (1 mL) was collected in a clean container. Drops of Dragendorff's reagent were added. A positive test was indicated by turbidity or precipitation.

Flavonoids

Ethyl acetate (10 mL) was added to powdered *C. macowanii* bulbs (0.5 g). This was heated for 3 min over a steam bath. The solution (4 mL) was taken and ammonia solution (1 mL) was added and this was shaken. A positive test was indicated by a yellow colour change that disappears after a while.

Steroids

C. macowanii bulbs ground powdered (0.5 g) was dissolved in chloroform (5 mL). The solution was filtered. Concentrated H_2SO_4 was added to the filtrate. Layers were allowed to separate. A positive test was indicated by a reddish brown colour forming a steroid ring.

Saponins

The water extract (10 mL) was shaken vigorously to allow the formation of a stable froth that lasted for at least 10 minutes. The froth was collected and mixed with 3 drops of olive oil. The solution was shaken vigorously and positive results were indicated by the formation of a stable froth and emulsion after the oil was added.

Cardiac glycosides

Powdered sample (0.5 g) was weighed into a beaker, glacial acetic acid (2 mL) containing 1 drop of $FeCl_3$ (1%) and concentrated H_2SO_4 (1 mL) was added. The phases were allowed to separate. A positive result was indicated by a brown ring at the interface (indicating deoxysugar characteristics of cardenolides). A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may have formed gradually throughout it.

3.2.4 Two Dimensional Gas Chromatography TOFMS (GC×GC-TOFMS) for the analysis of crude sample

GC×GC-TOFMS was used since plant samples are quite complex and characterizing possible compounds may prove difficult. GC×GC-TOFMS is sensitive even to small amounts and only little amounts are needed for the procedure. The use of gas chromatography (GC) for separation of metabolites and mass spectrometry (MS) for their quantification and identification makes this chromatographic technique advantageous over other techniques previously used.

Each extract was 1 mL weighed, reconstituted in HPLC grade solvent and filtered through cotton glass pipette. The filtrate was then placed into a capped GC×GC vial (Merk) and analyzed immediately. An Agilent 7683 ALS auto sampler equipped with 10 µl syringe was used for the injections with an injection volume of 1 µl per sample and three sample washes from 2 different solvents (chloroform and methanol) with no viscosity delay. The instrument used for the analysis was an Aligent Technologies 7890A GC equipped with a LECO cryo-modulator coupled to a Pegasus 4D TOF/MS. The settings used for the GC×GC-TOFMS are presented in Table 3.1.

Table 3.1: The GC×GC-TOFMS setting specification

Specification	Setting
Column	30 m x 0.25 m x 0.25 µm Rxi-5Sli Stabilwax
Injection volume	1 µl (2 injection per sample)
Carrier gas	Helium 1cm^3/min for entire run
Oven program	80 °C for 1 min increased to 250 °C at 15 °C/min, hot pulse time 0.80 sec
Modulation period	4 sec
Split	10:1

The samples analyzed were the organic phases of the solvent extracts of the plants' bulbs. The sample underwent a dual phase separation (GC columns) with two orthogonal phases separated by a cryo modulator and then various components of the plants were detected by a Time of Flight Mass spectrometer, generating individual mass spectra which were then compared to the NIST and Adams EO library databases (Ralston-Hooper *et al.*, 2008).

3.3 Results and discussion

3.3.1 Phytochemical screening

The bulbs showed a strong result in the test for alkaloids, confirming that the plant is rich in this specific group of compounds as has been previously cited in a number of reports. In the test for saponins a lot of persistent froth also formed, but the test is not quantitative, so no conclusions could be made on the amount of saponins present. Tannins, flavonoids, steroids and cardiac glycosides were also detected in the bulbs. This was done as a preliminary test to qualitatively check the compounds present.

Table 3.2: Phytochemical screening of crude bulb extract of *Crinum macowanii*.

Chemical Compound	Observation	Results
Tannins	Faint green colour	Present
Flavonoids	Yellow colouration	Present
Steroids	Red ring	Present
Reducing Sugars	Dark green colour	Present
Alkaloids	Turbidity/ Precipitation	Present
Saponins	Persistent froth	Present
Cardiac glycosides	Brown ring at interface	Present

The phytochemical screening in the present study revealed the presence of steroids, flavonoids, tannins, saponins, alkaloids, reducing sugars and cardiac glycosides as previously mentioned in Table 3.2. The different classes of these phytochemicals have been reported to have therapeutic properties (Chapter 2), hence the use of *Crinum macowanii* bulbs for medicinal purposes. The presence of steroids, flavonoids and alkaloids in *C. macowanii* bulbs corresponds with the findings of Asmawi *et al.,* (2011), where they reported the presence of the same classes of phytochemical compounds in other crinum species. To the best of our knowledge this is the first report noting the presence of tannins, reducing suagars, saponins and cardiac glycosides in *C. macowanii* bulbs. The presence of these phytochemical compounds in *C. macowanii* bulbs, with their different pharmacological properties as discussed in section 2.1 provides some form of justification and

rational behind the plant's traditional use in traditional medicine for the treatment of different ailments.

3.3.2 Phytochemical investigation by two dimensional gas chromatography (GC×GC-TOFMS)

Although a variety of plants (including *C. macowanii*) have been shown to contain more or less the groups of naturally occurring chemical compounds mentioned in Table 3.2, which can always be qualitatively identified, correlating the actual phytochemical classification (e.g alkaloid, flavonoid etc) requires column chromatography and in depth studies and analysis of comprehensive NMR data. GC×GC-TOFMS and in other instances LC-MS and its derivatives (where the volatile nature of the chemical constituent in question is not volatile) have become invaluable techniques. Since *C. macowanii* is known to be rich in alkaloids as shown in section 3.3.1, additional classes of compounds (saponins, reducing sugars, tannins and cardiac glycosides) were shown to be also present in the plant. GC×GC-TOFMS data (summarized in Table 3.3) allowed for the correlation of these classes to definitive compounds. Whilst literature has not been conclusive on the occurrence of nucleosides in crinum species, our results revealed xanthosine as a bioactive component in crinum bulbs. Xanthosine has a number of bioactivities that include interactions with epithelial cells (Table 3.3) and involvement in a variety of biosynthetic systems. Detection of lycorine, flexinine, trisphaeridine, dihydronormorphinone, *9(10H)*-Acridinone, 4-methoxy-, guanosine and xanthosine was no surprise as the plant is known for its alkaloid content. Nevertheless, literature search has not been very conclusive about the occurrence of xanthosine in crinum species. As such, we are inclined to suspect that evaluation of *C. macowanii* using GC×GC-TOFMS managed to reveal a phytochemical known to be associated with plants, but has not been described in *C. macowanii*. Given the bioactivity of alkaloids, this validates *C. macowanii* as a medicinal plant (although more GC×GC-TOFMS studies are needed as more compounds not known to associate with the plant may be unearthed).

Interestingly, an unexpected detection of dihydromorphinone (Table 3.3) was noted. Since morphine is known to be exclusively biosynthesized by *Papaver somniferum* and its related species (Wilson and Gisvold, 2009). Detection of this opiate was a surprise as crinum species are not known to contain this class of compounds. Even more so, dihydromorphinone is a semi-synthetic compound derived from morphine by a rearrangement involving heating of a morphine containing

solution in an acidic environment or by use of catalysts, thus, its detection from *C. macowanii* is a surprise. As such, this result will need to be reinvestigated and conclusively established. This is of interest since as far as ethno-pharmacology literature is concerned, *crinum* extracts are known to be analgesic or have been noted to be used as such. If indeed dihydromorphinone is contained in *C. macowanii*, this could be a further validation for its use as a medicinal plant for conditions associated with or requiring analgesics.

As much as compounds with a sterol scaffold are known to occur in a variety of plants, especially medicinal plants, literature search has not revealed a lot of information with regard to sterols in crinum species, except for Refaat *et al.,* 2009, who showed that *Crinum augustum* Rox from Egypt contains mixtures of β-sitosterol and stigmasterol over and above other phytochemicals known to be associated with crinum. Comparing their findings with the observations made in this study where β-sitosterol and campesterol were detected by GC×GC-TOFMS, it is therefore possible that a number of other crinum species may contain sterols.

Squalene, a triterpene known to occur in plants and functions as a biochemical intermediate that acts as a precursor to stigmasterol. Even though stigmastrol has been reported in criunum species before as stated by Refaat *et al.,* (2013), no information has been documented stating the presence of squalene in *C. macowanii* . This then indicates that GC×GC-TOFMS was able to identify compounds that naturally occur in the crinum species under investigation but have been missed by previous studies.

Fatty acids such as palmitic acid, stearic acid and linoleic acid have been identified in different crinum species as state by (Refaat *et al.,* 2013). With that being said *cis*-Vaccenic acid, a fatty acid commonly found in animals and mammals was detected as stated in (Table 3.3). This could have been caused by biosynthesis processes of other fatty acids present in the plant. Oleic Acid was detected and this supports literature, as Refaat *et al.,* (2013) noted its presence in a variety of crinum species (*C. bulbisperm*, *C. augustum* Rox and *C. americanum*).

Table 3.3: Volatile compounds isolated from *Crinum macowanii* bulbs crude solvent extract and identified by GC×GC-TOFMS.

No.	Peak #	Name of the compound	Area %	Biological/Pharmacological uses	Compound nature
1	825	Oleic Acid	28.844	An anti-inflammatory (Carrillo and Cavia, 2012)	Fatty acid
2	613	Hexadecanoic acid	2.8606	An anti-Inflammatory (Aparna *et al.*, 2012)	Fatty acid
3	654	9,19-Cycloergost-24(28)-en-3-ol,4,14-dimethyl-,acetate, (3á,4à,5à)-	0.24719	Antimicrobial (Singariya *et al.*, 2012)	Cycloartanol (steroids containing a cycloartanol moiety)
4	617	β-Sitosterol	0.19201	Antidiabetic (Tripathi *et al.*, 2013)	Sterol
5	567	9,12 Octadecadienoyl chloride, (Z,Z)-	0.18879	Anti-inflammatory (Anitha *et al.*, 2012)	Linoleic acid
6	808	*cis*-Vaccenic acid	0.18754	Antiviral (Rontani *et al.*, 2003)	Fatty acid methyl ester
7	34	1,2,3 Propanetriol	0.11308	Bacteriostatic (Aono *et al.*, 1999)	Polyol (sugar alcohol)
8	11	Ethanone,1-(4-hydroxy-3-methoxyphenyl)-	0.032397	Anti-inflammatory (Stefanska and Pawliczak, 2008)	Volatile oil

9	388	Squalene	0.028821	Anti-bacterial, Anti-oxidant Anti-tumor, Anti- HIV (Amudha and Rani, 2014)	Tri-terpene compound
10	432	Trisphaeridine	0.000079615	Antitumor (Zupko *et al.*, 2009)	Alkaloid
11	138	Dihydronormorphinoe	0.002388	Analegestic (Wilson *et al.*, 2004)	Alkaloid
12	26	Xanthosine	0.0024561	Increase mammary epithelial cell proliferation of lactating bovine (Choudhary, 2014)	Alkaloid
13	397	Lycorine	0.0025952	Anticancer (Wang *et al.*, 2014)	Alkaloid
14	531	dl-α-Tocopherol	0.0029011	Antioxidant (Nystrom *et al.*, 2006)	Steryl ferulates (ferulic acid esters of sterols)
15	668	Andrographolide	0.0091158	Anti-tuberculosis (Anju *et al.*, 2012)	Labdane diterpenoid
16	572	Campesterol	0.0097256	Anticarcinogenic (Choi *et al.*, 2007)	Sterol
17	37	Guanosine	0.013114	Anti-HIV (Taylor *et al.*,1996)	Alkaloid

18	193	*9(10H)*-Acridinone,4-methoxy-	0.00080801	Antiviral (Sepulveda *et al.*,2013)	Alkaloid
19	345	Flexinine	0.0034653	Anti-proliferative (Kuete *et al.*,2013)	Alkaloid
20	75	*p*-Dioxane-2,5-dimethanol	0.0036502	Widely used as a model cosolvent for solubility studies of drugs (Ruidaiz *et al.*, 2011)	Cyclic ether

Note: A number of known and unknown compounds were identified but only a few are represented in the table above

3.4 Conclusion

From the phytochemical screening results, compound groups such as alkaloids, saponins, tannins, flavonoids, steroids and cardiac glycosides were detected in the bulbs. Concentrations of each compound could not be determined as quantitative tests were performed. The compounds detected (by GC×GC- TOFMS) have been reported to have biological applications such as antimicrobial, antidiarrhoeal and anticancer activites. Phytochemical analysis of both extracts was performed by two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-TOFMS), where this chromatographic technique was the first to be carried out on *C. macowanii* bulbs. Phytochemicals from compound groups such as fatty acid, sterol, volatile oil, alkaloid, cyclic ether, phenolic aldehyde and flavonoid were identified from both crude extracts (solvent extracts and pressurized hot water extracts). The phytochemicals are known to have biological applications such as anti-inflammatory, anti-diabetic, anti-inflammatory, anti-cancer, anti-bacterial and anti-microbial activities. This can support and justify the traditional use for the plant for treatment of heart disease associated with kidney, bladder, tuberculosis, back pain, and skin problems (boils, sores and acne) ailments. Alkaloids were the most abundant from both crude extracts and this supports literature since the plant is known to be rich in alkaloids.

3.5 References

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CHAPTER 4

ANTIBACTERIAL INVESTIGATION OF *CRINUM MACOWANI* BULBS

Abstract

In vitro antibacterial activity of *C. macowanii* was investigated by disc diffusion method and microdilution method. The study demonstrated that crude extracts of *C. macowanii* bulbs have good antibacterial activity against *Bacillus cereus, Enterococcus faecalis, Staphylococcus epidermidis, S. aureus, Mycobacterium smegmatis, Escherischia coli, Klebsiella oxytoca, Bacillus subtilis* and *Pseudomonas aeruginosa*. Our results and observations are concerned, there seem to be a justification for the implied traditional medicinal uses of the plant bulb for the treatment of wounds, skin, stomach, gastro intestinal tract, urinary tract and respiratory tract infections. The highest activity was exhibited against Gram-positive strains for both types of antibacterial tests done and it was observed that the Gram-negative bacterial strains (*Enterobacter cloaca* 16 mg/mL, *Enterococcus faecalis* 8 mg/mL, *Escherichia coli* 8 mg/mL, *Klebsiella oxytoca* 4 mg/mL and *Enterobacter aerogenes* 16 mg/mL) had not been inhibited by the crude extract during the disc diffusion method but inhibition could be observed by the microdilution method. *C. macowanii* crude bulb extract possesses a potential in the development and discovery of new drugs that could help in combating resistant bacteria. Pharmacological and toxicological evaluations have to be conducted to confirm this.

Keywords: Disc diffusion, microdilution method, *C. macowanii* and antibacterial.

4.1 Introduction

The use of plants for the treatment of diseases dates back to the existence of human kind (Ncc and Fernandes, 2010). Traditional medicines in the form of plants have been used for many decades to treat common infections and ailments. The plants are ingested as decoctions or applied as poultice onto the infected wounds or burns (Mathur *et al.,* 2010). They are used for primary health care needs in developing countries and unsustainable harvesting, which eventually leads to extinction gradually decreasing this rich nonrenewable resource. Naidoo and Coopoosamy (2011) reported that about R270 million a year is generated through the trade of indigenous plants. Although phytochemicals have been reported to be responsible for the therapeutic properties of medicinal plants in most cases their chemical constituents and mode of action are not always known (Ncc and Fernandes, 2010) particularly in the traditional medicine market.

Due to the widespread use of *crinum* species in traditional medicine for the treatment of a variety of ailments associated with bacterial infections, a lot of studies have explored the different *crinum* species for anti-bacterial investigations, however, a few have focused on *C. macowanii*. There is a need to further study the anti-bacterial effects associated with the traditional uses of these plants. There is also a need to isolate, purify and identify the active principles that might be responsible for the observed bio activities.

In the current research fourteen bacteria were evaluated against the plant extracts of *C. macowanii* bulbs and these included *B. cereus* (ATCC10876), *B. subtilis* (ATCC19659), *E. faecalis* (ATCC13047), *S. epidermidis* (ATCC14990), *S. aureus* (ATCC25923) and *M. smegmatis* (MC2 155) for Gram-positive bacteria. *E. aerogenes* (ATTC13048), *E. cloacae* (ATCC13047), *E. coli* (ATCC25922), *K. oxytoca* (ATCC8724), *K. pneumonia* (ATCC13882), *P. mirabilis* (ATCC7002), *P. vulgaris* (ATCC6380) and *P. aeruginosa* (ATCC27853) for Gram-negative bacteria. These bacteria were chosen because they are associated with most diseases that were claimed to be cured by the plant extracts under investigation.

4.2 Materials and methods

4.2.1 Antibacterial analysis of *Crinum macowani* bulbs crude extract

Antibacterial activity tests were carried out in the microbiology laboratory of the University of Johannesburg. A fresh batch of crude extracts was prepared as discussed in Section 3.3.2 and extracts were analyzed within a week of extraction to maintain extract stability. The samples were tested against 14 bacterial strains, which were obtained from Davies Diagnostics (gram positive and gram negative) associated with gastrointestinal, skin, respiratory problems and urinary tract infections. These are shown in Table 4.1 with their reference strain numbers and the ailments they are commonly associated with. All strains were confirmed purity stock cultures and maintained in the Department of Biotechnology and Food Technology's laboratories of the University of Johannesburg. Mueller-Hinton agar and Mueller-Hinton broth were obtained from Oxoid. All bacterial strains were cultured overnight in sterile Mueller-Hinton broth. The turbidity of the culture solutions was adjusted to match a 0.5 McFarland standard within 15 minutes prior to antibacterial testing.

Table 4.1: Infections on body parts and the associated microorganisms.

Test Microorganism	Reference strain number*	Gram reaction	Pathogen primarily associated with infections related to:
Staphylococcus aureus	(ATCC25923)	Gram-positive	Mouth
Bacillus cereus	(ATCC10876)	Gram-positive	Gastro intestinal tract
Bacillus subtilis	(ATCC19659)	Gram-positive	Gastro intestinal tract
Enterococcus faecalis	(ATCC13047)	Gram-positive	Respiratory tract
Staphylococcus epidermidis	(ATCC14990)	Gram-positive	Skin
Mycobacterium smegmatis	(MC2 155)	Gram-positive	Soft tissue (muscles, ligaments)
Enterobacter aerogenes	(ATCC13048)	Gram-negative	Urinary tract
Enterobacter cloacae	(ATCC13047	Gram-negative	Urinary tract
Pseudomonas aeruginosa	(ATCC27853)	Gram-negative	Wounds
Escherichia coli	(ATCC25922)	Gram-negative	Gastrointestinal Tract

Klebsiella oxytoca	(ATCC8724)	Gram-negative	Wounds
Proteus vulgaris	(ATCC6380)	Gram-negative	Wounds
Proteus mirabilis	(ATCC7002)	Gram-negative	Eye
Klebsiella pneumonia	(ATCC13882)	Gram-negative	Respiratory tract

*Reference strain number, ATCC = American Type Culture Collection

*Reference strain number, MC= Microbial Culture

4.2.1.1 Antibacterial screening of crude sample by disc diffusion method

The disc diffusion method is a primary screening method used to check for possible inhibition of bacterial growth at a specific concentration of the crude extract. All disc diffusion tests were done in triplicate.

The crude extract (300 mg/mL) was dissolved in chloroform. The dissolved extract (15 µL) was inoculated onto 6 mm sterile discs and allowed to air dry for 10 minutes in a sterile environment. The disc inoculation process was repeated again to result in inoculant of 9 mg of extract per disc. Aliquots (20 mL) of hot nutrient agar was poured into petri dishes and allowed to solidify, and then later different bacterial strains were closely streaked onto the plates using sterile swabs. The paper discs containing the extracts were placed onto the plates that were streaked with the test organism. Extra 6 mm sterile discs were used for both the negative and positive controls. Sterile distilled H_2O was used as the negative control whereas the antibiotic streptomycin (0.96 mg per disc) was used as the positive control. The plates were incubated for 24 hours in an incubator at 37 °C after which the zones of inhibition were measured. The diameters of zone of inhibition produced by the extract were measured in mm. As described by Othman *et al.*, (2011) with minor modifications.

4.2.1.2 Antibacterial analysis of crude sample by Minimum Inhibitory Concentrations (MIC)

Micro serial dilution was used to check for the lowest inhibition concentration of the crude extract to specific bacterial species.

Crude extract preparation

The crude extract (0.176 g) was weighed into empty autoclaved McCartney bottles to ensure sterility. A minimal amount of dimethyl sulfoxide (DMSO) was used to dissolve the crude extracts and Mueller-Hinton broth was added to bring the volume of the dissolved crude extract to 5.5mL.

Serial dilution preparation

McCartney bottles (x 6) containing Mueller Hinton broth (5.5 mL) were prepared according to manufacturer's instructions and autoclaved at temperature of 121 °C, pressure of 15 psi for 15 minutes. These were used for the serial dilution processes to yield different concentrations of the crude extracts. Serial dilutions were carried out from 32 mg/mL to 1 mg/mL.

Experimental procedure

The procedure by Andrews (2001) was followed with minor modifications. Briefly, the experiment was done in five repeats using a 96-well micro titer plate. The outer wells of the plate were filled with sterile dH_2O. The inoculum (100 µL) was added into each well that did not contain the sdH_2O. The diluted crude extract samples (100 µL) were added in five wells horizontally and the concentrations decreased in vertical order from 32 mg/mL down to 1 mg/mL. The plates were covered with sterile aluminum foil and incubated overnight at 37 °C. After incubation, 10 µL of 0.02% (w/v) Resazurin sodium salt dye solution was added to the wells and incubated for another two hours. Colour changes were observed, where the blue colour showed inhibition of the bacteria and the pink colour showed bacteria were able to metabolize the indicator meaning they were viable and not affected by the extract.

4.3 Results and discussion

In the disc diffusion experiments *C. macowanii* showed the highest antibacterial activity against *B. subtilis* with a 12.67 mm zone of inhibition. Against *S. aureus* the disc diffusion results showed a 10.67 mm diameter zone of inhibition with MIC of 32 mg/mL. The plant extract displayed no activity against *K. pneumoniae*, *P. mirabilis* and *P.vulgaris* for both disc diffusion and MIC (Table 4.3). All three of these species are Gram-negative. From the results it is observed that the Gram-negative bacteria strains *E. cloaca*, *E. faecalis*, *E. coli*, *K. oxytoca* and *E. aerogenes* had not been inhibited by the crude extract during the disc diffusion method but inhibition could be observed by the microdilution method, this could be because the polarity of the natural compounds can affect

the diffusion of compounds onto the culture medium leaving hydrophobic compounds to diffuse less (Jiang, 2011). From the microdilution results, the crude extracts showed inhibition of *M. smegmatis* at 0.125 mg/mL and *B. cereus* at 0.5 mg/mL while *S. epidermidis* was inhibited at 0.0625 mg/mL.

Table 4.2: Antibacterial evaluation of *Crinum macowanii* crude bulb solvent extract

Bacteria species	**Disc diffusion Zone of inhibition (mm)**		**Minimum inhibitory concentration (mg/mL)**	
	Solvent crude extract	**Control Streptomycin**	**Solvent crude extract**	**Control Streptomycin**
Bacillus cereus	8.30	26.67	**0.50**	0.125
Bacillus subtilis	12.67	0.00	16.00	0.125
Enterococcus faecalis	0.00	22.33	**8.00**	0.125
Mycobacterium smegmatis	0.00	23.33	**0.125**	0.125
Staphylococcus aureus	10.67	24.67	**8.00**	0.032
Staphylococcus epidermidis	2.67	22.33	**0.0625**	0.5
Enterobacter aerogenes	0.00	27.00	16.00	8.00
Enterobacter cloacae	0.00	11.6	16.00	0.50
Escherichia coli	0.00	24.00	**8.00**	8.00
Klebsiella oxytoca	0.00	26.00	**4.00**	4.00
Klebsiella pneumoniae	0.00	26.67	>16.00	0.50
Proteus mirabilis	0.00	24.00	>16.00	0.50
Proteus vulgaris	0.00	26.67	>16.00	0.125
Pseudomonas aeruginosa	8.67	30.67	>16.00	8.00

Note: 0mm = No inhibition, Bold= low concentrations

C. macowanii showed inhibitory activities against both Gram-positive and Gram–negative bacteria, with more Gram-positive bacterial species showing susceptibility to the antibacterial compounds in the bulbs. Most Gram-negative bacteria are known to have multidrug-resistant pumps (as opposed to the Gram positive counterparts) which force drugs out of the outer

membrane, hence the observed inhibition of most Gram positive bacteria tested in this study (Iannello *et al.,* 2014).

From our results, *P. aeruginosa* was the only Gram-negative species that showed susceptibility to the bulb extract with a zone of inhibition of 8.67 mm for the disc diffusion method compared to 30.67 mm zone of inhibition for the positive control (Streptomycin). *Pseudomonas aeruginosa* (ATCC27853), a familiar cause of nosocomial infections involving the respiratory and the urinary tracts, and wounds is known to be resistant to crude extracts of crinum species since it is able to transform lycorine into its inactive metabolite 2-O-dimethylungiminorine (Iannello *et al.*, 2014), however, in this case the opposite was observed. This could have been due to that lycorine is able to damage cell membranes thus leading to the exosmosis of intracellular materials and therefore the absorption of alternative toxic substituents of the crude extracts (Cao *et al.,* 2013; Rossolini and Mantengoli, 2005). These results go a long way in justifying the traditional use of the *C. macowanii* bulb for the treatment of urinary tract problems.

The MIC values for the majority of Gram-negative bacteria could not be determined since the values were above 32 mg/mL which was the highest concentration tested. Bacterial species with the lowest MIC concentrations were *M. smegmatis* (MIC of 0.125 mg/mL), *B. cereus* (MIC of 0.5 mg/mL) and *S. epidermidis* (MIC of 0.0625 mg/mL). *M. smegmatis* is a Gram-positive bacterium which shares some virulence gene homology with *Mycobacterium tuberculosis* which is a causative agent of tuberculosis (Abidi *et al.*,2014). With an MIC inhibition value of 0.125 mg/mL (given that since 100 µg/mL is a set criterion for the activity of an anti-infective drug agent), encourages further research into the exploration *C. macowanii* extracts and to further establish how these extracts can be refined to enhance their efficacy towards successfully inhibiting this bacterium (Cos, 2006).

Bacillus cereus a Gram positive bacterium, which is a causative agent for most foodborne diseases and a contaminant if isolated from clinical specimen such as blood, wounds and sputum (Bottone, 2010;Tajkarmini, 2007). Kotirantaa *et al.,* (2000) noted that *B. cereus* is an emerging causative agent for nosocomial infections such as postoperative and posttraumatic wound infections and burns. With a minimum inhibition concentration of 0.5 mg/mL, this signifies a point of more

research effort towards establishing the application of this plant usage as an additive in bandages (for wound purposes) and treatment of rashes and boils (Elgorashi *et al.,* 2003; Nair *et al.,* 2000)

Before microdilution experiments could be undertaken, the disc diffusion experiment was attempted to evaluate which extracts contained bioactive principles. From the experiments conducted, different results were generated for the 50:50 methanol: dichloromethane crude bulb extract where *S. aureus* had a zone of inhibition of 10.67 mm, *S. epidermidis* (2.67mm) and *Bacillus subtilis* (12.67 mm) as compared to the water extracts which showed very small zones of inhibition (these are discussed in Chapter 5).

Staphylococcus epidermidis is known to cause hospital acquired infections found on the human skin (Mack *et al.,* 2013). Bek-Thomsen *et al.,* (2008) reported that *S. epidermidis* was isolated from acne vulgaris-affected skin. While the results of this study showed that the 50:50 methanol: dichloromethane crude bulb extracts inhibited *S. epidermidis* at a concentration of 0.0625 mg/mL. Rabe and van Staden (1997) on the other hand found that there was marginal inhibition (MIC 1 mg/mL) for *B. subtilis*, *S. epidermidis*, *S. aureus*, *Escherichia coli* and *K. pneumoniae* from the water and methanol crude bulb extract for the disc diffusion method. As such, this is evident that the methanol and dichloromethane extract contained more lethal constituents possessing more antibacterial activity as compared to the methanol or water crude extracts alone. The same observation was made by Rabe and van Staden (1997) where it was noted that when water was used as an extracting solvent the subsequent extracts lacked antibacterial activity as compared to the methanol: dichloromethane extract.

The MIC value is a quantitative measure of antimicrobial activity and it is defined as the lowest concentration at which bacterial growth is inhibited. The value is influenced by the method used, the microorganisms tested and the degree of solubility of each test-compound. Crude extracts are recognised as having antimicrobial activity when the MIC values range between 8 and 1 mg/mL with an MIC value below 1 mg/mL being a good indicator of the antibacterial properties of the crude extract (Smânia *et al.,* 2007). From the results six of the bacteria (*B. cereus*, *E. faecalis*, *S. epidermidis*, *S. aureus*, *M. smegmatis*, *E. coli*, and *K. oxytoca*) had MIC values between 8 mg/mL and 1 mg/mL which indicated the extracts as good antibacterial agents (as discussed before).

Section 3.3.2: Correlating antibacterial activity of the crude extracts to GCxGC-TOFMS data (chapter 3, table 3.3)

Given the antibacterial activities from the crude extracts listed in (Table 4.2), it could only be concluded that the agents contributing to the inhibition may be the alkaloids such as lycorine, which has been shown to inhibit *B. subtilis*, *E. coli*, *K. pneumoniae* and *S.s aureus* at concentrations above 0.25 mg/mL Maroyi (2016). Even though the crude extract was used for this study, the above statement could be relevant since the following MIC values were observed where *B. subtilis* was inhibited at 16 mg/mL, *E. coli* at 8 mg/mL, *K. pneumoniae* at more than 16 mg/mL and *S. aureus* at 8 mg/ML.

From the results obtained oleic acid, 9,12 octadecadienoyl chloride, (*Z,Z*)-, *cis*-vaccenic acid and hexadecanoic acid were also detected by GC×GC-TOFMS, these fatty acids have also been reported to have antibacterial activity. 9,12 Octadecadienoyl chloride and oleic acid have been reported to inhibit *Mycobacterium* species such as *M. smegmatis* (Salem *et al.*, 2014). The inhibition observed may have been due to the different active components acting in synergy or individually. This is a point of departure for future studies, to assess the syngergistic effects of these compounds.

Since *M. smegmatis* has been reported to cause human post-traumatic wound infections (Brown *et al.*, 1999) over and above its other effects on immune-compromised individuals, there is basis to validate the traditional use of *C. macowanii* given the inhibition it showed on various bacteria causing skin infections. To further support this, Singariya *et al.*, (2012) reported that 9,19-cycloergost-24(28)-en-3-ol,4,14-dimethyl-,acetate(3á,4à,5à)- contained in *C. macowanii* inhibited *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis*, while that β-Sitosterol was shown to inhibit *E. coli*, *P. aeruginosa*, *S. aureus* and *K. pneumonia* (Saeidnia *et al.*,2014). In another study by Parikh *et al.*, (2011), 9(10H) acridone derivatives were reported to have good antibacterial activity against *S. aureus*, *B. subtilis* and *E. coli*.

The antibacterial activities observed in Table 4.2 could be attributed to the components working in synergy, as stated by Olufunke (2012) that natural products may possess pharmacological activities when in mixture of compounds (synergy) as compared to when in isolation.

4.4 Conclusion

This study demonstrated that the crude extracts of *C. macowanii* have good antibacterial activity against *B. cereus, E. faecalis, S. epidermidis, S. aureus M. smegmatis, E. coli, K. oxytoca, B. subtilis* and *P. aeruginosa*. This further encourages more studies to explore the volatile extracts as antibacterial agents. A scientific justification for the traditional medicinal uses of *C. macowanii* bulb extracts was also provided by the results and observations made in this study for the treatment of wounds, skin, stomach, gastro intestinal tract, urinary tract and respiratory tract infections. The highest activity was exhibited against gram positive bacterial strains. *C. macowanii* crude bulb extracts have a potential for clinical applications while further pharmacological and toxicity evaluation are needed to confirm this hypothesis.

4.5 References

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CHAPTER 5

PRESSURIZED HOT WATER EXTRACTION (PHWE) OF *CRINUM MACOWANI* BULBS

Abstract

In vitro antibacterial activity of *C. macowanii* was investigated by disc diffusion method and microdilution method. The study demonstrated that the pressurised hot water crude extracts of *C. macowanii* bulbs have good antibacterial activity against *Staphylococcus epidermidis* (MIC 8.00 mg/mL), *Staphylococcus aureus* (MIC 8.00 mg/mL; disc diffusion 6.12 mm), *Bacillus subtilis* (MIC 4.00 mg/mL; disc diffusion 5.67 mm) and *Pseudomonas aeruginosa* (MIC 8.00 mg/mL; disc diffusion 4.45 mm). The highest activity was exhibited against Gram-positive strains for both antibacterial methods done and it was observed that the Gram-negative bacterial strains (*Enterobacter cloaca, Enterococcus faecalis, Escherichia coli, Klebsiella oxytoca* and *Enterobacter aerogenes*) had not been inhibited by the pressurised hot water extracts (PHWE) during the disc diffusion method and the microdilution method. A phytochemical probing of the plant was done using a Comprehensive Two dimensional gas chromatography coupled to time of flight Mass Spectrometry (GC×GC-TOFMS). From the GC×GC-TOFMS results, a number of compounds such as apocynin, epibuphanisine, buphandrin, crinan-3-ol,1,2-didehydro-(3á) and powelline were successfully identified. These compounds have been previously reported to have biological activities such as anti-inflammatory, anti-microbial and anti-cancer activities.

Key words: *Crinum macowanii*, GC×GC-TOFMS, Pressurized Hot Water Extraction and antibacterial activity

5.1 Introduction

A solvent is described as a substance that dissolves a solute resulting in a solution (Flórez *et al.,* 2014; Ghude *et al.,* 2013). While different solvents are used in various industries for different applications such as cleaning agents, dispersants and processing aids their implications on the economy, the environment and social wellbeing have to be taken into consideration (Monroy *et al.,* 2015). For plant extraction purposes, organic solvents can be toxic and should be removed if the extract is to be used for food or pharmaceutical applications whereas volatile organic solvents are usually flammable, contribute to smog formation and pose health hazards to people and are generally environmentally unfriendly (Flórez *et al.,* 2014; Mokgadi *et al.,* 2013).

Factors such as intrinsic environmental advantages related to performance, solubility, inertness, health, cost, and safety should be considered when selecting a solvent for purposes of extracts destined for human consumption (Flórez *et al.,* 2014). Despite water being so environmentally friendly, there are limitations to its use as a solvent for extraction processes such as poor extraction efficiency for most organic compounds such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and most pesticides at ambient temperature (Flórez *et al.,* 2014; Liang and Fan, 2013). The different states of water (liquid, gas and solid) are altered by a change in temperature and pressure. At low pressures, water cannot exist in the liquid state and goes straight to gas from solid by sublimation whereas at high pressures, the liquid and gas states cannot be separable a state called supercritical steam (Rovio *et al.,*1999). The dielectric constant (35.6) of water plays an important role in the solvent and solute interactions where; a high dielectric constant such as at room temperature favors the solubility of ionic and very polar compounds and at 350 °C and a dielectric constant of 14.3, water is able to extract non-polar hydrocarbons. When liquid water is heated above 100 °C, its dielectric constant decreases and its ionic forces increase. At a 200 °C, water has the same dielectric constant as that of methanol at room temperature. Benzene is completely miscible with water at 297 °C (Flórez *et al.,* 2014). The dielectric constant of steam is close to 1.00, which explains why many compounds are better extracted with steam than with liquid water (Rovio *et al.,* 1999). The properties stated above, have made water to regain attention as a solvent of choice in some instances. All of these factors combined allow water to mimic organic solvents when variables such as temperature and pressure are combined and applied at specific ratios, thus allowing for the increased extraction of biologically active compounds

responsible for the elimination of bacteria (Liang and Fan, 2013; Makita, 2014). The increase in pressure and temperature in the PHWE method that causes water to mimic similar properties to those of other organic solvents such as ethanol. Under a critical temperature of 373.98 °C and critical pressure of 224.8 kg/cm water can easily solubilize organic compounds from polar (at lower temperatures) to nonpolar (at higher temperatures) like phytochemicals which are normally insoluble in ambient water (Liang and Fan, 2013; Rovio *et al.*, 1999).

5.2 Method and material

5.2.1 Plant collection and Sample Preparation

Crinum macowanii bulbs were purchased at Faraday Muthi market in Johannesburg South Africa in January 2015. A voucher specimen (no. BTNST01) is available at the UJ herbarium. After purchasing, the bulbs were washed and chopped into smaller pieces and later air-dried at room temperature. The dried plant material was blended into fine powder by using a commercial blender (Yadav and Agarwala, 2011).

5.2.2 Sample Preparation and extraction by PHWE

Extraction of phytochemicals was achieved by a makeshift laboratory scale PHWE unit. The system consisted of a HPLC pump (Waters 6000 fluid controller, Waters Corporation, Manchester, UK), stainless steel extraction cell (70 × 30 mm and approximately 20 mL) fitted with a metal frit i.e. filter (3/8 in. diameter, 1/32 in. thickness and 2.0 μm pore size), refurbished GC 600 Vega Series 2 oven (Carlo Erba Instruments, Italy) with an automatic temperature controllable unit, stainless tubing (1.58 mm in outer dimension (OD) and 0.18 mm inner dimension (ID), back-pressure valve (Swagelok, Johannesburg, South Africa), and a collection flask.

Extraction: Ground bulbs powder (3 g) was mixed with diatomaceous earth (2 g) (Sigma, Munich, Germany), and placed inside the extraction cell. The oven temperature was maintained at 150 °C. Extraction was performed in dynamic mode using at different ratios of methanol–water mixture i.e. 0, 20, 40 and 60% composition of aqueous methanol (Romil Ltd, Waterbeach Cambridge). The solvent was delivered at a constant flow rate of 5 mL/min and a pressure of 1000 ± 200 psi was maintained using the back-pressure valve. Extracts were collected in a falcon tube up to the 50 mL

mark through an outlet coil immersed in a cooling water bath. Each extraction operation lasted for 10 min. The extracts were filtered using a 0.22 μm nylon syringe filter into a 2 mL HPLC capped vial and preserved at −20 °C prior to analysis (Khoza *et al.*, 2014).

Figure 5.1: PHWE extracts of *C. macowanii* bulbs

5.2.3 Antibacterial analysis of *Crinum macowanii* bulbs crude obtained by PHWE

The same procedure as described in **4.3.1.1** and **4.3.1.2** was followed.

5.2.4 Two Dimensional Gas Chromatography TOFMS (GC×GC-TOFMS) analysis of crude sample of *C. macwoanii* bulbs PHW extracts

The same procedures as described in **3.3.4**

5.3 Results and discussions

5.3.1 Antibacterial evaluation of *Crinum macowanii* bulbs with PHWE extract.

In the disc diffusion experiments *C. macowanii* showed the highest antibacterial activity against *Staphylococcus aureus* with a zone of inhibition of 6.12 mm. The lowest zone of inhibition was 4.45 mm for *P. aeruginosa*. The disc diffusion method of the plant extract displayed no activity against *E. faecalis, E. aerogenes*, *E. cloacae*, *E. coli, K. oxytoca, K. pneumonia, P. mirabilis, P. vulgaris, P. aeruginosa* and *M. smegmatis*. From the results it was observed that the Gram-negative bacteria strains *E. cloaca, E. faecalis, E. coli, K. oxytoca* and *E. aerogenes* had not been inhibited

by the crude extracts obtained through pressurized hot water extracts using the disc diffusion method, but inhibition could be observed by the microdilution method, this could be because the diffusion of the polar compounds onto the culture was more efficient, whereas, a medium containing compounds that are less polar diffused slower, hence the contradiction on the activities using the two methods (Jiang, 2011). *S. epidermidis* and *P. aeruginosa* were inhibited at 8 mg/mL using the microdilution method whereas the disc diffusion contradicted somewhat with inhibition concentrations slightly above than 8 mg/mL.

Table 5.1: Antibacterial evaluation of C. macwoanii bulbs extracted by PWE.

Bacteria species	**Disc diffusion Zone of inhibition (mm)**		**Minimum inhibitory concentration (mg/mL)**	
	PHW crude extract	**Control Streptomycin**	**PHW crude**	**Control Streptomycin**
Bacillus cereus	2.45	26.67	>16.00	0.125
Bacillus subtilis	5.67	0.00	**4.00**	0.125
Enterococcus faecalis	0.00	22.33	>16.00	0.125
Mycobacterium smegmatis	0.00	23.33	>16.00	0.125
Staphylococcus aureus	6.12	24.67	**8.00**	0.032
Staphylococcus epidermidis	0.00	22.33	**8.00**	0.50
Enterobacter aerogenes	0.00	27.00	>16.00	8.00
Enterobacter cloacae	0.00	11.6	>16.00	0.50
Escherichia coli	0.00	24 .00	>16.00	8.00
Klebsiella oxytoca	0.00	26.00	>16.00	4.00
Klebsiella pneumoniae	0.00	26.67	>16.00	0.50
Proteus mirabilis	0.00	24.00	>16.00	0.50
Proteus vulgaris	0	26.67	>32	0.125
Pseudomonas aeruginosa	4.45	30.67	**8.00**	8.00

Note 0mm=no inhibition Bold= low concentrations

C. macowanii PHWE showed inhibitory activities against both Gram-positive and Gram–negative bacteria, with more Gram-positive bacterial species showing susceptibility to the antibacterial compounds in the bulbs. As mentioned before, most Gram-negative bacteria are known to have multidrug-resistant pumps which force drugs out of the outer membrane hence poor inhibition of most species of Gram positive bacteria Iannello *et al.,* (2014). The pressurized hot water extract method is known to be selective in the extraction of compounds, especially polar compounds. Although water is used as an extraction solvent by traditional healers, its chemical and physical properties are different from other solvents like methanol and ethanol and can largely change when varying the temperature and pressure (Plaza and Turner 2015; Teo *et al.,* 2010). Also, factors such as the age of the plant, the amounts of bioactive compounds extracted affect the yield and effectiveness of bioactive compounds (Azmir *et al.*,2013).

The traditional preparation of medicinal plants is either done by mixing the plant parts with hot or cold water which is used to treat different ailments (Ndhlala *et al.*,2011; Ndhlala *et al.*,2011). However, from Table 5.1 the PHWE extract for both antibacterial methods failed to inhibit the majority of the bacteria which are considered to be pathogenic. Even though PHWE extract was able to inhibit some of the bacteria tested, it is not an ideal solvent for the extraction of bioactive compounds from *C. macowanii* bulbs. This is because the solubility of bioactive compounds differs in extracts and hence there was few antibacterial activities observed. Mensah *et al*., (2013) reported that water is not a good solvent for the extraction of solute which has inhibitory activity as it can be seen in Table 4.2 and Table 5.1, were *S. epidermidis* had different antibacterial activity of 0.0625 mg/mL for solvent extraction and 8.00 mg/mL for PWE. Alcoholic solvents extract a great number of natural products as compared to water even in different temperatures since active ingredients are more soluble in alcohol solvent than in water (Ogie-Odia *et al*., 2014). Ogie-Odia et al., (2014) reported that in most cases were the plant extracts are prepared with water, the antibacterial activities are low or most of the time not detectable, this explains the differences noted in Table 4.2 and Table 5.1.

5.3.2 Two Dimensional Gas Chromatography TOFMS (GC×GC-TOFMS) analysis of crude sample of *C. macwoanii* bulbs PHW extracts

From the GC×GC-TOFMS results obtained, compounds such as epibuphanisine, buphanisine and powelline (Table 5.2) have been found in *crinum* species and were in agreement with the reports

by (Elgorashi *et al.*, 2003; Nair *et al.*, 2000 ; Refaat *et al.*, 2012 ; Refaat *et al.* ,2013) and, where same compounds were obtained in *crinum* species on either the bulb, leaves or the whole plant

Table 5.2: Volatile Compounds isolated from *Crinum macowanii* bulbs crude PHWE extracts extract and identified by GC×GC-TOFMS

No.	Retention time (s)	Name of the compound	Area %	Biological/Pharmacological uses	Compound nature
1	357.1	Hexanoic acid	0.13686	Anti-Inflammatory activity (Aparna *et al.*, 2012)	Fatty acid
2	648.8	Indole	0.08078	Anti-cancer and anti-inflammatory (Kaushik *et al.*, 2013)	Aromatic heterocyclic organic
3	798.5	Apocynin	0.22573	Anti-inflammatory (Stefanska and Pawliczak, 2008)	Phenolic aldehyde
4	815.3	Dodecanoic acid	0.13285	Anti-bacterial (Dayrit ,2015)	Fatty acid
5	1373.3	Epibuphanisine	0.21424	Anti-inflammatory and antibacterial activity, and Acetylcholinesterase inhibitor (AChE) (Ndhlala and Finnie *et al.*,2011;Nair *et al.*,2013)	Alkaloid
6	1404.5	Crinan-3-ol,1,2-didehydro-(3á)	11.112	Anticancer (McNulty *et al.*, 2007)	Alkaloid
7	1461.2	Buphandrin	1.0384	Antibacterial (Maroyi, 2016)	Alkaloid
8	1558.6	3-Epimacronine	2.1817	Anti-acetylcholinesterase activity (Cortes *et al.*,2015)	Alkaloid
9	355.7	Pentanoic acid	0.078038	Antioxidant (Nichols,1997)	Carboxylic acid
10	596.1	2-Coumaranone	0.085023	Antimicrobial (Ververidis *et al.*, 2007)	Flavonoid
11	647.7	5H-1-Pyrindine	0.076275	Antiviral (Parikh *et al.*, 2011)	Alkaloid
12	1488.1	Powelline	6.3584	Antibacterial (Maroyi, 2016)	Alkaloid
13	1527.6	Pancracine,O_2-methyl-, (2á)-	0.6378	Antibacterial (Iannello, 2014)	Alkaloid

14	577.5	Thiophene,2,3-dihydro-	0.13589	Anti-inflammatory, antiviral, fungicidal and antibacterial activity (Rezanka *et al.,* 2006)	Heterocyclic compound

A number of known and unknown compounds were identified but only a few are repressed on the table above

Phospholipase A2 which induce inflammation has been reported to be inhibited by hexanoic acid (Aparna *et al.,* 2012). This will support the traditional use of the bulbs since the leaves are used as remedy for scrofula (Nair *et al.,*2000). Indole is an aromatic heterocyclic organic compound, it is reported to have diverse biological activities such as anticancer, sexual disorder and anti-inflammatory activities due to its heterocyclic nature (Kaushik *et al.,* 2013).

Apocynin has been reported to have entered clinical trials for anti-inflammatory effects and chronic obstructive pulmonary disease which justifies the traditional use of the plant for the treatment of tuberculosis (Nair *et al.,* 2000; Stefanska and Pawliczak, 2008). Furthermore, it has also been reported to act as a general anti-inflammatory agent (Kaushik *et al.,* 2013). Apart from the general commercial uses of dodecanoic acid (lauric acid), it has also been reported to have antimicrobial properties by (i) destroying the cell membrane of gram positive bacteria, (ii) interfering with cellular processes and (iii) stabilizing human cell membranes. Moreover, dodecanoic acid has been used as a remedy for skin problems such as boils, sores and acne (Chan *et al.,* 2010). Dayrit (2015) reported that lauric acid was the most active fatty acid against gram-positive bacteria such as *S. aureus*, *E. faecalis* and *C. perfringens*. Epibuphanisine has been reported to have biological applications such as anti-inflammatory, antibacterial and AChE (Nair *et al.,* 2013). AChE has been tested as a treatment for Alzheimer's disease (Nair and van Staden, 2013).

Buphandrin was reported by Maroyi (2016) to be active against *B. subtilis* and *S. aureus* (mouth infections) and two Gram-negative counterparts (*E. coli* which is known to cause gastrointestinal tract infections and *K. pneumoniae* associated with wound infections) stated in Table 4.2. The antibacterial complement of the pressurized hot water extracts may be due to a number of these individual compounds acting either alone or in synergy. This assertion is a subject for future work where these individual components could be isolated and their synergistic effects evaluated. The fact that the pressurized hot water extracts contain these bioactive substituents strengthens and validates the traditional use of *C. macowanii*, and this is supported by Elgorashi *et al.*, (2003) that the plant is successfully used by traditional healers to treat stomach diseases and remedy skin problems such as boils, sores and acne. Moreover powelline (an alkaloid) has been reported to be active against *B. subtilis*, *E. coli*, *K. pneumoniae* and *S. aureus* (Maroyi, 2016).

5.4 Conclusions

This study demonstrates that PHW crude extracts of *C. macowanii* bulbs have good antibacterial activity against *S. epidermidis*, *S. aureus*, *B. subtilis* and *P. aeruginosa.* The plant's bulb showed encouraging antibacterial activity against most test bacterial species with a potential or known to cause ailments and this could explain why the bulb is the preferred choice of plant part used in traditional medicine to treat a variety of common bacterial infectious agents. The PHWE inhibited fewer bacterial species as compared to the solvent extracts in Table 4.3, despite water being easily available it had less antibacterial activity as compared to the solvent extracts. Due to its easy access, water can still be used for herbal preparations. This provides a scientific justification for the traditional medicinal uses of the plant bulb for the treatment of skin, stomach and urinary tract infections. The results obtained indicated a number of phytochemicals present, which are associated with treating a number of ailments (bacterial, cancer and inflammation) caused by a number of problematic bacterial species. The GC×GC-TOFMS indicated the presence of compounds that have been reported to be used in most biological applications and this could help in drug development or drug recovery. Therefore, *C. macowanii* crude bulb extract possess a potential for clinical applications and further pharmacological and toxicity evaluation should be attempted.

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CHAPTER 6

INDUCTIVELY COUPLED PLASMA OPTICAL EMISSION SPECTROMETRY METAL ANALYSIS OF *CRINUM MACOWANII* BULBS

Abstract

In this chapter, the metal composition of *Crinum macowani* bulbs was detected. The metals present in plants could be from the soil or water. A screening method was carried out to assess the levels of metals in *C. macowani* bulbs, which were obtained from a Muthi market. The metals analysis performed were analysed by ICP-OES and metals such as calcium, cadmium, chromium, copper, iron, mercury, potassium, manganese, sodium, nickel, lead, strontium and zinc were detected. All metals with permissible limits were below those limits except for mercury, cadmium and chromium with values of 0.5443 ppm, 0.1261 ppm and 5.607 ppm respectively and the permissible limit is 0.20 mg/kg, 0.02 mg/kg and 0.60 mg/kg respectively. Metals such as iron, zinc and chromium are known to have pharmacological effects such as resistance to infections, wound healing and prevention of atherosclerosis. This supports the traditional uses of the plant for the treatment of heart disease and a remedy for skin problems such as boils, sores and acne. The lack of permissible limits available makes it difficult to determine the toxicity of the bulbs. Therefore, toxicity evaluation tests have to be done on the plant.

Keywords: ICP-OES, metals, *Crinum macowani*, permissible limits and toxicity.

6.1 Introduction

Contamination of the soil by heavy metals due to mining, steel and iron industry, smelting procedures, chemical industries, traffic, agriculture and domestic activities poses serious health and environmental problems. Due to their non-degradable ability, heavy metals persist in the environment for a long time and cause serious environmental pollution. Since much cannot be done about metal accumulation in the soil due to mines and human activities, the amount of metals in herbal/medicinal plants has to monitored and checked. This can be achieved by good manufacturing practices, such that proper sampling, storage and processing regulations are put into place for traditional healers to avoid metal contamination when preparing herbal medicines. This will help in improving the quality, safety, and efficacy of herbal drugs (Dzomba *et al.*, 2012). Even though efforts have been made by the Environmental Protection Agency of the United States (USEPA) to determine the total contents of heavy metals in soils by using concentrated nitric acid as an extractant, such did not seem to show good bioavailability to plants. In South Africa, a number of metal poisoning cases have been reported which were linked with the use of traditional medicines. The metals included arsenic (As), chromium (Cr) and magnesium (Mg). These cases led to poisoning, morbidity and mortality (Steenkamp *et al.*,2002). The South African government has a policy framework in place to promote chemical safety through the Strategic Approach to International Chemicals Management(SAICM), which is a united nations environment programme (UNEP) initiative (Steenkamp *et al.*,2002). This helps in the screening of traditional medicines for potentially harmful and toxic metal in order to protect consumers. Such an initiative recommends good agricultural and collection practices (GACP) and good manufacturing processes (GMP) as stated by WHO (2007), which will guarantee the quality and stability of herbal products. Therefore, there is a need to investigate the safety and authenticity of medicinal plant material sold in traditional medicinal markets in order to improve the quality assurance and safety (Okem *et al.*, 2014).

6.2 Method and material

The purpose of the present work was to perform a detailed validation of the analytical procedure and estimate the measurement uncertainty budget for determination of some essential (Fe, Mn, Zn, Cr, Cu, Al, Mg) and toxic (Pb, Cd, Ni, As) elements in the medicinal plants and their aqueous extracts. The method was followed according to (Şenila *et al.*, 2011)

6.2.1 Microwave digestion

Approximately 0.5 g of sample was digested with 10 mL of HNO_3 and 2 mL of H_2O_2 in microwave digestion system, according to the digestion program presented in Table 6.1.

Table 6.1 the Microwave digestion setting specifications.

Specification	Setting
Temperature °C	200
Power * 100 % power corresponds to 1400 W	1800.
Ramp Time /(min)	20 min.
Hold Time /(min)	10 min.
Pressure/ Pa	800.

The resulting solutions were cooled and diluted to 50 mL with distilled water. The resulted solutions were analyzed by ICP-OES.

6.2.2.ICP-OES determination

The operating conditions employed for ICP-OES determination were 1300 W RF power, 15 L min^{-1} plasma flow, 2.0 L min^{-1} auxiliary flow, 0.8 L min^{-1} nebulizer flow, 1.5 mL min^{-1} sample uptake rate. The axial view was used for metals determination, while 2-point background correction and 3 replicates were used to measure the analytical signal. The emission intensities were obtained for the most sensitive lines free of spectral interference. The calibration standards were prepared by diluting the stock multi-elemental standard solution (1000 mg L^{-1}) in 0.5% (v/v) nitric acid. The calibration curves for all the studied elements were in the range of 0.01 to 1.0 mg L^{-1}.

6.3 Results and discussions

Metals such as potassium 45.844 mg/kg, calcium 19.714 mg/kg, sodium 18.902 mg/kg (as seen in Table 6.2) have been detected at moderately high amounts in the bulbs and these are essential nutrients to the body. Gallium, rhodium and indium could not be detected from the bulbs. Rhodium and indium are toxic.

Table 6.2: Elemental composition in *Crinum macowanii* bulbs (mg/kg Dry Weight DW) detected by inductively coupled plasma optical emission spectrometry (ICP-OES). Results are presented as mean ± SE (n = 3).

Analyte	Symbol	Analytical wavelength (nm)	ICP-OES	Permissible limits (mg/kg)
Calcium	Ca	317.933	19.714 ± 1.465	N/A
Cadmium	Cd	226.502	0.126 ± 0.005	0.02 (Ogundele *et al.,* 2015)
Chromium	Cr	283.563	5.607 ± 0.114	1.30 (Ogundele *et al.,* 2015)
Copper	Cu	324.754	0.0483 ± 0.004	10.00 (Ogundele *et al.,* 2015)
Iron	Fe	239.562	5.662 ± 0.016	20.00 (Nazir *et al.,* 2015)
Gallium	Ga	141.444	ND	N/A
Mercury	Hg	253.652	0.544 ± 0.016	0.20
Potassium	K	766.491	45.844 ± 4.349	N/A
Manganese	Mn	257.611	0.059 ± 0.001	N/A
Sodium	Na	588.995	18.902 ± 0.943	N/A
Nickel	Ni	221.648	5.626 ± 0.004	10.00 (Ogundele *et al.,* 2015)
Lead	Pb	172.680	0.873 ±0.019	2.00 (Ogundele *et al.,* 2015)
Rhodium	Rh	343.489	ND	N/A
Strontium	Sr	421.552	3.83 ± 0.012	N/A
Zinc	Zn	206.200	0.037 ±0.007	0.60 (Ogundele *et al.,* 2015)
Indium	In	230.606	ND	N/A

Keywords: ND = Not detected, N/A= Not available, Bold = significantly high values compared to permissible limits

Potassium had the highest value 45.844 mg/kg. This chemical element is known to include relief from stroke, blood pressure, heart and kidney disorders (WHO, 2012). This justifies the traditional use of the bulb for the treatment of urinary tract problems (Nair *et al.,* 2000; Taylor *et al.,* 2003).

Iron was 5.662 mg/kg and it is vital for metabolic processes such as DNA synthesis and oxygen transport to cells and for treating chronic disorders like renal failure anemia (Okem *et al.,*2014). Ca present in *C. macowanii* might be therapeutic since its known to be responsible for metabolic processes such as cell division and the regulation of cell proliferation (Okem *et al.,*2014). It is also vital in regulating and strengthening bone mass. Cd and Pb are known to be toxic at low concentration and they were detected at 0.126 mg/kg and 0.8 mg/kg respectively and their World Health Origination WHO permissible limits are 0.3 and 10 mg/kg, respectively (Okem *et al.,* 2014). Hg was measured at 0.544 mg/kg and its safety limit is 2 µg/kg (Okem *et al.,*2014) therefore precautions has to be taken when consuming the plant for medicinal purposes.

Manganese is needed in small amounts in the body since it's a co-enzyme in antioxidant processes, however, extremely high levels above 13 mg/kg, which is the safety limit of this essential elements can be toxic. High explosion levels of Mn may cause irritation of the lungs which could lead to pneumonia (Steenkamp *et al.,* 2006).

Nickel is known to promote breast milk production. Exposure to high levels of Ni compare to that normally found in water and food has been reported to cause lung disease and affects stomach and kidneys in dogs and rats. A common reaction to contact with nickel is a skin rash. An exposure level of more than 0.1 mg/L in drinking water is considered toxic (Agency for Toxic Substances and Disease, 2005). Na is an essential nutrient that aids in heart performance, nervous system and glucose absorption. Health effects such as hypertension, cardiovascular disease and bone disease are caused by high consumption of sodium (Doyle, 2008). Cr which causes irritation of the skin, damage to kidneys, liver, circulatory and nerve tissues, respiratory problems and nose bleeds. Cr was detected at 5.607 mg/kg and the safety limit is 27 mg/kg in Cr (VI) (Steenkamp *et al.,* 2006; Okem *et al.,* 2014).

Previous studies have shown elevated levels of toxic heavy metals in some important South African medicinal plants obtained from muthi markets. For instance, (Steenkamp *et al.*,2006) reported high levels of manganese (Mn) and chromium (Cr) in some medicinal plant materials used in South African traditional medicine. In a similar study, (Ndhlala *et al.*, 2013) reported high levels of arsenic (As) and cadmium (Cd) above the WHO recommended levels in bulbs of some frequently used South African medicinal plants obtained from outdoor-street markets. *Hypoxis hemerocallidea* accumulated high levels of aluminium (Al) which indicated that it could be a hyperaccumulator of this element (Okem *et al.*,2014). On the contrary, (Mtunzi *et al.*,2012) reported levels below the recommended safety limits of metal concentrations in some medicinal plants used in South African traditional medicine (Okem, 2014).

6.4 Conclusions

In conclusion, the WHO reported that up to 80% of the world's population depends on some form of traditional medicine for their primary healthcare needs (Brusotti *et al.*,2014), wherein the use of herbal products as the first choice in self-treatment is the preferred and available choice. To ensure the safety of plants, as an herbal remedies, permissible limit has to be set for the different metals found in the plant. It has been concluded from this study that estimation of heavy metals is highly essential for raw drugs or plant parts used for the preparation of compound formulation drugs. The metals detected in *C. macowanii* bulbs are within the permissible limits except for mercury, cadmium and chromium. Further investigations have to be done on the safety and quality of the plant since its collected from the wild and used for medicinal applications.

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CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

7.1 General Conclusion and Recommendations

Introduction

This chapter gives a concise summary of the results and the main outcomes that have risen from this study. Recommendations for future work are also presented.

Conclusion

The set objectives of the study were explored and from the obtained results the following conclusions can be drawn:

- Solvent extraction gave the highest yield as compared to the pressurized hot water extract. This could be because solvents (dicholoromethane and methanol) are known to break plant cell walls thus releasing chemical compounds. This could suggest that the bulbs contain high levels of polar phytochemicals.
- Different compound groups such as alkaloids, saponins, tannins, flavonoids, steroids and cardiac glycosides were detected in the bulbs. The concentrations could not be determined as the tests done was quantitative tests and not qualitative tests. Where the phytochemicals are reported to have biological uses such as antimicrobial, antidiarrheal and anticancer. The bulbs showed a strong result in the test for, confirming that the plant is rich in this specific group of compounds. In the test for a lot of persistent froth also formed, but the test is not quantitative, so no conclusions could be made on the amount of saponins present.
- The chromatographic technique was the first to be done on *C. macowanii* bulbs. For both crude extracts (solvent extracts and Pressurized Hot Water Extracts). Different compounds such as fatty acid, sterol, volatile oil, alkaloid, cyclic ether, phenolic aldehyde, flavonoid which they are known to have biological applications such as anti-inflammatory, anti-diabetic, anti-inflammatory, anti-cancer, anti-bacterial and anti-microbial, such supports the traditional use for the plant for treatment of heart disease, treatment of kidney and bladder diseases, treatment of tuberculosis, compress for back pain and remedy for skin problems such as boils, sores and acne. From the results, more alkaloids compounds were detected and thus supports literature since the plant is known to be rich in alkaloids.

- The antimicrobial tests on *C. macowanii* crude bulbs extracts, demonstrated that the bulb exhibit good antibacterial activity against *B. cereus*, *E. faecalis*, *S. epidermidis*, *S. aureus* and *P. aeruginosa*. This provides an important basis for justifying the traditional medicinal uses of the plant against skin, stomach, respiratory and kidney and bladder infections. The PHWE had inhibited few bacterial strains as compared to the solvent extracts. Most negative bacterial species were not inhibited by both extracts and both test methods were used, and the highest activity is exhibited against gram positive strains. The crude extracts, mostly the solvent extract hypothetically possess a potential for clinical applications and further pharmacological and toxicity evaluation could be necessary to confirm this hypothesis.
- The metals analysis was able to detect metals such as calcium, cadmium, chromium, copper, iron, mercury, potassium, manganese, sodium, nickel, lead, strontium and zinc. All metals with permissible limits were below those limits except for mercury. Some of the metals such as iron, zinc and chromium are known to have pharmacological effects such as resistance to infections, wound healing and prevention of atherosclerosis, this supports the traditional uses of the plant for the treatment of heart disease and a remedy for skin problems such as boils, sores and acne. The lack of permissible limits available makes it difficult to determine the toxicity of the bulbs and therefore toxicity evaluation tests have to be done.

Recommendations

From the outcomes of the study the following recommendations for future work can be made:

- The crude extracts have shown good antibacterial activity and hypothetically possess a potential in the development and discovery of herbal drugs. Further research should be done on the toxicity and mode of action to confirm the efficacy and safety of the plant.
- Isolated *C. macowanii* phytochemicals should be further investigated through animal testing for pharmacological effects and clinical applications for assessing the in assessing the efficacy of *C. macowanii* compounds for the development and discovery of pharmaceutical products and drugs in preclinical and clinical trials.
- A detailed research assessing the toxicological and poisonous properties of *C. macowanii* plant parts and the isolated phytochemicals. Factors such as superficial irritation, safety

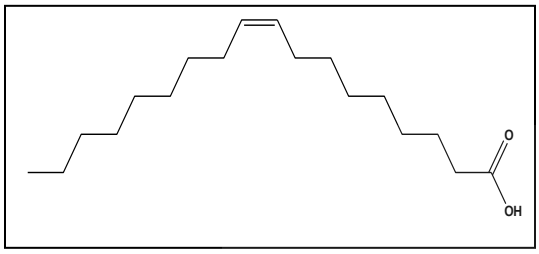
oral intake of decoctions and dermatotoxicity should be evaluated to investigate side effects and/or toxicity associated with intake of *C. macowanii* herbal products.

- A detailed research assessing the metal content present in *C. macowanii* plant parts and the soil where they are grown. This will assist in the toxicological and poisonous properties to ensure the safety of the plant as herbal preparation.
- Proper cultivation strategies should be put in place to ensure sustainable utilization of *C. macowanii*. Such could include micropropagation techniques and more planting and restricted harvesting from the wild.

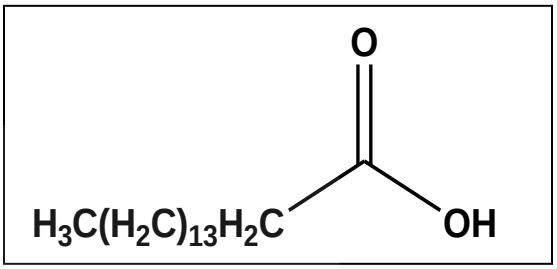
APPENDICES

1.Chemical structures of volatile compounds isolated from crude solvent *Crinum macowanii* bulbs extracts

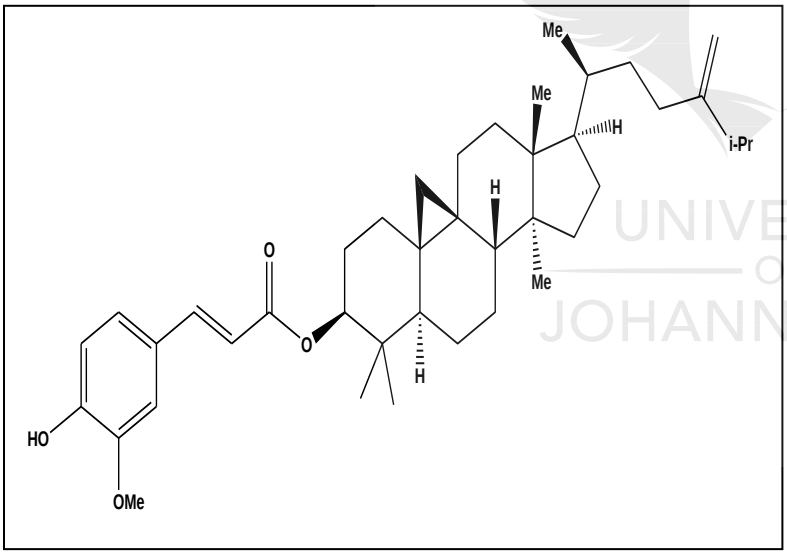
1. Oleic acid



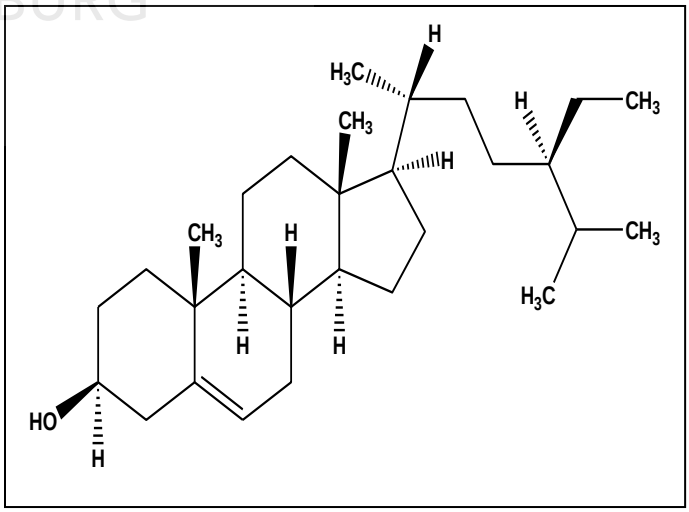
2. Hexadecanoic acid



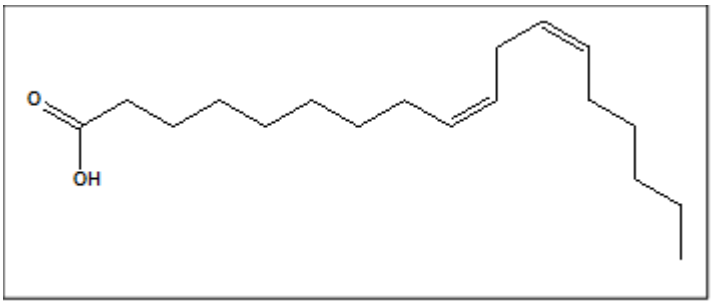
3. 9, 19-Cycloergost-24(28)-en-3-ol, 4, 14-dimethyl-, acetate, (3á, 4à, 5à)-



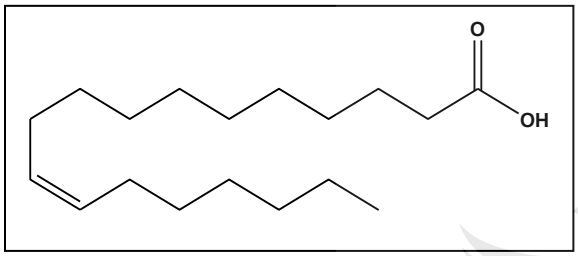
4. β-Sitosterol



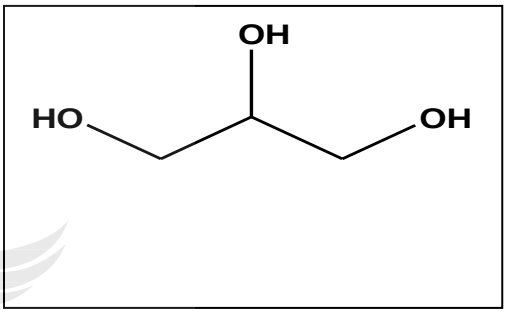
5. 9,12 Octadecadienoyl chloride, (*Z*,*Z*)-



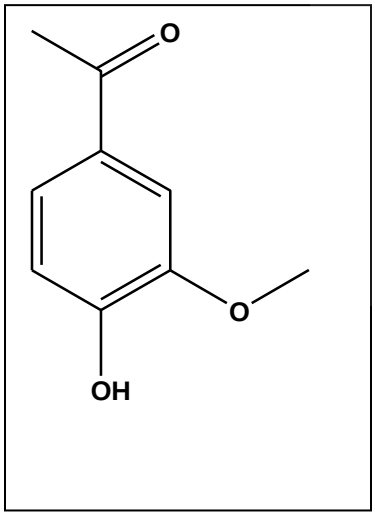
6. cis-Vaccenic acid



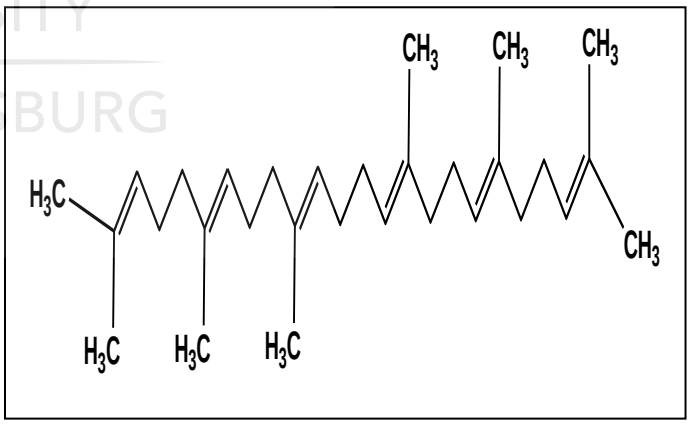
7.1,2,3 Propanetriol



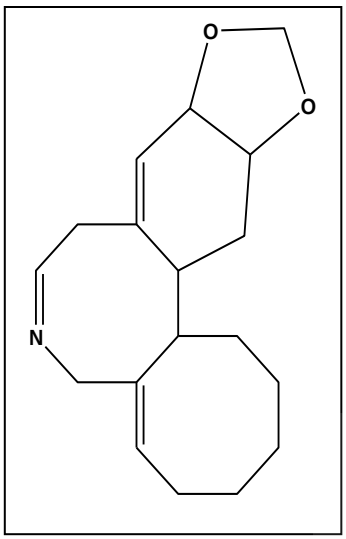
8. Ethanone, 1-(4-hydroxy-3-methoxyphenyl)-



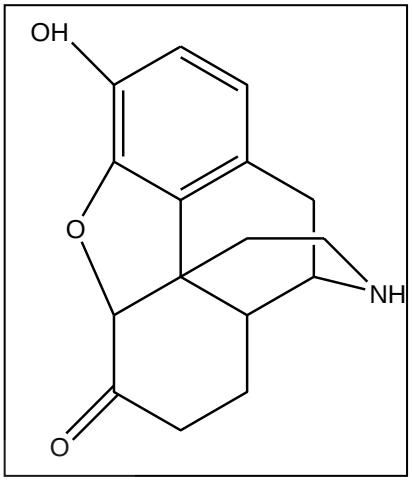
9. Squalene



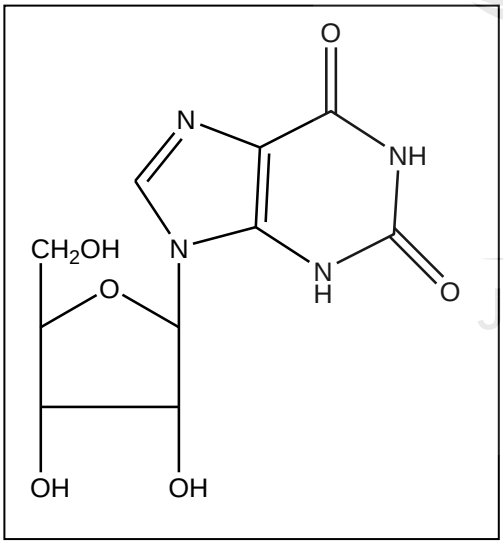
10. Trisphaeridine



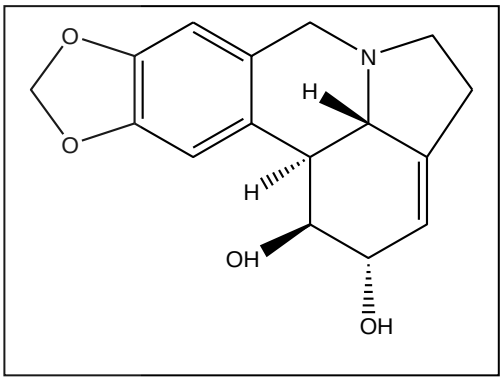
11. Dihydronormorphinone



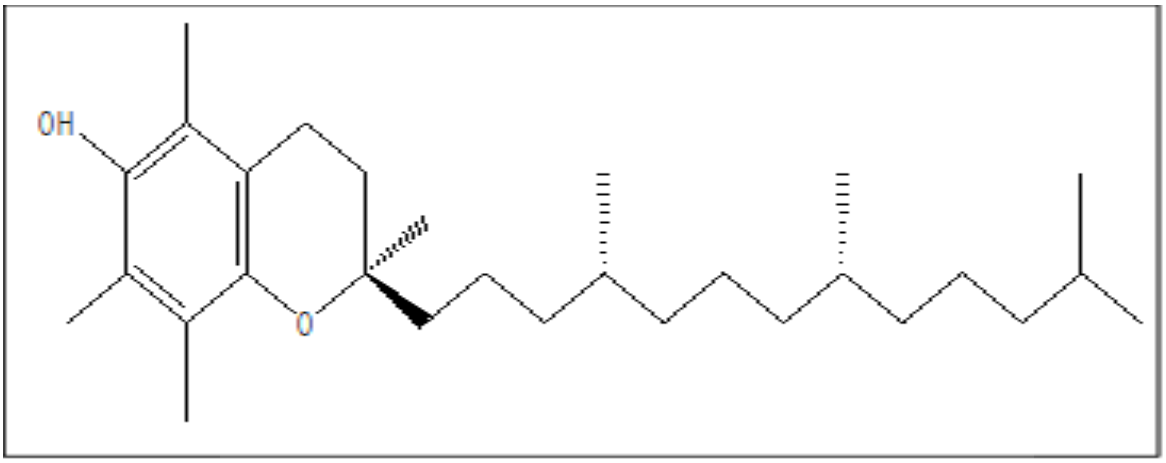
12. Xanthosine



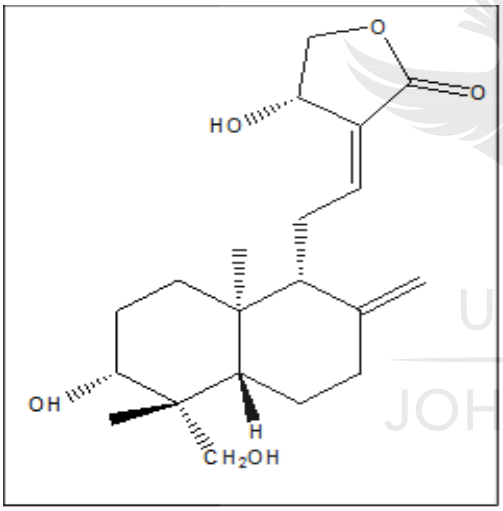
13. Lycorine



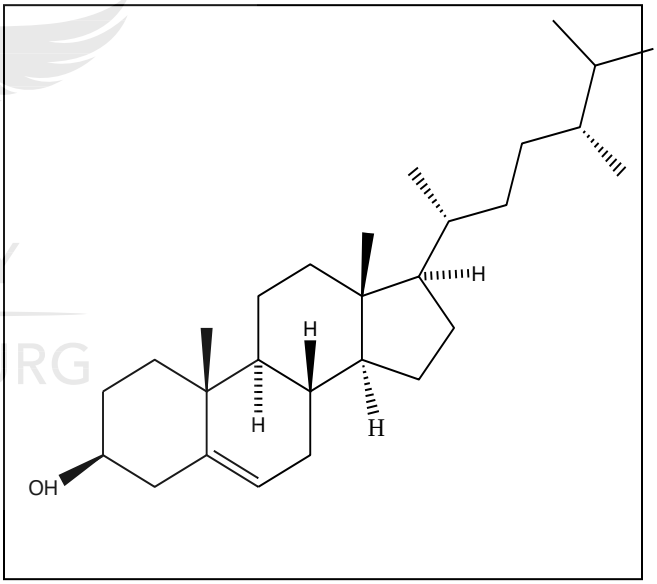
14. dl-à-Tocopherol



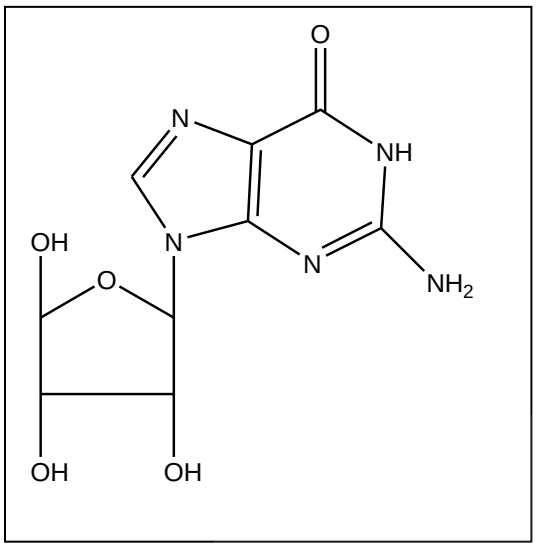
15. Andrographolide



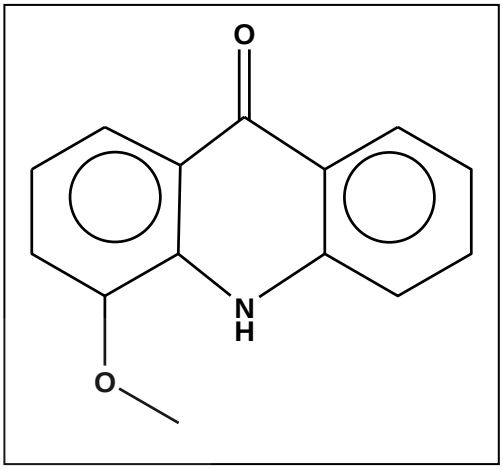
16. Campesterol



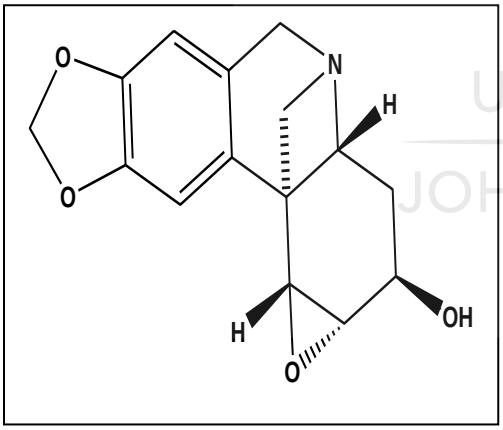
17. Guanosine



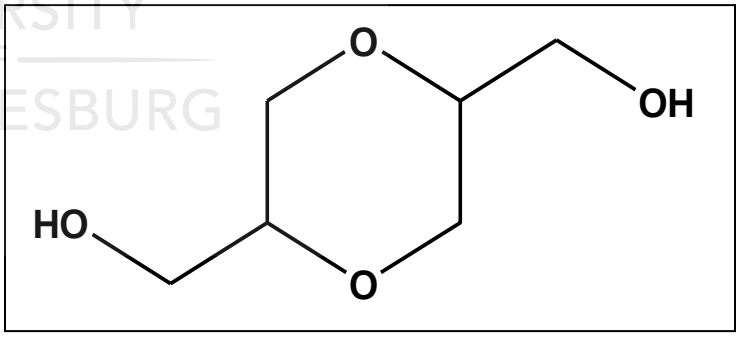
18. 9(10H)-Acridinone, 4-methoxy-



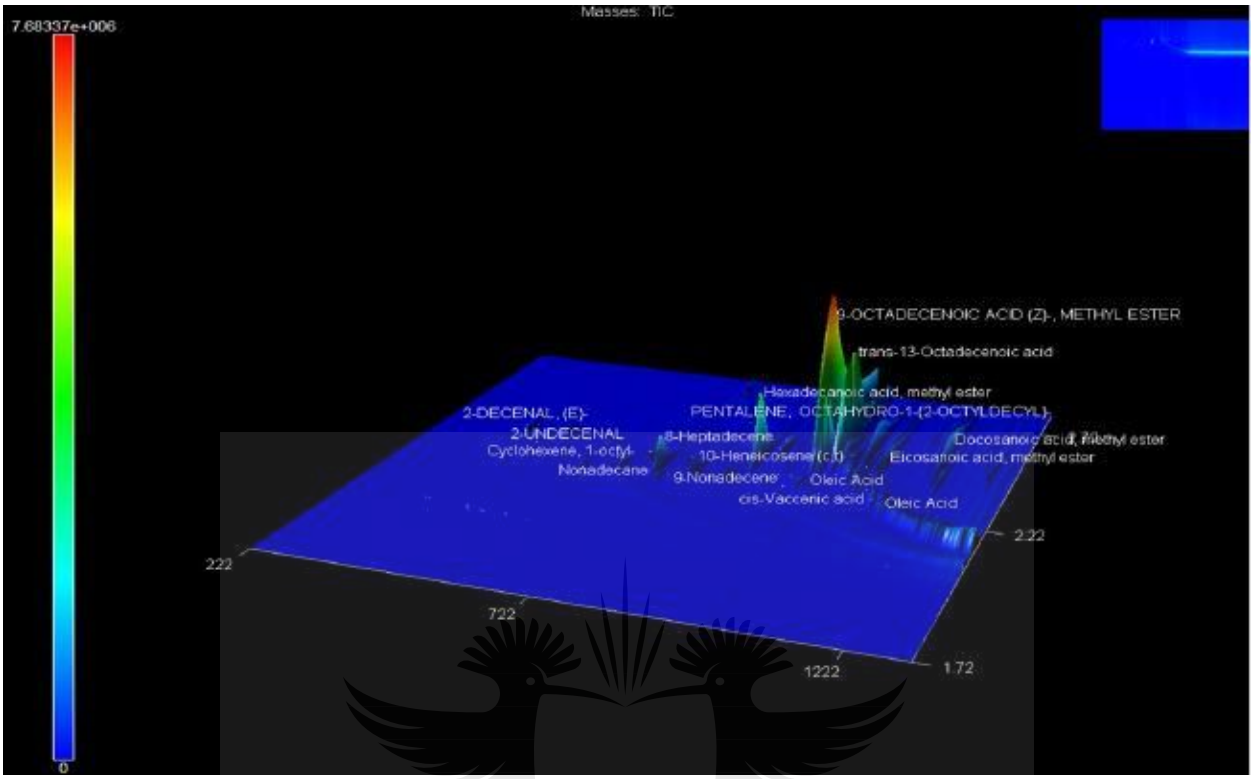
19. Flexinine



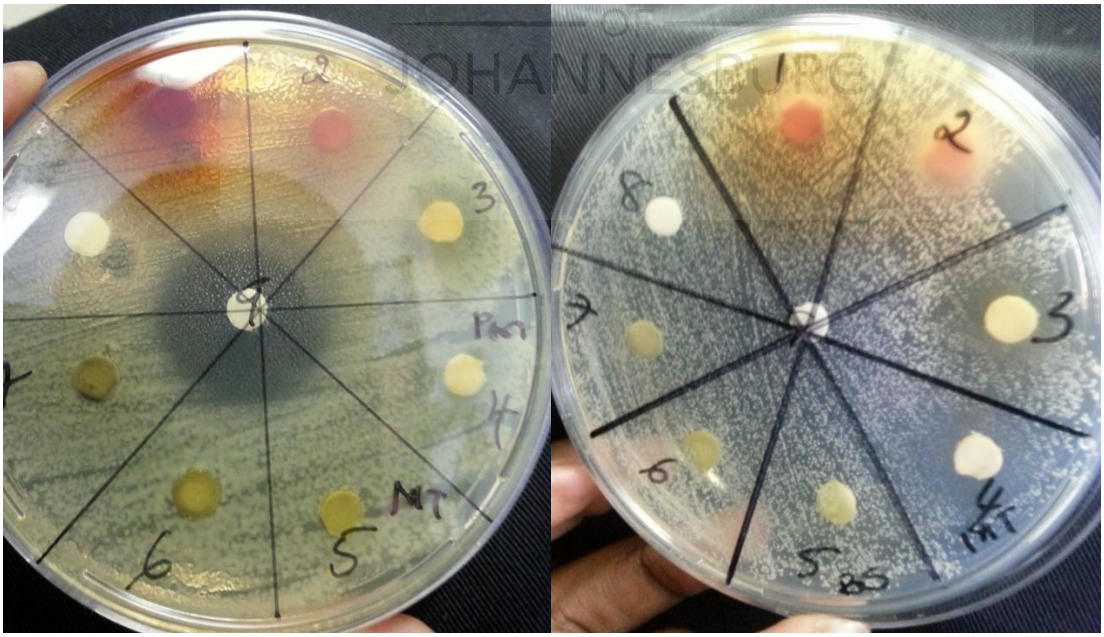
20. p-Dioxane-2, 5-dimethanol



2. Chromatogram of the PHW extracted identified by GC×GC-TOFMS



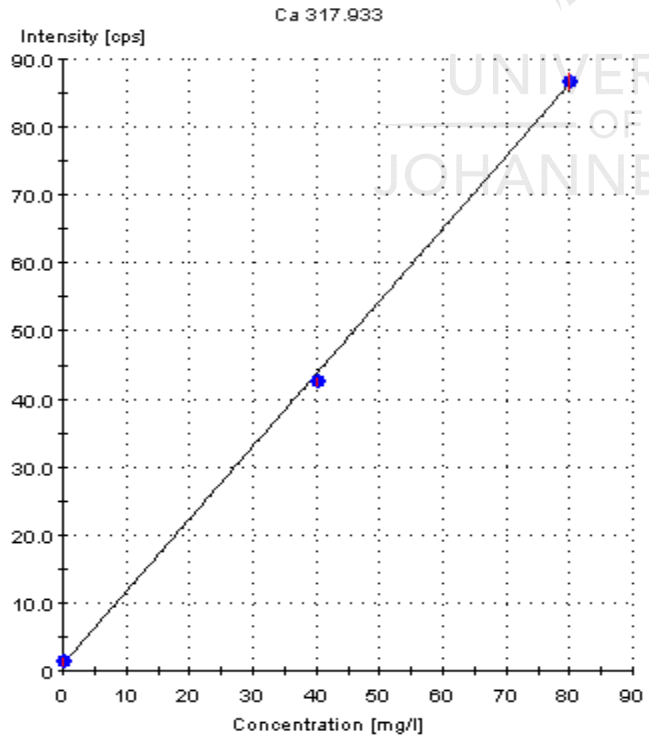
3. Agar plates used for the disc diffusion tests



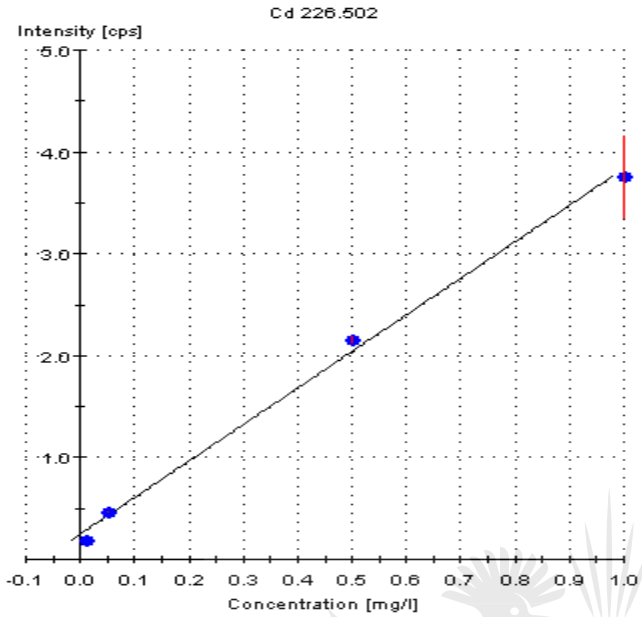
4. 96-well microdilution plate used for the MIC test

5. Regression line graphs showing the different wavelengths for the elemental analysis (Table 6.2)

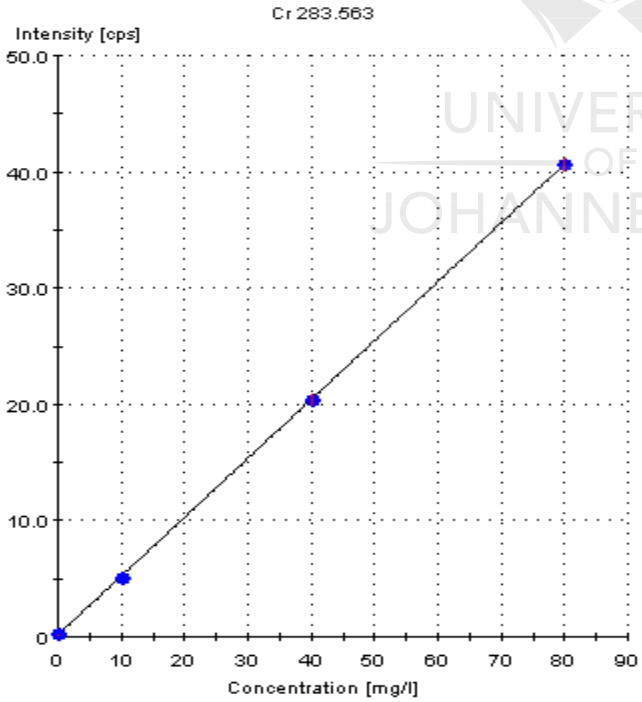
Calcium



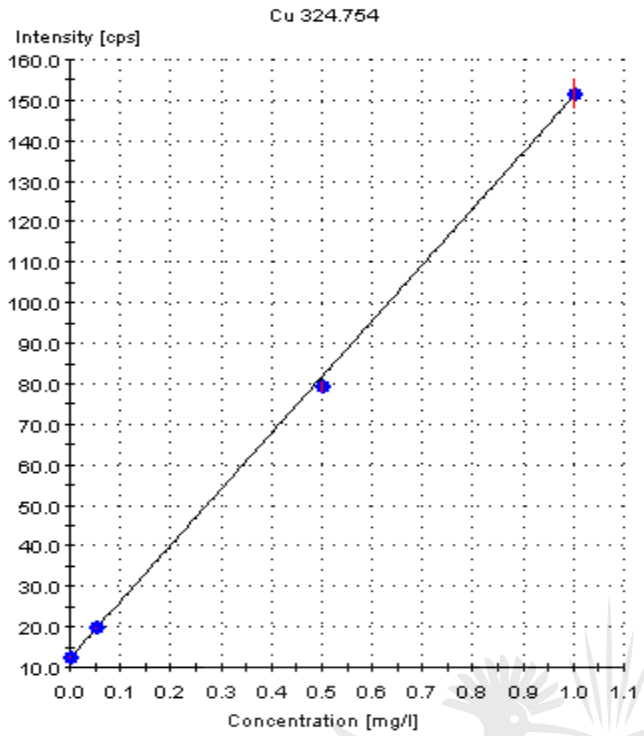
Cadmium



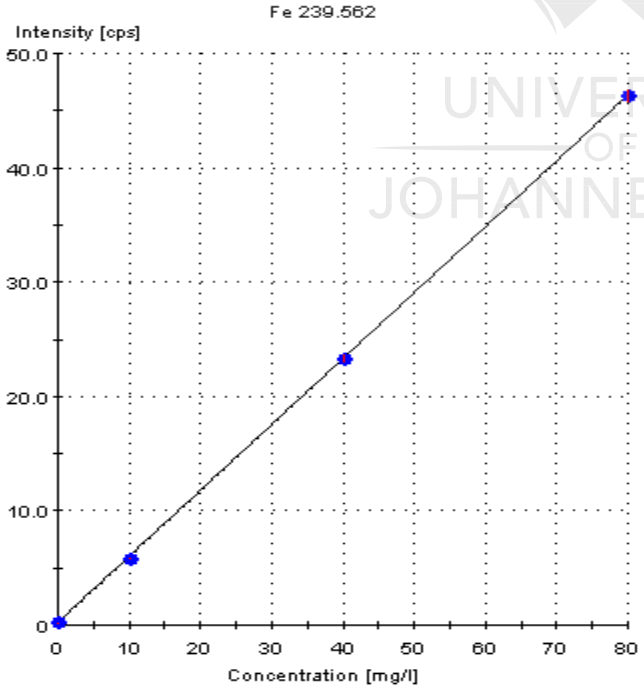
Chromium



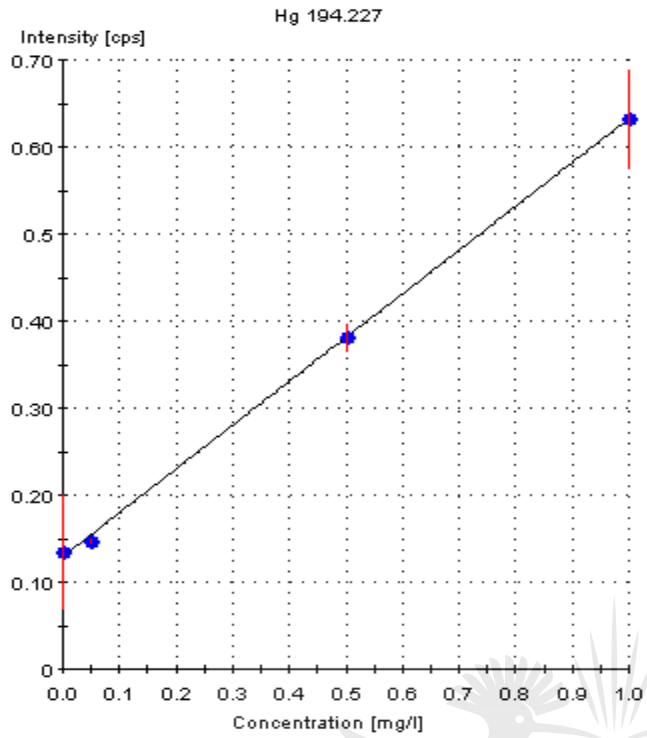
Copper



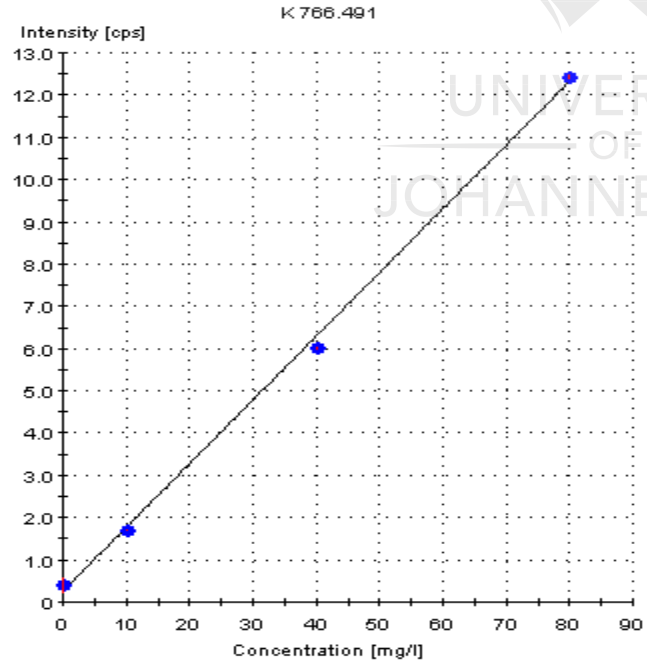
Iron



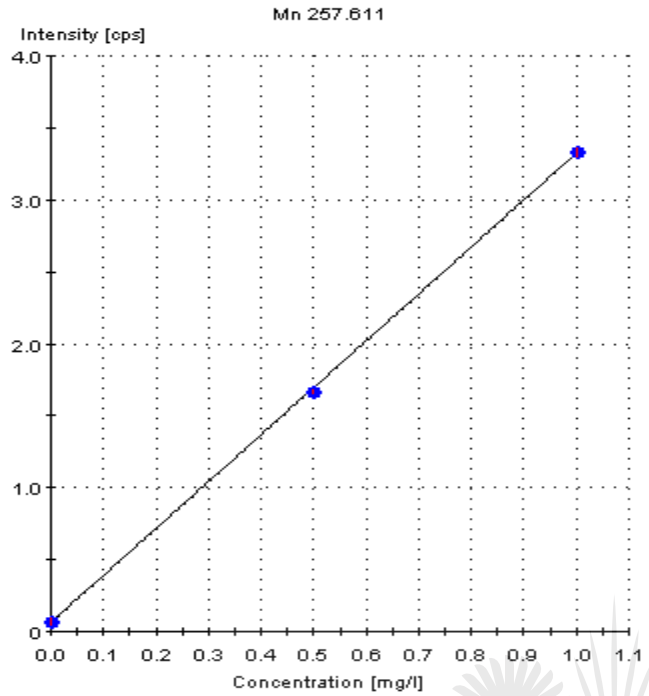
Mercury



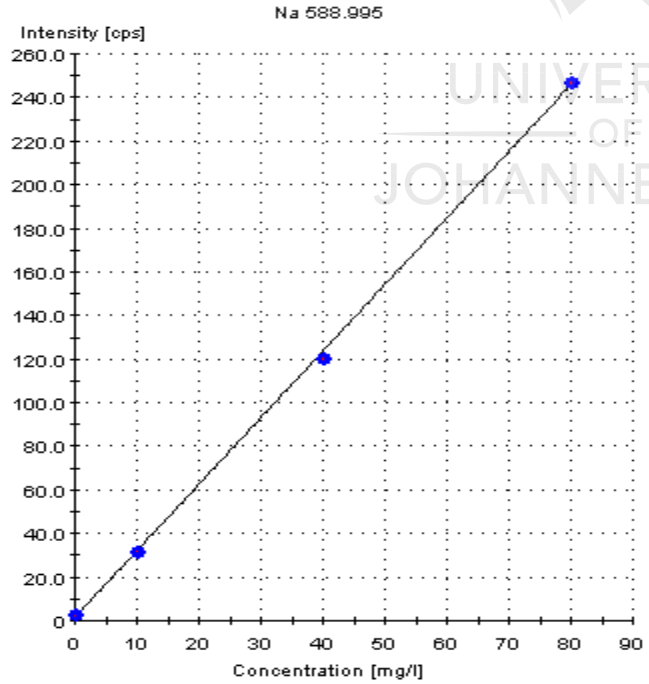
Potassium



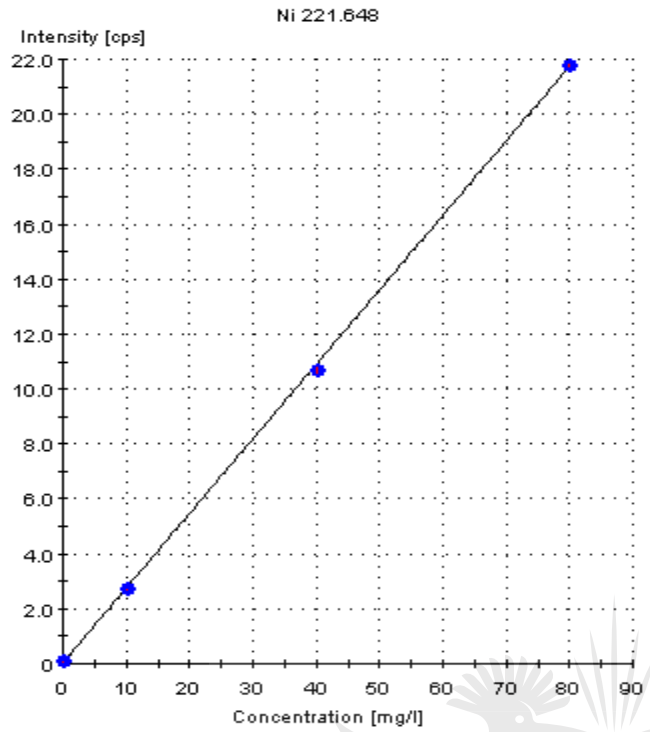
Manganese



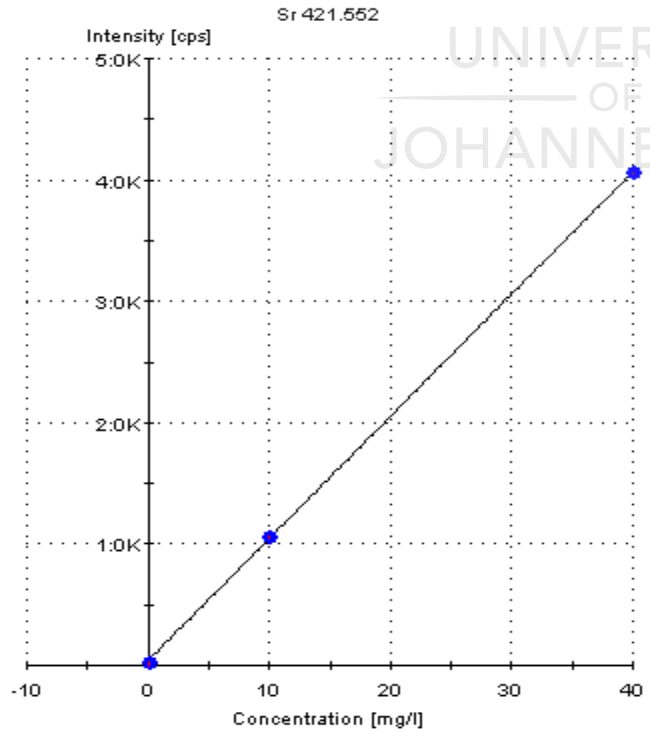
Sodium



Nickel



Strontium



Zinc