Antiviral, antibacterial and cytotoxic activities of South African plants containing cardiac glycosides

by

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Declaration

I hereby declare that this dissertation submitted for the degree Master of Science at the University of Pretoria, is my own work and effort and has not been submitted to any other University. Where other sources of information have been used, they have been acknowledged.

Signed:

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April 2013

Conference contribution from this research

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Abstract

South Africa has one of the richest and diverse floras in the world with over 30000 species of higher plants. There are approximately 3000 species of medicinal plants in South Africa. The discovery of active compounds in medicinal plants plays a strategic role in the phytochemical investigation of crude plant extracts. Secondary metabolites of medicinal plants are a major source of drugs for the treatment of various health disorders. Cardiac glycosides are one of the subgroups of steroids modified from terpenoids. The existence of cardiac glycosides in some plant species often indicates toxicity. Among the contagious elements, diseases caused by viruses are one of the major causes of death, disability, and social and economic disruption for millions of people. Viruses also cause many important plant diseases and are responsible for huge losses in crop production and quality in all parts of the world. According to literature, plants containing cardiac glycosides demonstrate potential for the discovery of more effective new drugs in the treatment of infection especially viral contagions. The main objectives of this study were to evaluate the antiviral (HSV-2 and PVY^{N}), antibacterial and cytotoxic activities of South African plants containing cardiac glycosides. Furthermore, isolation and identification of compounds with an emphasis on cardiac glycosides was carried out. Eight plants known to contain cardiac glycoside compounds were selected. Leaves of *Gomphocarpus fruticosus, Nerium oleander, Cotyledon orbiculata,* the fruits and leaves of *Strophanthus speciosus,* the bulbs of *Bowiea volubilis* and *Merwilla plumbea* were selected to examine their antimicrobial activities, toxicity, antiviral and to isolate potential pure compounds.

The ethanol extracts of all selected plants were screened for antibacterial activity against two Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and two Gram-negative (*Escherichia coli* and *Klebsiella pneumonia*) pathogens. Plant extracts showed promising antibacterial action against Gram-positive bacteria. The most active extract against both Gram-positive bacteria was *C. orbiculata* with the MIC value of 1.25 mg/ml.

Cell toxicity was monitored by determining the effect of the ethanolic crude extracts on human embryonic kidney cell line (HEK 293) using the XTT method. All extracts exhibited high toxic effects with IC_{50} < 100 µg/ml on the tested cell line. The XTT assay was used to determine of the antiviral activity of crude ethanolic extracts on the kidney epithelial cells of African Green Monkey (Vero). The results revealed that the crude ethanolic extracts of all

selected plants exhibited a cytotoxic effect on Vero cells at concentrations lower than their EC_{50}. Consequently, the determination of antiviral activity of the selected plant extracts was not successful.

Based on the chromatographic and bioassay results from the six plants selected for this study, the fruit extract of *S. speciosus*, belonging to the Apocynaceae family, was chosen for the isolation of compounds, particularly cardiac glycosides. All plant extracts were tested for antibacterial activity against *E. coli* (Gram-negative) and *E. faecalis* (Gram-positive) by using bioautography. The general TLC test showed more variety of compounds in the fruit extract of *S. speciosus*. The results of the bioassay showed promising activity of the fruit extract of *S. speciosus* and the bulb extract of *B. volubilis* against Gram-positive bacteria. The chromatographic investigation of the *S. speciosus* fruit extract led to the isolation of three pure compounds including a cardiac glycoside. The three compounds were identified based on NMR (1D and 2D) and HRMS. The isolated compounds were identified as: a triterpene (ursolic acid methyl ester), a sugar: *myo*-inositol methyl ester and an unidentified cardiac glycoside. According to literature, it is the first report of the isolation of ursolic acid methyl ester and *myo*-inositol-methyl ether from *S. speciosus* fruit extract.

The isolated cardiac glycoside exhibited no inhibitory activity at 1.25 mg/ml (the highest concentration tested) against all four tested bacteria (*S. aureus*, *E. faecalis*, *E. coli* and *K. pneumonia*). The cytotoxicity and anti-HSV-2 screening of the isolated cardenolide demonstrated the highly toxic effect of this compound on the HEK 293 cell line with 4.62 µg/ml IC_{50} value and < 25 µg/ml IC_{50} of Vero cell line. No evidence could be found in the literature of the cytotoxic activity of cardiac glycoside compounds on the HEK 293 cell line.

The ethanolic extracts of all plant extracts and isolated cardenolide were tested against the PVY^N *in vivo* and *in vitro*. The results revealed that the high concentration (50 mg/ml) of *M. plumbea*, *N. oleander*, *B. volubilis* (fresh bulb), *C. orbiculata* and isolated cardenolide reduced the PVY^N symptoms on tobacco plants in an *in vivo* experiment. In *in vitro* analysis, the high concentration (50 mg/ml) of *S. speciosus* (leaves & fruits), and especially *M. plumbea* (dry bulb) showed significant antiphytoviral activity. *In vivo* and *in vitro* results demonstrated that *M. plumbea* has potential antiphytoviral activity.

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List of Abbreviations

^{13}C NMR	Carbon nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
ACV	Acyclovir
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ARC	Agricultural Research Council
ATCC	American Type Culture Collection
ATL	Adult T cell leukaemia
ATP	Adenosine triphosphate
AZT	Azidothymidine
BC	Human breast cancer cell
BSE	Bovine spongiform encephalopathy
BYMV	Bean Yellow Mosaic Virus
Ca	Calcium
CC_{50}	50% cytotoxicity concentration
Cd	Cadmium
CEC	Crop Estimated Committee
CMV	Cucumber Mosaic Virus
CO_2	Carbon dioxide
CVB3	Coxsackie virus B3
DAS-ELISA	Double antibody sandwich-enzyme linked immunosorbent assay

DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ds-	Double-stranded
EBV	Epstein-Barr Virus
EC_{50}	50% effective concentration
ED_{50}	50% effective dose
ELISA	Enzyme-linked immuno sorbent assay
FAO	Food and Agriculture Organisation
FAOSTAT	Food and Agriculture Organisation Corporate Statistical Database
FXYD	Proteins act as channels or as modulators of ion channel
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HEK 293	Human embryonic kidney cells
HIV	Human Immunodeficiency Virus
HLTV-1	Human T Lymphotrophic Virus-1
HMBC	Heteronuclear Multiple Bond Correlation
HPV	Human Papilloma Virus
HRMS	High resolution mass spectrometry
HSK	Herpetic Stromal Keratitis

HSV	Herpes Simplex Virus
IC_{50}	50% inhibitory concentration
ICTV	International Committee on Taxonomy of Viruses
INT	Iodonitrotetrazolium chloride
IPP	Isopentenyl diphosphate
IQ	Intelligence Quotient
KB	Oral human epidermoid carcinoma cells
LD_{50}	50% lethal dose
LDH	Lactate dehydrogenase
MeOH	Methanol
MEP	Methyl-erythritol phosphate
Mg	Magnesium
MIC	Minimum inhibitory concentration
MMTV	Murine Mammary Tumour Virus
mRNA	messenger RNA
MTT	3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide
Na^+/K^+-ATPase	Sodium-potassium adenosine triphosphatase
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NICD	National Institute for Communicable Diseases
NMR	Nuclear magnetic resonance spectroscopy
O_2	Oxygen
OH	Hydroxyl groups

PC-3	Human prostate cancer cell line
pH	Potential hydrogen
PLRV	Potato Leaf Roll Virus
PMS	N-methyl dibenzopyrazine methyl sulphate
PTNRD	Potato Tuber Necrotic Ring Spot Disease
PVA	Potato Virus A
PVX	Potato Virus X
PVY	Potato Virus Y
PVY^{N}	Potato Virus Y Necrotic
RDDP	RNA-dependent-DNA polymerase
RNA	Ribonucleic acid
SARS	Severe Acute Respiratory Syndrome
SI	Selectivity Index
SINV	Sindbis Virus
ss-	Single-stranded
STD	Sexually transmitted disease
TK	Thymidine kinase
TLC	Thin layer chromatography
TMV	Tomato/Tobacco Mosaic Virus
TNV	Tobacco Necrosis Virus
TSWV	Tomato Spotted Wilt Virus
TYLCV	Tomato Yellow Leaf Curl Gemini-Virus

UNAIDS	United Nations Programme on HIV/AIDS
UV	Ultra violet
vCJD	Variant Creutzfeldt-Jakob disease
Vero	Kidney epithelial cells of the African Green Monkey
VK	Vervet monkey kidney cells
WHO	World Health Organization
XTT	Sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino carbonyl]-2H-tetrazolium inner salt
ZYMV	Zucchini Yellow Mosaic Virus

Chapter 1

General introduction

1.1 Background and motivation

Natural products (secondary metabolites) are present in all higher plants and produce a wide range of organic compounds. They have an essential role for survival and reproduction of plants but they do not participate directly in growth and development of the plants (Wink, 2003). According to the biosynthetic origin of plants, their natural products are divided into three major groups: terpenoids, alkaloids and phenolic compounds. Terpenes are the largest class of secondary metabolites (Zwenger & Basu, 2008). According to the numbers of the C5 units, terpenes are classified into six groups of which the triterpenes (C30) is one of these groups (Taiz & Zeiger, 2006). A steroidal structure assembles the core with an A/B and C/D *cis*-conformation of this compound which is called an aglycone. This steroidal core is double substituted with an unsaturated lactone ring at position 17 and a sugar portion at position 3. The type of lactone moiety distinguishes the subgroup of the cardiac glycosides (Prassas & Diamandis, 2008). According to the nature of the lactone ring, there are two major groups of cardiac glycosides namely, the bufadienolides and the cardenolides (Croteau *et al.*, 2000). Cardenolides have a five member unsaturated butyrolactone ring, whereas bufadienolides contain a six member unsaturated pyrone ring (Prassas & Diamandis, 2008). A wide variety of sugars are attached to natural cardiac glycosides. Interestingly the sugars themselves have no activity, but the addition of sugars to the aglycone portion affects the pharmacodynamic and pharmacokinetic profile of each different type of cardiac glycoside (Prassas & Diamandis, 2008).

According to Khan *et al*. (2005), for a long time, medicinal plants have been used for the treatment of many infectious diseases. Plants contain a large number of biologically active chemicals and some of these have been found to be extremely useful for treating various human and animal diseases (e.g. digitoxin and atropine). At present, there is an increasing emphasis on investigating and determining the scientific evidence for the use of plants and their isolated compounds as strong medication against viral pathogens (Khan *et al*., 2005). The presence of chemical substances such as cardiac glycosides and alkaloids in some species

can often be related to toxicity. In some cases, poisonous plants may contain active compounds with useful biological activities (McGaw & Eloff, 2005). There are recent interesting results from plants containing cardiac glycosides which exhibit therapeutic antiviral activity (Khan *et al*., 2005; Dodson *et al.*, 2007).

Cardiac glycosides are found in limited plant families and the animal kingdom (Knight & Walter, 2002). South African poisonous plants which contain cardiac glycosides are distributed in the following families: Apocynaceae, Hyacinthaceae, Crassulaceae, Iridaceae, Melianthaceae and Santalaceae (Van Wyk *et al.*, 2005).

There are several biological activities of cardiac glycosides which among them, the use as an antiarrhythmic agent for congestive heart failure treatment is the most well known function. The mechanism of action of this compound is decreasing the Na^+/K^+-ATPase and increasing the intracellular calcium concentration, in other words cardiac glycosides are able to bind to and inhibit Na^+/K^+-ATPase (Winnicka *et al.*, 2006). This ubiquitous enzyme is a membrane protein which uses energy derived from adenosine triphosphate (ATP) hydrolysis to drive the active transport of potassium ions into cells and sodium ions out of cells. Unexpected results from epidemiological studies demonstrated significantly lower mortality rates of patients with cancer receiving cardiac glycosides and led to investigate the anticancer properties of cardiac glycosides. There are *in vitro* and *in vivo* evidences to confirm the antiproliferative and apoptotic effects of the cardiac glycosides in several cancer cell lines, such as breast, prostate, melanoma, pancreatic, lung, leukaemia, neuroblastoma and renal adenocarcinoma (Prassas & Diamandis, 2008). Accordingly the first generation of cardiac glycoside based drugs are currently in clinical trials (Prassas & Diamandis, 2008). The vast therapeutic potential of cardiac glycosides was further demonstrated by Srivastava *et al.* (2004) who revealed the potential of cardiac glycosides in the treatment of cystic fibrosis lung inflammation. Furthermore, according to Su *et al.* (2008), digitoxin and its analogues demonstrate the anti-Herpes Simplex Virus-1 and -2 activities (HSV-1 and HSV-2) with a 213.2 selectivity index (SI). Brazilian scientists showed the anti-HSV-1 and -2 activity of a cardenolide compound via inhibition of viral protein synthesis and the blockage of virus release and the reduction of viral cell to cell spread (Bertol *et al.*, 2011).

With regards to the importance of the impact of viruses on the economic condition, public health and also human and plant populations, there is now an urgent need to investigate and elucidate the novel type of treatments that can improve viral treatment strategies and give more successful results.

Viruses are minute infectious agents that can only replicate inside living cells so they require host cell enzymes to aid replication, transcription and translation (Wagner *et al.*, 2008). Infectious diseases create major risks to the health and safety of global human and animal populations. Humans are directly at risk from infection and indirectly at risk through the impact on their living conditions such as food supply. The risks associated with food supply include economic losses and the unavailability of food due to real or suspected contamination. These risks have the potential to disrupt global supply chains and further damage human health. One recent example is Severe Acute Respiratory Syndrome (SARS), which eradicated nearly 800 lives and imposed a devastating $50 billion in global losses. So, the life sciences, food and agriculture, and health care industries face the greatest risk from the impact of diseases caused by viruses (Marsh *et al*., 2008).

According to Blower & Volberding (2002), along with the increasing ability to treat viral infections there is a concern about the confrontation of these pathogens to the recognized drugs. The ability of pathogenic agents to rapidly develop drug resistance is a complexity of the treatment of the diseases which they produce. Viruses have developed numerous resistance mechanisms that allow them to evade the antiviral treatments (Blower & Volberding, 2002). As a result, many viruses have become resistant to almost every available antiviral agent. This problem is becoming increasingly acute and developing new techniques and also new active medication into ways to combat this problem is essential. In some cases the Human Immunodeficiency Virus (HIV) is already resistant to the current treatments. Therefore, it makes the procedure of HIV treatment much more complicated. It may cause concern that this problem occurs for other viral infections like HSV-2 (the cause of genital herpes) and the Hepatitis B Virus (HBV) (Blower & Volberding, 2002). Furthermore, some types of viruses have the ability to produce secondary oncogenic diseases in humans such as; HSV, HIV and HBV that cause breast and liver cancers (Talbot & Crawford, 2009).

Viruses also cause many important plant diseases and are responsible for huge losses and reduction in crop production and quality in all parts of the world. Also, protection of plants against the virus pathogen is often costly. Potato Virus Y (PVY) is an important virus as it spreads easily and can decrease yield greatly when the incidence is high and the cultivar is sensitive. Economically, PVY is the most important virus in cultivated potatoes grown throughout the world. Potato Virus Y mostly infects plants in the family Solanaceae, including potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.), tobacco (*Nicotiana tabacum* L.) and certain Solanaceous weeds (Jones *et al.*, 2003). Based on the presented symptoms in tobacco plants, PVY were divided into two major pathology groups: the ordinary strain, PVY^{O} that induces mosaic or vein clearing symptoms in tobacco; and the necrotic strain, PVY^{N}, which induces systemic vein necrosis in tobacco (Hu *et al*., 2009). Potato is an important vegetable product all around the world and according to the Crop Estimated Committee (CEC) for 2011 in South Africa the total production of the potato crop was 2,072,000 tonnes with a value of R4,718,791. Unfortunately, there has been an increase in the PVY infection rate of the potato crops in South Africa (Crops and Markets, 2011).

Six plants from different families that are recognized to contain cardiac glycoside compounds based on information in literature were selected, namely: leaves of *Gomphocarpus fruticosus* (L.) W. T. Aiton. [Synonym: *Asclepias fruticosa* L.]; *Nerium oleander* L.; *Cotyledon orbiculata* L.; the fruits and leaves of *Strophanthus speciosus* (Ward. & Harv.) Reber; the bulbs of *Bowiea volubilis* Harv. and *Merwilla plumbea* (Lindl.) Speta (Van Wyk *et al.*, 2005). These selected plants were investigated to identify the possible antibacterial activity against the Gram-negative (*Escherichia coli* and *Klebsiella pneumonia*) and Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) bacteria. Furthermore since they contain cardiac glycosides, and could therefore be antiviral agents, they were investigated to determine their cytotoxicity and anti-HSV-2 activity. The selected plants were also evaluated to determine the best plant extract to prevent PVY^{N}.

1.2 Aim and objectives of the study

The discovery of natural based antibacterial and antiviral agents has increased globally in past decades. Many investigations confirmed the potential of plant extracts and their isolated substances to prevent the microbial agents. There is much concern about the effective antibacterial and antiviral agents, the drug resistance problem and also the health and

economic damages by viruses in the human and plant world. For these essential reasons the development of novel antibacterial and antiviral agents is being strongly demanded. Furthermore, cardiac glycosides have been shown to contain antiviral activity and could therefore be developed as antiviral agents.

Thus, the aim of this study was to investigate the antiviral, antibacterial and cytotoxic activities of selected South African plants containing cardiac glycosides. Therefore the extract with the least cytotoxicity and best bioactivity should be considered as a new approach to novel antibacterial and antiviral medication.

The specific objectives of this study were:

- To determine the antiphytoviral activity of selected plant extracts against PVY^N virus
- To determine the antibacterial activity of the plants extracts
- To investigate the cytotoxicity and anti-HSV-2 activity of the extracts using cell lines
- To isolate and identify potential cardiac glycosides from the selected plant extracts

1.3 Chapter layout

Chapter 2 An introduction to plant primary and secondary metabolites, terpenes, cardiac glycosides, distribution of cardiac glycosides, cardiac glycosides and South African poisonous plants is given. Furthermore, the chapter gives a brief overview of the chemistry and biochemical mechanism of cardiac glycosides, therapeutic value of cardiac glycosides, antibacterial and antiviral activity of cardiac glycosides and its toxicity, cytotoxicity and phytotoxicity. A concise review of human and plant viruses, taxonomy, structure and classification of viruses as well as a review of antiviral potential of medicinal plants is provided.

Chapter 3 The extraction procedure of the selected plant parts is described in this chapter. The antibacterial activity of the plant extracts is investigated against Gram-negative (*E. coli* and *K. pneumonia*) and Gram-positive (*S. aureus* and *E. faecalis*) bacteria.

Chapter 4 The cytotoxicity of selected plants against the HEK 293 (human embryonic kidney) and Vero cell line (kidney epithelial cells) is determined in this chapter. The antiviral activity of selected plant extracts against HSV-2 virus is evaluated by the XTT method.

Chapter 5 The isolation and identification of three compounds from *S. speciosus* fruit extract using various isolation and identification procedures such as column chromatography, thin layer chromatography, nuclear magnetic resonance (NMR) and high resolution mass spectrometry (HRMS). The antibacterial activity and cytotoxic activity of the isolated compounds are also evaluated.

Chapter 6 This chapter focuses on the *in vitro* and *in vivo* antiphytoviral evaluation of the selected plant extracts against PVY^{N}.

Chapter 7 The general discussion and conclusions of the study are presented to interpret and explain the results of the study, with future recommendations and considerations made.

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Chapter 2

Literature review

2.1 Introduction to primary and secondary metabolites in plants

Organic molecules are compounds that contain carbon and hydrogen. Carbon, because of its unique ability to form links with itself, plays a special role in the natural world. In organic molecules, there are particular groups of atoms in which characteristic chemical reactions take place. These are called functional groups (Hoffman, 2003). Their addition to a carbon frame results in the formation of a new compound with different functions and reactions. The chemical constituents in plants are often classified in two major groups: primary and secondary metabolites (Hoffman, 2003).

2.1.1 Primary plant metabolites

Sugars, amino acids, common fatty acids, nucleotides, and the polymers obtained from them [such as polysaccharides, proteins, lipids, Ribonucleic acid (RNA) and Deoxyribonucleic acid (DNA)], are all derived from similar metabolic pathways for the synthesis and use of essential chemicals in all organisms. These compounds, which are essential for the survival and security of the organism, are primary metabolites. These compounds are responsible for the primary life processes of respiration, photosynthesis, growth, development, and other essential functions (Croteau *et al.*, 2000).

Carbohydrates as a major group of primary metabolites are the first products of photosynthesis. The definition of carbohydrates has been modified to incorporate polyhydroxy aldehydes and ketones, alcohols and acids, and simple derivates of these compounds, as well as those formed by their condensation into oligosaccharides or polysaccharides. In biochemistry, sugars and carbohydrates are commonly termed *saccharides*. This name is derived from the Latin word *saccharum*, meaning “sugar”. These compounds are classified according to their size and solubility. Most carbohydrates in plants are bound as oligosaccharides or polysaccharides or attached to a range of different aglycones as glycosides (Hoffman, 2003).

2.1.1.1 Glycosides

There are a wide variety of glycosides in plants. These compounds play a critical role in many biological activities. The glycosides contain a sugar unit attached to a noncarbohydrate molecule that it is called an aglycone, i.e. sugar+aglycone= glycosides. The glycosides are more water soluble than the respective aglycones and a molecules hydrophilicity is increased by attaching with the glycosidic moiety. This effect influences pharmacokinetic properties of the respective compounds, such as circulation properties and concentration of it in the body fluids (Kren & Martinkova, 2001).

Glycosides are classified into three groups according to their chemical substances (Hoffman, 2003):

By the sugar unit. A glycoside based on glucose is called a glucoside. In nature, a 5-carbon pentose sugar or 6-carbon hexose sugar produce most types of glycosides.

The nature of the aglycone-sugar linkage. There are many different chemical bonds' possible by aglycone-sugar linkages. These bonds have different chemical and physiological effects. The most important linkages are O-glycosides, C-glycosides, S-glycosides and N-glycosides, that in each respectively, oxygen, carbon, sulphur and nitrogen atoms act as the link.

By the specific aglycone. This category is used for phytotherapy purposes. Phenolic glycosides, flavonoid glycosides, anthraquinone glycosides and steroid and saponin glycosides are the examples of these types of specific glycosides.

Glycosides are associated with many biological active compounds. According to the activities of the aglycone and the respective glycosides there are different types of glycosides:

- **Glycosidic antibiotics**: In this group the sugars have a very important role. The uncommon deoxy and amino sugars are a typical characteristic of a large group of glycosidic antibiotics (Kren & Martinkova, 2001; Kren, 2008).

- **Glycosides of vitamins**: These types of glycosides have an advantage over the respective aglycones in their superior water solubility, stability against ultra violet (UV) light, heat and oxidation, reduction of the bitter taste and aroma, and resistance

to an enzymatic action. There are both hydrophilic and lipophilic types of these vitamins in nature (Kren & Martinkova, 2001; Kren, 2008).

- **Alkaloid glycosides:** Most of these types of glycosides are prepared artificially for pharmacological reasons. There are only few examples of natural alkaloid glycosides and the biological activity of them has not been studied to a large extent (Kren & Martinkova, 2001; Kren, 2008).

- **Polyphenol glycosides:** Most polyphenols are produced by plants. Many of these compounds are used as important components of traditional medicines. Phenolic hydroxyl (OH) groups are targets of biological glycosylations. The minute alterations in the structure of polyphenolic glycosides, for example, positional changes of the OH group situations, can cause dramatic changes in their biological effects. Due to such minor changes sometimes the glycosylations of these compounds changes completely (Kren & Martinkova, 2001; Kren, 2008).

- **Terpenoids and steroid glycosides:** This group is one of the large groups of physiologically active compounds; the activity of which is largely dependent on the complete structure, including the glycosidic moiety. Saponins and cardiac glycosides are two important subgroups of triterpenoid natural products. The name saponin implies natural soap-like and detergent properties. These physico-chemical properties are responsible for their haemolytic toxic activity of some saponins, which is caused by damage to the erythrocyte membrane (Kren & Martinkova, 2001; Kren, 2008).

  According to the studies of Kren & Martinkova (2001), cardiac glycosides consist of a five-ring cardenolide aglycone, which is known as genin, which bears one or more attached sugar residues. The cardenolides and bufadienolides are two related groups of C_{23} and C_{24} steroids of triterpenoid origin. These types of compounds are toxic to many vertebrate herbivores, are cardio active and possess anticholesterolemic agents of pharmacological significance. Cardiac glycosides will be discussed in further detail in Section 2.2.

2.1.2 Plant secondary metabolites

During the centuries, humans used plants as an important source of food and shelter. Plants are a valuable resource of a wide variety of natural products, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, bio-pesticides and food additives. Over 80% of the approximately 30000 known natural products are of plant origin. In 1985, of the 3500 new chemical structures identified, 2600 originated from the higher plants (Rao & Ravishankar, 2002).

Vascular plants contain a large and diverse assortment of organic compounds. The large majority of these compounds don't have a function directly related to growth and development of plants. These substances are known as secondary metabolites, secondary products or natural products. Specific secondary metabolites are often found in only one plant species or related groups of species. In contrast, the primary metabolites, such as phytosteroles, acyl lipids, nucleotides, amino acids, and organic acids are found in the entire plant kingdom and their metabolic roles are essential for plants (Croteau *et al.*, 2000; Taiz & Zeiger, 2006).

In the past, secondary compounds were viewed as functionless end products of metabolism or metabolic wastes. Studies by organic chemists during the last decades have established the role of secondary metabolites in defence against herbivores, pests and pathogens. This defensive role can involve deterrence or anti-feedant activity, toxicity or the secondary metabolites may act as precursors to physical defence systems (Bennett & Wallsgrove, 1994).

According to Taiz & Zeiger (2006), general ecological functions of natural products are protection of plants against being eaten by herbivores and against being infected by microbial pathogens. On the other hand, these products are serving as attractants like smell, colour and taste for pollinators and seed dispersing animals. They also function as agents of plant-plant competition and plant-microbe symbioses. The difference between the molecules of primary and secondary metabolites is not easy recognizable; but can be identified on the basis of originator molecules, chemical structures or biosynthesis origins. For example, the diterpenes (C_{20}) and triterpenes (C_{30}) are derived from both primary and secondary metabolites; kaurenoic acid and proline are primary metabolites, while the closely related compounds abietic acid and pipecolic acid are recognized as secondary metabolites. The

similarity and difficulty in distinguishing between the primary and secondary metabolites is illustrated in the Figure 2.1 (Croteau *et al.*, 2000).

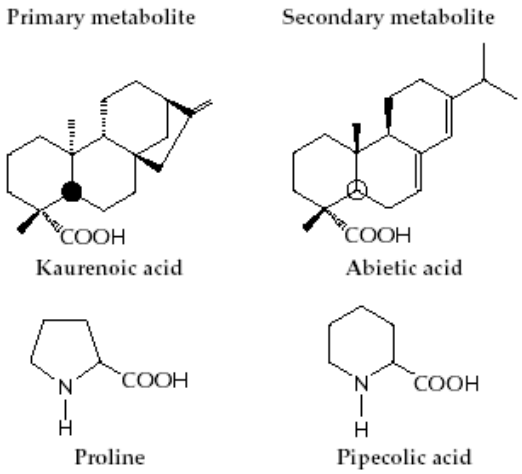


Figure 2.1: Comparing the similarity and difference between primary and secondary metabolites (Croteau *et al.*, 2000).

Plant secondary metabolites, based on their biosynthetic origin, can be derived into three main groups: the terpenoids, the alkaloids (nitrogen-containing compounds) and the phenolic compounds. Figure 2.2 shows the major pathways of secondary metabolites biosynthesis and their inter-relationships with primary metabolism (Croteau *et al.*, 2000; Taiz & Zeiger, 2006).

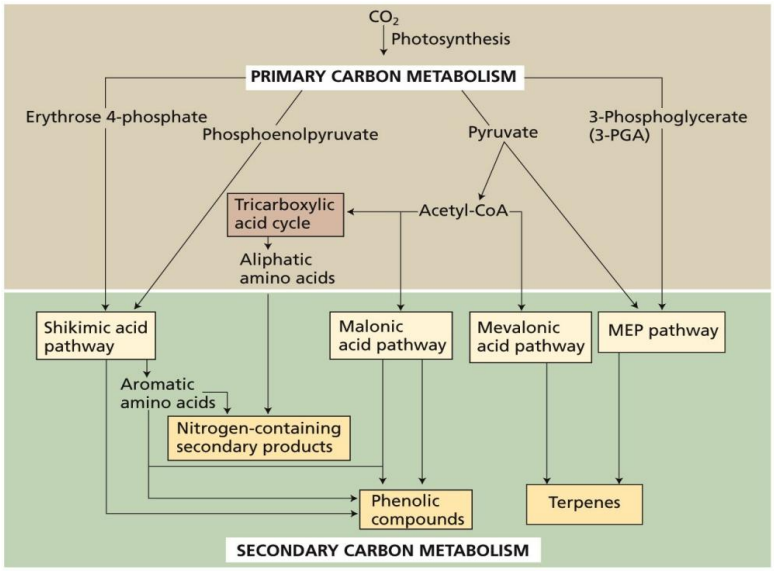


Figure 2.2: The major pathways of secondary metabolite biosynthesis (Taiz & Zeiger, 2006).

More than 12000 known alkaloids contain between one or several nitrogen atoms. They are biosynthesized primarily from amino acids. The shikimic acid pathway or malonate/acetate pathway leads to the constitution of more than 8000 phenolic compounds. Finally, all primary metabolites and along with over 25000 secondary compounds of terpenoids are derived from the five-carbon precursor isopentenyl diphosphate (IPP) (Croteau *et al*., 2000).

2.1.2.1 The Terpenes

The terpenes or terpenoids are the most structurally varied as well as being the largest class of secondary products (Figure 2.3). The name of these substances is derived from the first members of the class isolated from turpentine. Isoprene units are the basic structural elements of terpenes. Terpenes are derived by repetitive fusion of branched five-carbon isoprene units, in other words, terpenes are multiples of C_5 units linked together in the position of head to tail (Torssell, 1997; Croteau *et al*., 2000).

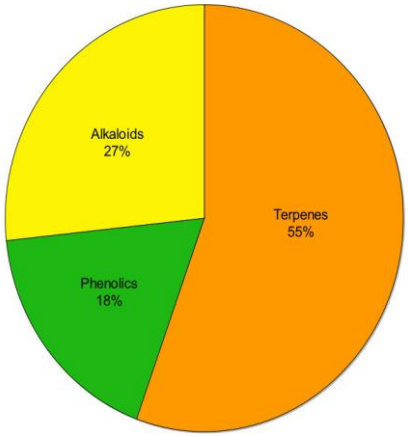


Figure 2.3: The major groups of plant secondary metabolites. Terpenes are the most numerous and structurally diverse plant natural products (Zwenger & Basu, 2008).

According to the number of C_5 units, the terpenes are classified into 6 groups: monoterpenes (C_{10}); sesquiterpenes (C_{15}); diterpenes (C_{20}); sesterterpenes (C_{25}); triterpenes (C_{30}); and tetraterpenes (C_{40}). The basic C_5 units are synthesized by two different pathways: mevalonic acid pathway and methyl-erythritol phosphate (MEP) non mevalonate pathway (Torssell, 1997; Taiz & Zeiger, 2006). The terpenes are biosynthesized from primary metabolites via these two pathways: acetyl-CoA or glycolytic intermediates. The fusion of three molecules of acetyl-CoA leads to the formation of the mevalonic acid pathway. The six-carbon

intermediate is then pyrophosphorylated, decarboxylated, and dehydrated to yield isopentenyl diphosphate (IPP). The IPP is the activated five-carbon building block of terpenes (Taiz & Zeiger, 2006).

The second system of the biosynthesis of IPP is the formation of intermediates in glycolysis or the photosynthetic carbon reduction cycle by a separate set of reactions which is called the MEP pathway. This pathway operates in the chloroplasts and other plastids in plants. Condensation of the glycerol aldehyde-3-phosphate and two carbon atoms of pyruvate form the five-carbon intermediate 1-deoxy-D-xylulose-5-phosphate. The rearrangement and reduction of this molecule leads to 2-C-methyl-D-erythritol 4-phosphate (MEP). The MEP is reformed to the IPP (Taiz & Zeiger, 2006).

2.1.2.2 Triterpene secondary modification and biosynthetic origin of cardiac glycosides

The common modifications of triterpenes as a resource of cardiac glycosides include additional hydroxyl and olefinic groups, oxidation of alcohol functions to carbonyl groups and side chain alkylation by S-adenosyl methionine (Torssell, 1997). The demethylation of C_4 and C_{14} are the fundamental secondary modification of terpenoids that leads to steroid formation. The steroids include a large number of extensive compounds which are divided into subgroups, according to side chain functionality: sterols, sapogenins, cardiac glycosides, bile acids, adrenal steroids and sex hormones (Torssell, 1997).

In cardiac glycosides, the side chain has been converted to an α,β-unsaturated γ-lactone. The formation of the cardiac glycoside, e.g. digitoxigenin from cholesterol, is via the cleavage of the side chain of cholesterol at the first C_{20} in a cytochorome P450 catalysed reaction (O_2, NADPH) to provide 4-methylpentanal and pregnenolone which then is oxidized to progesterone. The condensation of progesterone with an acetate unit finally leads to the digitoxigenin. The other alternative system in the butenolide ring of cardiac glycosides is the cleavage of the side chain at C_{23} to get norbile acids and then oxidation and lactonization (Torssell, 1997).

2.2 Cardiac glycosides

The first reports of using cardiac glycoside containing plants occur in ancient manuscripts dating back more than 1500 years ago (Newman *et al.*, 2008). Despite the toxicity of these specific compounds, they have been used in the past as arrow poisons, abortifacients, emetics, diuretics and as a treatment for leprosy, malaria, ringworm, indigestion, venereal disease, heart tonics and even as an agent in suicide (Langford & Boor, 1996; Newman *et al.*, 2008).

These types of compounds increase cardiac contractility and act as antiarrhythmic agents to control atrial fibrillation. The mechanism of action of cardiac glycosides for the treatment of congestive heart failure is via the inhibition of Na^+, K^+- ATPase and increasing the intracellular calcium concentration (Newman *et al.*, 2008). There are two different types of cardiac glycosides. The five membered lactone rings are known as cardenolides, while those with six membered lactone rings are known as bufadienolides.

2.2.1 Distribution of cardiac glycosides

Cardiac glycosides are found in limited plant families including the Apocynaceae, Asclepiadaceae, Brassicaceae, Celastraseae, Crassulaceae, Fabaceae, Hyacinthaceae, Iridaceae, Liliaceae, Melianthanceae, Moraceae, Ranunculaceae, Santalaceae, Scrophulariaceae, Sterculiaceae and Tiliaceae (Steyn & Van Heerden, 1998; Knight & Walter, 2002). Cardiac glycosides may be found throughout the plant, but except for a few rare cases, the concentrations are low (lower than 1%).

There are also cardiac glycosides in the animal kingdom: bufadienolides occur in *Bufo* (toads) (some frog-poisons contain bufadienolides- bufalin, marinobufagenin); *Photibus* (fireflies) that led to the isolation of certain compounds that render the fireflies distasteful to birds, and snakes belonging to the genus *Rhabdphis*, which are common in Japan and Southern East Asia. The cardenolides are also found in Lepidoptera (butterflies) (Bruneton, 1995; Steyn & Van Heerden, 1998; Winnicka *et al.*, 2006).

2.2.2 Cardiac glycosides and South African poisonous plants

The plant kingdom has proved to be most valuable in the treatment of ailments. South Africa has one of the richest and diverse floras in the world, containing about 3000 species of plants with medicinal properties (Van Wyk *et al*., 2009). According to Stafford *et al.* (2008), it is estimated that about 80% of the black population consult traditional healers. In South Africa, traditional healers believe in the therapeutic powers of plants based on the accumulated practical knowledge. Around 147 plant families are used traditionally for medicinal purposes by the Zulu, Xhosa and Sotho people of South Africa (Louw *et al*., 2002). The lists of South African poisonous plants which contain cardiac glycosides are presented in Table 2.1 & 2.2. The selection of the plants for this project was done according to literature and availability (Van Wyk *et al*., 2005).

Table 2.1 List of South African poisonous plants which contain cardiac glycosides (Van Wyk *et al*., 2005)

Plant name	Type of cardiac glycosides
Acokanthera oppsitifolia (Apocynaceae)	Cardenolide
Adenium multiflorum (Apocynaceae)	Cardenolide
Gomphocarpus fruticosus (Apocynaceae)	Cardenolide
Bowiea volubilis (Hyacinthaceae)	Bufadienolide
Cotyledon orbiculata (Crassulaceae)	Bufadienolide
Drimia robusta (Hyacinthaceae)	Bufadienolide
Homeria pallida (Iridaceae)	Bufadienolide
Kalanchoe rotundifolia (Crassulaceae)	Bufadienolide
Melianthus comosus (Melianthaceae)	Bufadienolide
Merwilla plumbea (Hyacinthaceae)	Bufadienolide
Moraea polystachya (Iridaceae)	Bufadienolide
Nerium oleander (Apocynaceae)	Cardenolide
Strophanthus speciosus (Apocynaceae)	Cardenolide
Thsium lineatum (Santalaceae)	Bufadienolide
Tylecodon wallichii (Crassulaceae)	Bufadienolide
Urginea sanguine (Hyacinthaceae)	Bufadienolide

Table 2.2 Exotic garden plants containing cardiac glycosides in South Africa (Van Wyk *et al*., 2005)

Plant name	Type of cardiac glycoside
Digitalis purpurea (Scrophulariaceae)	Cardenolide
Thevetia prituviana (Apocynaceae)	Cardenolide

According to Van Wyk *et al.* (2000), an analysis of the most important South African medicinal plants has revealed that approximately a third of the most commonly used indigenous plants are tree species. The other third can be classified as herbaceous plants or shrubs and the rest of the spectrum is divided between rhizomatous, succulent or leafy, and bulbous plants. Bulbous plant species belong to the monocotyledonous plants. The presence of steroids, cardiac glycosides and alkaloids in some species can often indicate toxicity.

The plants selected for this study include: the leaves of *Gomphocarpus fruticosus* (L.) W. T. Aiton. [Synonym: *Asclepias fruticosa* L.]; *Nerium oleander* L.; *Cotyledon orbiculata* L.; the fruits and leaves of *Strophanthus speciosus* (Ward. & Harv.) Reber; the bulbs of *Bowiea volubilis* Harv. and *Merwilla plumbea* Planch.

- ***Gomphocarpus fruticosus* (L.) W. T. Aiton. [Synonym: *Asclepias fruticosa* L.]**

  Gomphocarpus fruticosus belongs to the Asclepiadaceae family (Figure 2.4). It is indigenous to South Africa and its common names are milkweed, wild cotton (English); gansie, melkbos (Afrikaans); lebegane (Sotho); umsinga-lwesalukazi (Zulu) (Van Wyk *et al.*, 2005). This plant is a multi-stemmed small shrub containing latex with proteolytic activity. All the plant parts produce white latex when broken. It grows to a height of 2 m. The stems of this plant are long, thin and narrow with opposite leaves.

  The colours of the flowers are greenish-yellow followed by large, bladdery fruits with wiry hairs on the surface. *Gomphocarpus fruticosus* is toxic to cattle and sheep. The type of poison and major compounds of *G. fruticosus* are cardiac glycosides

(cardenolide), gomphoside and afroside. Powdered leaves and roots of this shrub have been used as a snuff to treat headaches and tuberculosis (Van Wyk *et al.*, 2005).

The leaves of *G. physocarpus* E. Mey, are used to strengthen the body, and the powdered leaves are used as a sedative (Stafford *et al.*, 2005). According to the study of Madureira *et al.* (2012) on the antibacterial activity of some African medicinal plants, which were used traditionally against infectious diseases, it showed that the growth of *Pseudomonas aeruginosa* was significantly inhibited (MIC value: 31 µg/mL) by the n-hexane and methanol extracts of *G. fruticosus* fruits. Another evaluation of anticancer and antimicrobial potential of sixty four Yemeni plants used as folk medicine illustrated that *G. fruticosus* leaf extract showed a significant growth inhibitory effect against three human cancer cell lines with IC_{50} values < 50 µg/ml. The antibacterial assay however showed that *G. fruticosus* leaf extract was not successful against all tested bacterial strains (Mothana *et al.*, 2009). The anticancer assay of *G. fruticosus* study supports the use of this plant, for the treatment of cancer diseases and is worthy of further investigation. The tested leaf extract of *G. fruticosus* extract was not very successful against the infectious diseases (McGaw *et al.*, 2000; Mothana *et al.*, 2009).

Figure 2.4: *Gomphocarpus fruticosus* leaves and flowers (Asclepiadaceae).

- ***Nerium oleander* L.**

Nerium oleander belongs to the Apocynaceae or Dogbane family (Figure 2.5) (Van Wyk *et al.*, 2005). The common names of *N. oleander* are oleander (English) and

selonsroos (Afrikaans). Oleander is a perennial, evergreen flowering shrub or small tree up to 6-10 m high. It has simple, narrow, sharply pointed, leathery leaves. The attractive white, pink or red flowers with five or more petals are produced in the spring and summer. Fruits pods contain many small, brown seeds, each with a bunch of reddish-brown hair (Knight & Walter, 2002; Van Wyk *et al.*, 2005).

All parts of the oleander plant are extremely poisonous to man, animals and certain insects. So it used sometimes as rat poison and as an insecticide. More than 30 different cardiac glycosides are found in the leaves and seeds of oleander. Oleandrin is the main compound. This plant occurs naturally in a large area, from the Mediterranean region to Western China (Van Wyk *et al.*, 2005).

The therapeutic usage of oleander despite the toxicity of this plant, have been as an abortifacient, diuretic and a treatment for dropsy (congestive heart failure), leprosy, malaria, ringworm, indigestion, venereal disease, and even as a suicide contrivance (Langford & Boor, 1996). Extracts are still used in homeopathy (Van Wyk *et al.*, 2005).

Figure 2.5: *Nerium oleander* shrub & leaves (Apocynaceae).

The leaves and the flowers of *N. oleander* are a cardio tonic, diaphoretic, and diuretic, emetic expectorant and sternutatory. A decoction of the leaves is applied externally in the treatment of scabies and to reduce swellings. Oil prepared from the root bark is used in the treatment of leprosy and diseases of a scaly nature

(Rajendran, 2011). The results obtained of the antimicrobial and anticancer activity of *N. oleander* are encouraging the significant anticancer and slightly antibacterial potential of oleandrin (Alkofahi *et al.*, 1990; Hussain & Gorsi, 2004).

- ***Strophanthus speciosus* (Ward & Harv.) Reber**

Strophanthus speciosus belongs to the Apocynaceae family. Its common names are common poison rope (English) and gewone giftou (Afrikaans) (Van Wyk *et al.*, 2005). This plant is a shrub or woody tree growing up to 10 m in height. It has bright, green, hairless leaves, which are usually arranged in groups of three at each node (Figure 2.6).

Figure 2.6: *Strophanthus speciosus* shrub, leaves & fruits (Apocynaceae).

The beautiful yellow and orange flowers with long slender petals appear at the end of the branches. There are complex mixtures of cardiac glycosides in this plant. The distribution of *S. speciosus* is mostly in the eastern parts of South Africa. The extracts of crushed seeds of *Strophanthus* spp. are best known as a source of arrow poisons and are dangerous to humans and animals. The seeds of *Strophanthus* spp. contain a glucoside called strophanthin, which is a cardio-active agent. In some species the ground seeds are used for cardiac insufficiency, while the roots or leaves of other types (e.g. *S. hispidus, S. kombe, S. gratus, S. welwischii, S. preussii*, etc.) are used as treatment of venereal diseases, intestinal parasites and serious skin diseases such as scabies (Cousins & Huffman, 2002; Van Wyk *et al.*, 2005). There is no motivating report on the antibacterial and antiviral potential of *S. speciosus.*

Because this species of plants are well known to have different types of cardiac glycoside compounds most of the medical investigation focuses on the potential of this plant to treat heart failure.

- ***Cotyledon orbiculata* L.**

Cotyledon orbiculata belongs to the Crassulaceae family. The common names of *Cotyledon* are pig`s ears (English), plakkie, kouterie (Afrikaans), imphewula (Xhosa) and seredile (Sotho and Tswana) (Van Wyk *et al.*, 2005; Amabeoku *et al.*, 2007). It is a succulent, small shrub with fleshy leaves. The colour of these leaves is bright green to grey, usually with a reddish margin and covered with a waxy layer on the surface (Figure 2.7). The types of cardiac glycosides that have been isolated are bufadienolides. The fleshy leaves are used to treat corns and warts. The juice of the leaves is used as drops for earache and toothache, and as a hot poultice for boils and inflammation (Amabeoku *et al.*, 2007).

Figure 2.7: *Cotyledon orbiculata* leaves (Crassulaceae).

The distribution of *Cotyledon* is throughout southern Africa (Van Wyk *et al.*, 2005). The results obtained by Amabeoku *et al.* (2007), suggest that *C. orbiculata* has anticonvulsant activity and provides pharmaceutical justification for the use of the plant extract by traditional medicine practitioners in the treatment of epilepsy. Some antibacterial activity of *C. orbiculata* has been reported (Aremu *et al.*, 2010).

- ***Bowiea volubilis* Harv.**

This bulbous plant is a member of the Hyacinthaceae family (Figure 2.8). It is known by these common names: climbing potato (English); knolklimop (Afrikaans);

igibisila (Zulu) and umagaqana (Xhosa). The bulb is globose up to 150 mm in diameter. The stems are inflorescence and succulent with leafless flowering that forms the main above-ground part of the plant.

Figure 2.8: *Bowiea volubilis* flowers and bulb (Hyacinthaceae).

The flowers are small and greenish. Even with the poisonous activity of *Bowiea*, it is used as a purgative, a remedy for dropsy, female fertility and as a heart stimulant (Hannweg *et al.*, 1996; Van Wyk *et al.*, 2005). Some investigations illustrated the promising antibacterial activity of *B. volubilis* (Stafford *et al.*, 2005; Buwa & Van Staden, 2006).

- ***Merwilla plumbea* (Linl.) Septa [Syn. *Merwilla natalensis* (Planchon) Septa (Syn. *Scilla natalensis* Planchon)]**

  This attractive bulbous plant belongs to the Hyacinthaceae family (Figure 2.9). Blouberglelie, Blousangkop (Afrikaans), Wild Squill, Blue Squill (English) and Inguduza (Zulu) are the common names of *M. plumbea.* This species produces papery bulbs which can be seen above or occurs below the ground. Its leaves are broad and have distinct veins. The beautiful blue flowers are arranged in small clusters situated on robust stalks in spring (Van Wyk *et al.*, 2005).

  This species is widely used as a medicinal plant: the Zulu people use them as purgatives and to facilitate labour. The Sotho eat the cooked bulbs as aperients, use bulb decoctions as an enema for internal tumours and cattle lung sickness, or rub

powdered bulbs over sprains, boils, sores, fractures, joints and back problems (Crouch *et al.*, 1999).

Figure 2.9: *Merwilla plumbea* (Hyacinthaceae).

The bulb of *M. plumbea* is used for treating internal tumours and applied topically on boils and wounds. It is known as a treatment for strains, sprains, fractures and cancers (Louw *et al.*, 2002). Aqueous extracts accelerated healing of eczema and abscesses in humans (Sparg *et al.*, 2002). A number of researches indicated the slightly antibacterial activity of *M. plumbea* (Sparg *et al.*, 2002; Stafford *et al.*, 2005). The type of cardiac glycosides of *M. plumbea* is bufadienolides. It grows in a wide area in the eastern parts of South Africa (Van Wyk *et al.*, 2005).

Natural products provide mankind with more environmentally friendly alternatives to commercially produced medicines. Despite the important traditional therapeutic values, the incorrect usage of the plant as medicine may cause injury or even death. Traditional uses of plants containing cardiac glycosides are considered to have therapeutic activity, which can lead to novel pharmaceutical developments.

2.2.3 Chemistry of cardiac glycosides

The cardenolides and bufadienolides are two related steroidal aglycone groups of C_{23} (cardenolides) and C_{24} (bufadienolides) of triterpenoid origin and a sugar moiety, most often an oligosaccharide (Figure 2.10). The efficacy of these substances was already known to the ancient Egyptians as they used Squill, a bufadienolide containing plant as the base of an expectorant, diuretic and remedy for cough. Bufadienolides are distinguished from

cardenolides by the lactone ring substituted at C_{17} in the steroid nucleus which is 6-membered, rather than 5-membered (Bruneton, 1995; Krenn & Kopp, 1998; Hoffman, 2003).

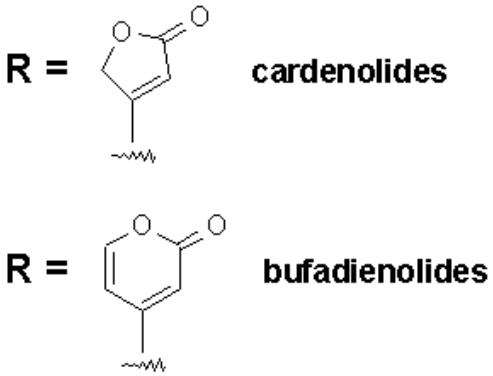


Figure 2.10: Two classes of cardiac glycosides have been observed in nature - the cardenolides and the bufadienolides (Desai, 2000).

- **Structure of the aglycones:** The classic, tetracyclic and steroidal nucleus are common in all of the aglycones. There are a *cis-trans-cis* and less often, a *trans-trans-cis* configuration at the A,B,C and D rings. The presence of two hydroxyl groups: one 3β secondary alcohol and the other one a 14β tertiary alcohol, are also common to the aglycones (Bruneton, 1995).

- **Structure of the sugar moiety:** There are a number of attached monosaccharides that often include deoxysugars. The most common sugars found in the cardiac glycosides are L-rhamnose, D-glucose, D-digitoxose, D-digitalose, D-digginose, D-sarmentose, L-vallarose, and D-fructose. These sugars mostly exist in the β-conformation in cardiac glycosides. The lipophilic character and the kinetic properties of the entire glycosides are due to the presence of the acetyl group on the sugar (Bruneton, 1995; Desai, 2000).

- **Structure of the glycosides and structure-activity relationship:** The sugar moiety is linked to the aglycone at the C_3 position, but in the Asclepiadaceae family, it occurs at both C_3 and C_2 with a 1,4-dioxane ring as a result. This sugar moiety is made up of a monosaccharide or often an oligosaccharide (Figure 2.11) (Bruneton, 1995).

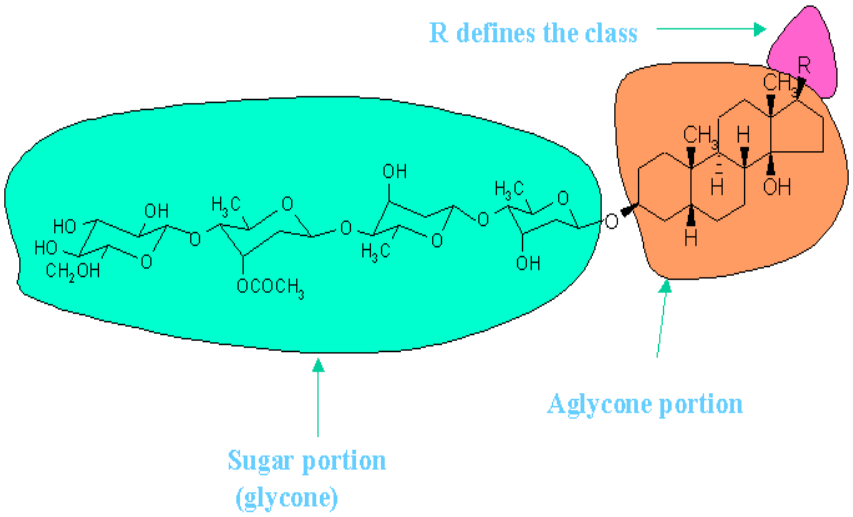


Figure 2.11: Cardiac glycosides are composed of two structural features: The sugar (glycoside) and the non-sugar (aglycon-steroid) moieties (Desai, 2000).

The relationships of the structure activity are understood. The aglycone is responsible for the cardiac activity. The sugar moiety doesn't contribute directly to activity, but its presence improves the activity and modulates it by modifying the polarity of the compound (Bruenton, 1995).

2.2.4 The biochemical mechanism of action of cardiac glycosides

In 1957, Skou described the sodium- potassium- ATPase (Na^+-K^+-ATPase) or Na^+ pump for the first time. This pump is an energy-transducing ion pump (Xie *et al*., 2003). The Na^+-K^+-ATPase enzyme consists of two types of subunits, catalytical α and regulatory β, in addition to a signal-transmembrane-spanning protein, FXYD. The FXYD protein family is a family of small membrane proteins that share a 35-amino acid signature domain. Four α, three β and seven FXYD subunits variation have been identified. The responsibility of the α subunit is the binding of magnesium (Mg^{2+}), Adenosine triphosphate (ATP), Na^+, K^+ to cardiac glycosides and it is considered the catalytic subunit of the enzyme. The glycoprotein β subunit is responsible as an adhesion molecule that regulates gap junction proteins; which is involved in structural and functional maturation of the holoenzyme (complete and fully functional enzyme molecule, consisting of the enzymatic subunit (apoenzyme) and any prosthetic group, cofactor, or regulatory either accessory protein subunits required for full regulated function). It facilitates transport of the α subunit to the plasma membrane and

maintenance of the enzyme in the lateral membrane of epithelial cells. The regulation of the enzyme function is the FXYD protein responsibility. The most acceptable function of Na^+,K^+-ATPase is to use ATP as an energy source to drive excess Na^+ out of cell in exchange for K^+, thereby maintaining essential ionic and osmotic conditions (Lawrence, 2005; Newman *et al.*, 2008).

The process of muscle contraction is shown in Figure 2.12. The simple explanation of the process of muscle contraction demonstrates that the movement of three cations, Na^+, calcium (Ca^{2+}), and K^+ controls the process of membrane depolarisation/repolarisation, in and out of the cell. On the outside of the cell membrane the concentration of Na^+ is high at the resting stage. On membrane depolarization Na^+ fluxes-in leading to an immediate elevation of the action potential. Elevated intracellular Na^+ triggers the influx of free Ca^{2+} that occurs more slowly. The higher intracellular Ca^{2+} results in the efflux of K^+. The re-establishment of the action potential occurs later by the reversal Na^+-K^+ exchange. The exchange of Na^+/K^+ needs energy, that it is provided by the enzyme, Na^+/K^+ ATPase (Desai, 2000; Pretorius *et al.*, 2005). Cardiac glycosides are proposed to inhibit this enzyme with the result of reduced Na^+ exchange with K^+ that leaves increased intracellular Na^+. This results in increased intracellular Ca^{2+}, which leads to a series of intracellular biochemical events that ultimately increase the force of the myocardial contraction or a positive inotropic effect (Desai, 2000).

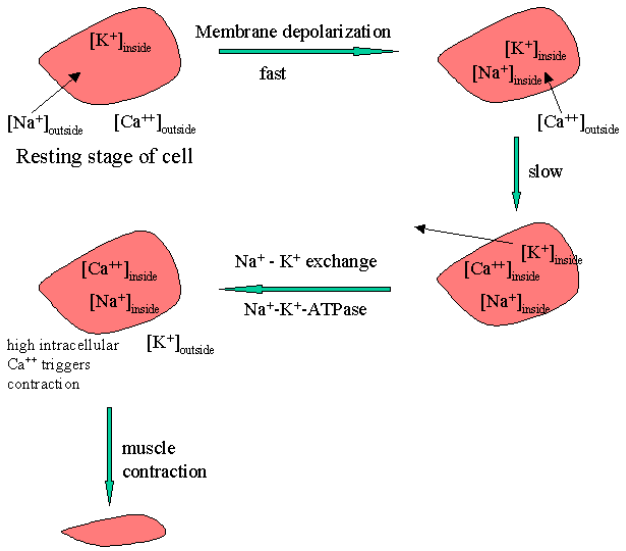


Figure 2.12: The process of muscle contraction (Desai, 2000).

2.2.5 Therapeutic value of cardiac glycosides

As mentioned previously, the use of cardiac glycoside containing plants for medicinal purposes was first reported in ancient texts more than 1500 years ago. They have been used traditionally as arrow poisons, abortifacients, emetics, diuretics, and heart tonics. It is the latter pharmacologic activity that cardiac glycosides are most commonly associated with, and after 200 years, compounds such as digitalis and digoxin are still prescribed by western doctors for control of congestive heart failure (Newman *et al.*, 2008).

Their use began after a meticulous analysis of a local herbalist's formula in 1775 by the English physician and scientist William Withering. He found that the patients with dropsy (congestive heart failure) improved after administration of an extract containing foxglove (*Digitalis purpurea* L. (Plantaginaceae)) which contains cardenolides (Newman *et al.*, 2008). According to Newman *et al.* (2008), concoctions containing digitalis have been used in European traditional medicine for centuries. The use of the plant in heart conditions was formally described in 1785 for treatment of dropsy or congestive heart failure (Newman *et al.*, 2008). In the 1960s inhibition of malignant cells by cardiac glycosides *in vitro* was reported and since then other anticancer effects of cardiac glycosides have been observed (Winnicka *et al.*, 2006). The therapeutic effect of cardiac glycosides has been known among breast cancer patients when the recurrence of the cancer among the patients who did not take cardiac glycosides was 9.6 times more than in the patients taking digitalis (Winnicka *et al.*, 2006).

According to the studies of Winnicka *et al.* (2006), cardiac glycosides induce apoptosis in the human prostate cancer cell line PC-3. Cardiac glycosides inhibit prostate cancer through four of the prostate target genes including transcription factors Hoxb-13, hPSE/PDEF, hepatocyte nuclear factor-3α, and the inhibitor of apoptosis. These activities propose that the membrane Na^+, K^+-ATPase may directly affect transcriptional regulation of prostate transcription factors (Winnicka *et al.*, 2006).

2.2.6 Antibacterial activity of cardiac glycosides

Infectious diseases originating from bacterial pathogens are still one of the major intimidations to humankind health. During the past, research on new acute antibacterial substances has continued. Over the past decade, investigation on herbal medicine showed

the antibacterial potential of medicinal plants and their isolated pure compounds. There are some reports on the antibacterial activity of cardiac glycosides isolated from natural resources.

Two types of bufadienolides isolated from the skin secretion of Brazilian toad *Bufo rubescens*, telocinobufagin (402.1609 Da) and marinobufagin Brasilia (400.1515 Da), illustrated the antibacterial activity against *Escherichia coli* and *Staphylococcus aureus.* The minimum inhibitory concentrations of telocinobufagin were 64.0 µg/ml for *E. coli* and 128 µg/ml for *S. aureus.* The marinobufagin MIC values against the *E. coli* and *S. aureus* were, respectively, 16.0 µg/ml and 128 µg/ml (Filho *et al.*, 2005).

Proceragenin, a type of cardenolide isolated from the succulent and erect shrub *Calotropis procera* (Ait.) R. Br. illustrated activity against both Gram-negative and Gram-positive organisms (Akhtar *et al.*, 1992). Biological screening of the isolated new cardenolide from the roots of *N. oleander*, 12β- hydroxy- 5β- carda-8, 14, 16, 20 (22) – tetraenolide, illustrated antibacterial activity and digoxin-like cardiac activities (Huq *et al.*, 1999). According to study of Puglisi *et al.* (2009), the bufadienolides compound isolated from *Helleborus bocconei* Ten. Subsp. *siculus* showed antibacterial activity against microorganisms responsible for the respiratory infections. This compound has the highest inhibitory activity against both Gram-negative and Gram-positive tested bacteria. The best inhibitory result of isolated bufadienolides was against the *Moraxella catarrhalis* and *Streptococcus pneumonia* with the MIC value of 100 µg/ml (Puglisi *et al.*, 2009).

2.2.7 Antiviral activity of cardiac glycosides

Medicinal plants have been traditionally used for the treatment of a broad range of ailments including infectious diseases. The treatment of infectious disease with currently available antiviral drugs leads to the problem of viral resistance against these agents and moreover, new viral pathogens may be encountered. So, there is an increasing need for substances with more effective antiviral activity, leading to the continuing search for new anti-infective agents (Bennett & Wallsgrove, 1994).

Herpes simplex virus type 1 (HSV-1) is an enveloped DNA virus. This type of virus can establish hidden infections in the nervous system and usually leads to life-threatening

diseases in immunocompromised individuals upon reactivation. In most cases, using the nucleoside analogues such as acyclovir (ACV), pencyclovir and their orally bioavailable drugs like, valacyclovir and famciclovir are effective for treating this ailment. But drug-resistance may arise due to prolonged treatment in immunocompromised individuals. Another drug, foscarnet, which acts directly on HSV DNA polymerase, can increase the rate of mutations in the DNA polymerase gene upon prolonged usage and the resulting mutants are often resistant to combination chemotherapy with existing compounds. Su *et al.* (2008) identified that digitoxin inhibited HSV-1 replication with a 50% effective concentration (EC_{50}) of 0.05 µM. The other analogues of digitoxin such as digoxin, ouabain octahydrate and G-strophanthin also showed anti-HSV activity (Su *et al.*, 2008).

According to the study by Prinsloo *et al.* (2007), the isolated digitoxigenin glucoside from *Elaeodendron creceum* (Thunb.) DC., an indigenous South African plant, showed toxicity of 20% at a concentration of 25 µg/ml on Vero cells and the active concentration of the compound against human immunodeficiency virus (HIV) is lower than 100 ng/ml with an inhibition of about 90% of the recombinant virus.

In a screening of Nepalese medicinal plants, *Carissa carandas* L. was active at lower concentrations than any of the other crude plant extracts. The extracts of *Carissa* species roots are poisonous and contain cardiac glycosides showing activity at a concentration of 12 µg/ml against all three viruses; Sindbis virus (SINV), Polio Virus 1 and HSV-1 (Taylor *et al.*, 1996).

These results establish the opportunity of cardiac glycosides to be more effective new drugs in the treatment of viral infections. Advanced analysis about the cardiac glycosides properties against the viruses may lead to the investigation of novel, safer and more effective drugs for treating the infectious diseases or even the drug resistant viruses in humans and plants.

2.2.8 Toxicity of cardiac glycosides

Plant poisoning in animals is usually accidental and most frequently occurs during unfavourable conditions when pastures are depleted due to drought, veldt fires, overstocking and trampling of the grazing. Plant poisoning in humans may be accidental or intentional by

confusing poisonous plants with edible ones, contamination of food with poisonous plants; or by using plants as remedies for medicinal purposes. Poisoning with the use of traditional medicines is the second most common cause of acute poisoning representing 12.1%, mostly of plant origin (Botha & Penrith, 2008).

It has been estimated that 15% of people in South Africa poisoned by medicinal plants will die, as compared to 2% suffering from acute, non-plant-induced poisoning; making the toxic effects of poisonous plants, particularly chronic effects, not always easy to reverse. The history, clinical syndrome observed, investigation of lesions, evidence that plants have been grazed, and remains of toxic plants in the gastrointestinal tract help to diagnose plant poisoning (Botha & Penrith, 2008).

Digitalis glycosides (cardiac glycosides) are defined as allosteric inhibitors of Na^+, K^+ ATPase and these types of compounds are used for treating heart disorders and a minute change in Na^+ is able to have a large effect on the contractile force of the heart. When the concentration of digitalis glycosides reaches toxic levels, the enzyme inhibition is too high (> 60%). So to return to the normal levels during diastole, the level of Na^+ and K^+ transfer must be decreased before the next depolarisation (Singh, 2004).

According to Van Wyk *et al*. (2005), symptoms of cardiac glycoside poisoning in rats are nausea, vomiting, vision disorder and extra-systoles. In severe cases complete heart block with bradycardia occurs. Death occurs due to ventricular fibrillation. The oral LD_{50} of digitoxin in rats is 8.3 mg/kg (Van Wyk *et al*., 2005).

2.2.8.1 Cytotoxicity of cardiac glycosides

According to studies, well-known drugs such as digoxin, digitoxin, and ouabain have antiproliferative effects on tumour cells. The studies of Johnson *et al*. (2002) have exhibited the ability of cardiac glycosides to induce apoptosis in the human prostate cancer cell line PC-3.

Studies show that cardiac glycosides in non-toxic concentrations are able to induce apoptosis in different malignant cell lines *in vitro*. Cardiac steroids induce the cytotoxicity via a series of morphological and biochemical changes that are characteristic for apoptosis, such as

phosphatidylserine externalization, internucleosomal DNA fragmentation and mitochondrial membrane potential disruption (Winnicka *et al.*, 2006).

Almost all isolated cardiac glycosides compounds of the methylene chloride extract of *Cerbera odollam* seed exhibited cytotoxic activity against oral human epidermoid carcinoma cells (KB), human breast cancer cell (BC) and human small cell lung cancer with IC_{50} ranges of 0.3-0.6 µg/ml (Laphookhieo *et al.*, 2004). The 50% cytotoxicity concentration (CC_{50}) of digitoxin is 10.66 µM (Su *et al.*, 2008).

According to the study done by Winnicka *et al.* (2006), the cardiac glycosides can be used as antineoplastic agents. The water soluble oleander extract, which is called Anvirzel, consists of several compounds, including complex polysaccharides, proteins and individual sugars. The two compounds of Anvirzel are cytotoxic. Clinical studies have shown that this oleander extract has strong activity against a variety of human malignant cell lines including melanoma, breast, and lung cancer. The experiments have demonstrated that the required concentrations of Anvirzel for achieving cytotoxicity are relatively non-toxic, but the clinically active functioning of cardiac glycosides in cancer therapy has been troubled by their related cardiotoxic action. Finding the mechanism of action of these types of compounds without cardiac activity but with cancer specific cytotoxicity is crucial (Winnicka *et al.*, 2006). Perhaps this complexity will also be a challenge when testing these compounds against viruses, therefore demonstrating the antiviral activity of cardiac glycosides and determining their toxicity as antiviral drugs may be a milestone in antiviral therapy.

2.2.8.2 Phytotoxicity of cardiac glycosides

The term of allelopathy was defined in 1937 by Molisch as biochemical interactions among plants (Uludag *et al.*, 2006). Allelopathy is a phenomenon defined as a direct or indirect harmful or beneficial effect of one plant on another through the production of chemical compounds released to the environment either from the aerial or underground parts in the form of root exudation, leaching by dews and rains, and volatilization or decaying plant tissue (Fujii *et al.*, 2003). Many plant-plant, plant-animal and plant-pathogen interactions have been described as regulatory functions in the environment. Allelopathy, classically defined as the inhibition of the growth or germination of one plant by another through the

release into the environment of selectivity toxic metabolic by products, is one of the less studied interactions that may occur among plant species growing together, which may account for the maintenance of high plant diversity (Fugii *et al.,* 2003).

Screening of 239 medicinal plant species for their allelopathic activity using the sandwich method was done by Fugii *et al.* (2003). The Apocynaceae family species (10 species) were one of the highest numbers of different plant species in a family in this test. According to the result the plants belonging to this family did not show great inhibition to lettuce (*Lactuca sativa* L.) radical growth (Fugii *et al.*, 2003).

The studies of Uludag *et al.* (2006), demonstrated that *N. oleander* showed different variation of allelopathic effects in weed control. The aqueous extract of *N. oleander* inhibited germination of weeds such as *Digitaria sanguinalis* (L.) Scop., *Lolium multiflorum* Lam., *Prosopis stephaniana* (M. Bieb.) Kunth ex Spreng., etc. A pot study with soil incorporation showed that growth of cotton (*Gossypium herbacium L.*), maize (*Zea mays* L.) and soybean (*Glycine max* L. Merril) was promoted by *N. oleander*. Plant parts of *N. oleander* significantly reduced weed density in maize fields and maize yield was increased compared to the weed control. Allelopathic potential of fresh plant parts and/or an aqueous extract of *N. oleander* was tested against the *Orobanche* spp. which is an obligatory parasite and infects many crops such as tobacco (*Nicotiana tabacum* L.), sunflower (*Helianthus annuus* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medik) and tomato [*Solanum lycopersicum* L. (Syn. *Lycopersicon esculentum* Mill.)] in greenhouses and in the field. *Nerium oleander* was stimulated by the number of *Orobanche ramosa* tubercules on tomato plants (Uludag *et al.*, 2006).

Allelopathic effects of root, stem, leaf and bud extracts and their mixtures of oleander were tested on germination and early seedling growth of bean (*Phaseolus vulgaris* L.) and wheat (*Triticum aestivum* L.) in Petri dish assays. Beans were more affected than wheat; the bud extracts had an especially negative effect on beans. Extracts stimulated wheat germination but reduced plumule and radicule length. Root extracts were the most effective on early seedling growth of wheat (Uludag *et al.*, 2006).

Another study was done by Amruth Kumar and co-workers (2011), to determine the allelopathic effect of *N. oleander* leaves on *Parthenium hysterophorus*. The aqueous and methanol extracts of *N. oleander* leaves showed inhibitory effect on the seed germination and early growth of *P. hysterophorus* in a concentration dependent manner. Methanol extracts of a white flowered variety illustrated higher inhibition compared to that of the pink flowered variety. Rutin and its aglycoside, Quercetin, are isolated compounds of *N. oleander,* and when mixed with soil inhibited seed germination, root and shoot lengths of *P. hysterophorus. Nerium oleander* extracts tested on seed germination and early growth of crop plants, had no effect on wheat, Ragi, Green gram and Soya gram seeds even at 1:3 dilution of the stock preparation (Amruth Kumar *et al.*, 2011).

Anjum *et al.* (2010) researched the allelopathic effects of 14 medicinal plants by using the sandwich method on lettuce seeds. *Nerium oleander* was one of the plants which was investigated. Different concentrations of *N. oleander,* had almost no effect on growth of the lettuce radical, but 80-85% inhibition of hypocotyls growth was observed (Anjum *et al.*, 2010). A study of Uygur & Iskenderoglu (1995) shows that the water extracts of *N. oleander* did not have any adverse effect on maize growth under field conditions. However, its residue increased maize yield and decreased weed coverage area.

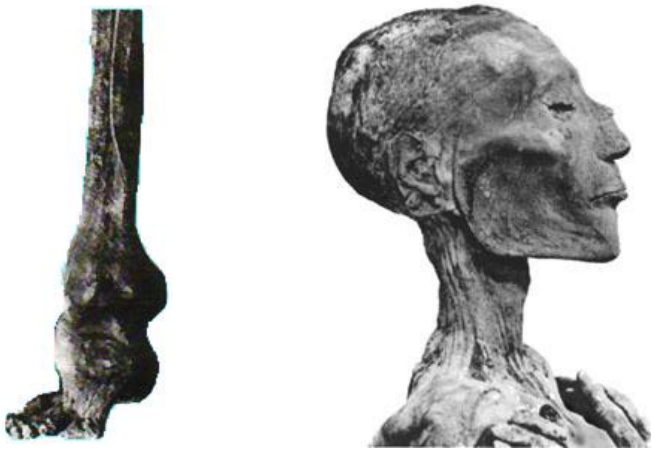
Cardiac glycosides (cardenolides) are endemic in the milkweed genus (*Asclepias*). Harry-O`kuru *et al*. (1999) tested two milkweed species (*A. syriaca* and *A. speciosa*) as nematicides and against armyworm. Germination inhibition assays were conducted and the result of these tests indicated no inhibition of wheat, maize, and soybean or hemp (*Cannabis sativa* L.) seed germination. Only tomato plant growth appeared to be retarded by 4% milkweed seed-meal added to soil. It seems that milkweed does not have a highly toxic effect on plants at the concentration levels tested (Harry-O`kuru *et al*., 1999).

2.3 Introduction to human and plant viruses

According to Henderson's Dictionary of Biology, a "virus" is a minute, intracellular obligate parasite, visible only under the electron microscope. A virus particle consists of a core of nucleic acid, which may be deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), surrounded by protein coat and in some viruses a further lipid/glycoprotein envelope. It is unable to multiply or express its genes outside a host cell as it requires host cell enzymes to

aid DNA replication, transcription and translation (Lawrence, 2005). This type of parasite is an interesting example of the "selfish gene". There are schemes for virus replication enciphered in the genetic code of the virus genome. These must be decoded by the molecular system of an infected cell. In essence, viruses are intracellular parasites dependent on the metabolic and genetic functions of living cells. The main purpose of a virus is to deliver its genome into the host cell to be expressed by the host cell (Gelderblom, 1996; Koonin *et al.*, 2006; Wagner *et al.*, 2008). In general, viruses are classified on the basis of morphology, chemical composition, and mode of replication. The type of genetic material (whether DNA or RNA) is an important factor in the classification of any given virus into groups. The arrangement of protein around the nucleic acid and also, symmetry and dimensions of the viral capsid are the other important factors to classify the viruses. The presence or absence of the envelope helps to organize the viruses as certain groups (Wagner *et al.*, 2008).

It would appear that the first evidence of viral infection originates from hieroglyphs found in Memphis, the capital of ancient Egypt, about 1400 BC, where a typical clinical sign of paralytic poliomyelitis was shown by a temple priest, called Ruma. The horse hoof-like withered leg and rigidly extended foot of the Pharaoh Siptah, who ruled in Egypt from 1200-1193 BC, shows the classical paralytic poliomyelitis. Further evidence of viral infection in ancient Egypt is the pustular lesions on the face of the mummified Pharaoh Ramses V, who was affected by smallpox (Figure 2.13) (Cockburn *et al*., 1998; Cann, 2005).



(A) (B)

Figure 2.13: A) The horse hoof -like of Pharaoh Siptah's mummified body. B) The mummy of Pharaoh Ramses V who was affected by smallpox (Cockburn *et al*., 1998).

According to historical evidence and studies by scientists, smallpox was endemic to China by 1000 BC, and survivors of smallpox epidemics were protected from subsequent infection (Cann, 2005). At the end of the 18th century, the English physician, Edward Jenner, who studied the occurrence of smallpox, obtained a liquid from pox pustules in cattle, which he called "vaccine virus". He then proceeded to inoculate people to protect them against smallpox with this obtained liquid (vaccine virus). Two pioneer virologists, Louis Pasteur and Robert Koch, showed that the microorganisms (germs) were responsible for the occurrence of infectious diseases in humans and animals. Pasteur believed that viruses are autonomous microorganisms and a living infectious entity of corpuscular, cellular nature. He called them *contagium vivum fixum*, but in spite of this, he could not discriminate between bacterial and other agents of disease (Van der Want & Dijkstra, 2006).

Until 1833, plant diseases were still considered to be caused by irregularities in chemical processes through unfavourable conditions. As a result of these aberrations, spontaneous development would occur in the organisms' entities. At the end of the 19th century, Burrill described a bacterium causing pear blight, and Wakker showed a bacterium to be the causal agent of the yellow disease of hyacinth (Van der Want & Dijkstra, 2006).

In February of 1892**,** Dmitri Iwanowski, a Russian botanist, presented a paper to the St. Petersburg Academy of Science which showed that extracts from diseased tobacco plants could transmit disease to other plants after passing through ceramic filters fine enough to retain the smallest known bacteria. This is generally recognised as the beginning of Virology. He believed that a type of toxic substance was excreted by bacteria or the bacteria were so small that they were able to pass through the filter pores (Cann, 2005; Van der Want & Dijkstra, 2006).

According to the report of Freidrich Leoffler and Paul Frosch in 1898, a similar agent was responsible for foot-and-mouth disease in cattle (Cann, 2005; Van der Want & Dijkstra, 2006). In 1909, poliomyelitis was recognized as the first human disease to be caused by a 'filterable agent' (Cann, 2005). Fredrick Twort and Felix d'Herelle were the first people to observe that viruses could also infect bacteria in 1915 to 1917 (Cann, 2005).

All pathogenic or non-pathogenic viruses and other microorganisms are important members of the biosphere and have an important impact on human life and future activities. According to Weiss and McMicheal (2004), fifty years ago, with the advent of antibiotics, vaccinations, insecticides and improved surveillance of microorganisms, it was noted that the rate of some recognized infectious diseases were receding and some of them were never eradicated. By the late twentieth century, many parts of the world witnessed an increase in the emergence and re-emergence of infectious diseases. About 30 new diseases have been identified, such as Human Immunodeficiency Virus (HIV/AIDS); Hepatitis C; Bovine Spongiform Encephalopathy (BSE) (a group of neurodegenerative diseases of humans and animals in which the brain has a characteristic sponge-like appearance after death); Variant Creutzfeldt-Jakob Disease (vCJD) (a rare fatal neurodegenerative disease of humans, a prion-caused spongiform encephalopathy), Nipah Virus, several strains of viral hemorrhagic fever, and most recently, Severe Acute Respiratory Syndrome (SARS) and Avian Influenza (Weiss & McMicheal, 2004; Lawrence, 2005). The emergence of these diseases and manifestation of a new generation of viruses can be due to consequences of various changes in human ecology, such as migration of the rural population to urban society; facilitating and increasing long-distance trade; the social consequences of wars; changes in personal behaviour, and global changes such as deforestation or climate changes. The other factors which increase the threat of infectious diseases such as HIV/AIDS are political issues, lack of medical technology, drug resistant microbes and contaminated equipment or biological medicines (Weiss & McMicheal, 2004).

2.3.1 Importance of virus infections in humans

Habitat damage and new contacts between wild flora and/or fauna and humans cause an increasing amount of human infections. There are many reports of cross-species infection and the emergence of crop or animal infectious diseases in humans. One of the remarkable examples of cross-species infection which is the establishment of piggeries close to the tropical forest in northern Malaysia in 1998, where the Nipah Virus first crossed over from fruit bats (flying foxes, *Pteropus* spp.) to pigs and then to pig farmers. Rodent-borne Hantavirus, a re-emerging infection, is prevalent in agricultural systems in South America and East Asia, as well as the grasslands of North America. In mid-1993, in the south-western United States, an epidemic of acute, occasionally fatal, respiratory disease occurred in humans known as `Hantavirus pulmonary syndrome` which was first found in animals

(Weiss & McMicheal, 2004). The Irish potato famine in 1845 and the English foot-and-mouth disease epidemic in 2001 underscored the consequence of disease emergence in crop and livestock for human societies (Weiss & McMicheal, 2004). In most countries around the world, life hope has increased during the last 50 years (Weiss & McMicheal, 2004). The most important exception occurs in the regions where HIV infection is widespread. Human viruses are one of the most important pathogens that challenge health around the world. Human Immunodeficiency Virus is one of the greatest viral challenges for humans in this century.

The United Nation Program on HIV/AIDS (UNAIDS) (2006) reported that HIV has infected 40.3 million people around the world, and that South Africa has the highest prevalence at 5.5 million HIV-infected people. The influenza A epidemic between 1918 to 1919 temporarily increased the death rate due to infectious disease. It was estimated that the 50 million deaths caused by the pandemic represented about 2% of the global population at that time, and is twice as much as the cumulative AIDS mortality of the past 20 years (Weiss & McMicheal, 2004).

Herpes Simplex Virus (HSV) is a member of Herpesviridae family. It is a double stranded DNA virus and is common human pathogen, classically causing oral-facial (mostly HSV-1) or genital (HSV-2) infections (Lecluse & Bruijnzeel-Koomen, 2010). Herpes simplex virus-2 may increase the risk of HIV-1 transmission through disruption of the barrier and inflammation of genital ulcer diseases. In sub-Saharan Africa, where HIV-1 is spread mainly by heterosexual transmission, HSV-2 infections are common (Wasserheit, 1992; Johnson & Laga, 1998).

Mankind's life is always threatened with an unknown influenza type or aberrant viral disease or epidemic, therefore access to the appropriate vaccine and/or acute drug treatment is vital. Figure 2.14 shows that the rate of infectious diseases (respiratory and other infectious diseases) has decreased from 1909 to 1999; but on the other hand, the rate of cancers has increased dramatically (Weiss & McMicheal, 2004).

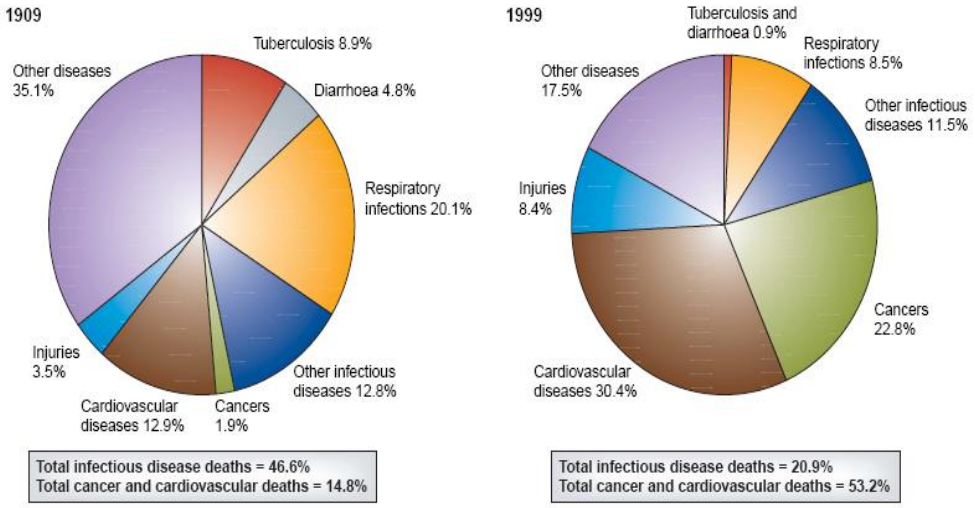


Figure 2.14: Proportion of total deaths from major cause of death categories, 1909 and 1999 (Weiss & McMicheal, 2004).

The latest investigation in the field of oncology showed that about 15% of human cancers can be attributed to viral infection. Only 7% of this data originates in the developed world, while in developing countries it exceeds 22%. This means that there is a global total of 1.2 million new cases of cancer per year (Weiss & McMicheal, 2004).

There are two major mechanisms in which oncogenic viruses form tumours: direct oncogenesis, that the virus infects a progenitor of the clonal tumour cell population, and usually perseveres in the tumour cells. Indirect oncogenesis occurs when the virus does not necessarily infect the tumour progenitor cell, but exerts an indirect effect on cell and tissue turnover or on the immune system, influencing tumour development (Table 2.3) (Kuper *et al.*, 2000; Talbot & Crawford, 2009).

Direct oncogenesis includes Herpes Viruses, Papilloma Viruses, Hepatitis B Virus (HBV) and retroviruses. Herpes viruses called EBV: Epstein-Barr Virus was the first human tumour virus discovered, in Burkitt`s lymphoma cells. This virus is responsible in some cancers such as gastric cancer, various AIDS-associated complications, including lymphoma and oral hairy leukoplakia.

Table 2.3 Viruses with oncogenic potential in humans (Talbot & Crawford, 2009)

	Human tumour	Non-malignant disease	Experimental tumours in animals
Papovaviridae			
• **Human papillomavir**	Cervical cancer	Warts	Skin cancer
• **BK**	None	Cystitis immunocompromised	Multiple tumours rodents
• **JC**	None	Progressive multifoc leucoencephalopathy	Multiple tumours rodents
Adenoviridae			
• **Adenovirus**	None	Gut and respirato infections	Sarcomas, carcinomas rodents
Herpesviridae			
• **Epstein-Bar virus**	Nasopharyngeal carcinoma, Immunoblastic lymphoma,'Hodgkin`s lymphoma	Infectious mononucleos	Lymphoma in monkeys
• **Kaposi`s sarcoma-associated herpesvirus**	Kaposi`s sarcoma, prima effusion lymphoma	Multicentric castlerman`s disease	Not known
Retroviridae			
• **Human T cell lymphotrophic virus 1**	Adult T cell leukaemia	Tropical pastic paraparesis	Adult T-cell leukaemia in rabbits
Murine mammary tumour virus		Not known	
• **HIV (indirect)**	Breast cancer	Bcell lymphom Kaposi`s sarcoma	Mammary tumours mice
Hepadnaviridae			
• **Hepatitis B virus**	Liver cancer	Hepatitis, cirrhosis	Not known
Flaviviridae			

• **Hepatitis C virus**	Liver cancer	Hepatitis, cirrhosis	Not known

Papilloma viruses, HPV: Human Papilloma Virus cause lesions of the genitals, upper respiratory and digestive tracts, and cutaneuos lesions at various sites. Cervix cancer was identified for the first time as a sexually transmitted disease in 1834, and in mid-1970, it was identified that HPV are associated with invasive carcinoma. Different types of this virus occur in other anogenital cancers and may lead to cancer formation at other sites of the body, such as the head and neck, oesophagus and skin (Talbot & Crawford, 2009). More than 250000 new cases of Hepato Cellular Carcinoma (HCC) arise each year. The main mechanisms involved in the development of HCC are viruses and the immune system, through induced chronic liver damage and the regeneration of hepatocytes (Kuper *et al.*, 2000; Talbot & Crawford, 2009).

Retroviruses are single-stranded RNA viruses which are associated with human leukaemia caused by the Human T Lymphotrophic Virus-1 (HLTV-1). According to the study of Talbot & Crawford (2009), HTVL-1 is endemic in Japan, the Caribbean and Africa. These viruses are able to transcribe to double-stranded DNA, using viral reverse transcriptase, and randomly integrate in to the host`s genome. Molecular studies illustrates that the virus is present in almost all cases of Adult T Cell Leukaemia (ATL). Murine Mammary Tumour Virus (MMTV) has been implicated in the development of some human breast cancers. The existence of this virus in an infected population increases the risk of breast tumours up to 42%; in contrast, a healthy population has a rate of just 1-2% of breast tumours (Kuper *et al.*, 2000; Talbot & Crawford, 2009). Indirect oncogenesis in general, consists of Hepatitis C Virus (HCV) and other viruses. Hepatitis C virus is a RNA virus that belongs to the Flaviviridea family. The rate of infection is highest in Africa (especially in Egypt), Japan and other Asian countries. Worldwide, 0.5-2% of the population has current or had a past infection with HCV. The period of HCV infection to development of cancer is 10-50 years. There is a strong association between chronic HCV infection, cirrhosis and hepatocellular carcinoma (Talbot & Crawford, 2009).

2.3.3 Plant viral diseases

Plant viruses are highly contagious among plants and the impacts of viral diseases are seen primarily as reductions in crop yield and quality and its effects on market prices. Economic

costs also arise significant through the need to take protective measures or to implement control strategies to avoid a disaster. Because of the high variation of potential yield and damage to the crop, the exact measure of the losses caused by any disease is not easy and sometimes it is even impossible to measure unless the crop is completely destroyed (Bos, 1982). In developing countries the harmful impact of plant viral diseases may be particularly severe because reduced crop yields can seriously affect the livelihoods of impoverished people and even threaten food security. In the 1990s, the outbreak of cassava mosaic disease in Uganda led to starvation in some districts in the country and is estimated to have resulted in a loss of US$60 million per annum during the height of the epidemic (Thresh & Cooter, 2005). Table 2.4 summarizes the main forms of damage that plant viruses can cause that lead to economic loss.

Table 2.4 Some types of direct and indirect damage associated with plant-virus disease (Bos, 1982)

1) Reduction in growth	• Yield reduction (even with symptom-less reduction) • Crop failure
2) Reduction in vigour	• Increased sensitivity to frost and drought • Increased predisposition to pathogens and pests
3) Reduction in quality market value	• Defects of visual attraction: size, shape, colour • Reduced keeping quality • Reduced consumer quality: grading (including size ar shape), taste, texture composition (e.g. protein conter sugar content) • Reduced fitness for propagation
4) Costs of attempting maintain crop health	• Cultural hygiene on the farm including vector control • Production and certification of virus-tested propagatic material • Checking of propagation material and other commoditi for virus freedom on export (health certification) ar import (inspection and quarantine) • Regional or national eradication of sources of infection • Breeding for resistance • Research, extension and education

According to Wutscher (1997), since elimination of a viral infection is not usually possible, most infected crops will remain diseased to a certain extent. High-value trees infected with a virus may have to be removed to prevent the spread to neighboring healthy trees, and it may take several years before replacement trees become productive. The devastating outbreak of citrus tristeza disease, in America is an example of the serious economic consequences that

may result due to a viral outbreak (Wutscher, 1977). A particular challenge is present with crops that propagate vegetatively, as viruses can spread rapidly through infected planting material. If adequate precautions are not taken, spread of viruses through human involvement of infective vegetative propagules can take place over much longer distances than would be possible through natural means (Thresh, 1986).

Epidemics of viral diseases in annual crops can develop extremely rapidly and cause total yield loss in a particular growth season. As with vegetative propagules, transfer of infected seed can result in the introduction of viral disease to new areas. Seed-borne infection leads to early onset of disease and therefore creates the potential for significant epidemics to arise in new plantings, assuming that the virus has other modes of transfer from plant to plant (Thresh, 1980). However, rapid viral disease spread may also occur where there are no introduced foci of infection through infected planting material. Non-persistent viruses that are transmitted by arthropod vectors can be spread very quickly within a crop due to the short feeding periods required for virus acquisition and inoculation. Semi-persistent viruses are transmitted less rapidly than non-persistent viruses, but they are retained for longer periods in the vector and can potentially be carried over longer distances to initiate new infections. Semi-persistent viruses, such as Sugar Beet Yellow Virus; continue to cause serious losses in sugar beet crops in Europe and North America (Thresh, 1980).

According to the study of Thresh (1980), the annual losses incurred by sugar beet (*Beta vulgaris* L.) growers due to the disease are in the region of £4.2 million. The studies of Thresh (1980) illustrated that the curly top of sugar beet and the swollen shoot of cacao (*Theobroma cacao* L.) have particularly disastrous effects. It nearly caused destruction in the sugar beet industry in the western United States in 1920s, and the swollen shoot of cacao killed millions of cacao trees in West Africa. Consequently to prevent further spread of the virus, many of the infected trees were also destroyed. By 1977,162 million cacao trees had been cut down in Ghana and the rate of eradication had risen to 15 million a year, which was equivalent to an area of 9400 ha (Thresh, 1980).

Since 1960, the potato (*Solanum tuberosum* L.) has been becoming an important source of food and its cultivation provides rural employment and income for the growing population in developing countries (Huaman & Schmiediche, 1999). The study of Blanco-Urgoiti *et al.*

(1998) demonstrates that Potato Virus Y (PVY) and also Potato Leaf Roll Luteo-Virus are two of the most economically important viruses affecting the potato industry. One of the most disturbing diseases of cultivated tomato is Tomato Yellow Leaf Curl Gemini-Virus (TYLCV), which is transmitted by the whitefly *Bemisia tabaci* (*Gennadius*). The quantitative and qualitative economic yield losses of the TYLCV often reaches up to 100% in the tomato crop in many tropical and subtropical regions and continues to spread towards new areas (Pico *et al*., 1996). Viruses affect plants to different extents and not all infections are harmful.

When considering the important nutritious source and economical effects of crops and agricultural products in both rural and urban society, these mentioned examples in the last pragraphs provide evidence for the necessity of the research and attainment of the most effective new drugs against the viral pathogens.

2.3.3 Taxonomy, structure and classification of viruses

2.3.3.1 Viral taxonomy

Studies conducted by Mayo and Pringle (1998), indicated that the taxonomy of viruses enable scientists to characterize the viruses into the related groups and identify the degree of relatedness. The International Committee on Taxonomy of Viruses (ICTV) was organized in 1973. The Species, Genus, Family and Order are four principal taxa recognized by ICTV. The principles of virus taxonomy must have four properties; namely stability, utility, acceptability and flexibility. According to the Sixth Report of the ICTV, viruses are classified into 184 genera, of these, 161 are classified in 54 families (Mayo & Pringle, 1998).

2.3.3.2 Viral structure

The structure of a virus consists of viral genomes, viral capsid and viral envelope:

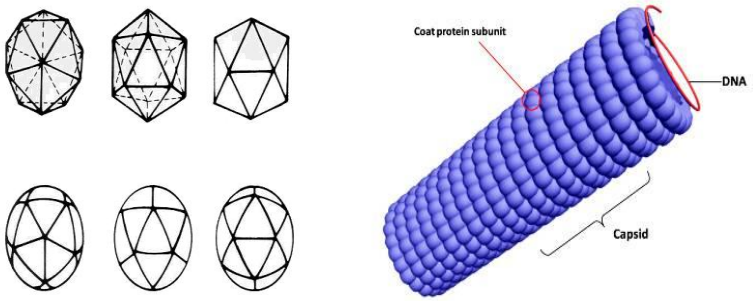
- **Viral genomes**

  All free living cells utilize only double-strand DNA as genetic material, but viruses are able to utilize other type of nucleic acid. The nucleic acid core of a virus has either DNA or RNA genes and is termed either DNA virus or a RNA virus, respectively. A viral genome, irrespective of nucleic acid type, is either single-stranded (ss-DNA or ss-

RNA) or double-stranded (ds-DNA or ds-RNA). The replication of the genome in DNA and RNA viruses does not occur in the same way. The DNA viruses use the infected cell's nucleus as the site of genome replication. These viruses share many common patterns of gene expression and genome replication as those occurring in the host cells. The replication of RNA viruses requires expression of specific enzymes that are not present in the uninfected host cell, because the cell does not have the mechanism of RNA-directed RNA replication. Virus genes encode the required proteins for replication and these proteins and the cellular proteins have analogous functions, but viral and cellular proteins are not identical. The encoded proteins for replication by viruses are enzymes, which are capable of both nucleic acid replication and the expression and regulation of viral genetic information. Viruses are able to encode enzymes and proteins involved in modifying the cell and optimize the cell for virus replication (Wagner *et al.*, 2008).

- **Viral capsid**

  A fully assembled infectious virus is called a virion. The simplest virion consists of two basic components: nucleic acid (ss or ds of RNA or DNA) and a coat or capsid. The structure of a capsid is made up of many identical subunits of viral protein, which is known as a capsomer. It functions as a protein shell providing a stable and secure environment for the chemically viral genome. The capsomer also protects the viral genome from nucleases and during infection attaches the virion to specific receptors exposed on the host cell (Gelderblom, 1996; Wagner *et al.*, 2008). Multiple protein copies of the capsomer must self assemble to form the continuous three-dimensional capsid structure. The result from self assembly of viral capsid is two basic regular shapes: helical symmetry, where in which the protein subunits and the nucleic acid are arranged in a helix, and icosahedral symmetry, where the capsomers form a regular solid structure that covers the nucleic acid-containing core (Figure 2.15).



(A) (B)

Figure 2.15: A) Icosahedral models, left to right: different fold axes of rotational symmetry (Gelderblom, 1996). B) The helical structure of a virus (www.en.academic.ru/dic.nsf).

Each type of virus has a unique shape and structural arrangement according to the nature of the capsid proteins and conditions of their interactions. Therefore, a capsid shape is a fundamental criterion in the classification of viruses (Gelderblom, 1996; Wagner *et al.*, 2008). Important information and structural features of the viruses are detected by X-ray crystallography.

- **Viral envelopes**

  In some virus species, which infect bacteria, plant and animals, the capsid is the outer layer. However, viruses can have an additional complex structure covering, which the capsid is surrounded by. This external layer is called the envelope, and can act as an additional protective coat. The envelope is made up of a lipid bilayer. This envelope is modified from the cell host membrane with the virus replicates and forms virus-encoded membrane-associated proteins (Wagner *et al.*, 2008). The virus envelope is constituted through budding from the plasma or the other intracellular membranes, which results in the lipid composition being virtually almost the same as its particular host membrane. Identifying the composition and structure of the outer capsid and also the envelope protein in the viruses, can determine the host range and genetic composition of the virion (Gelderblom, 1996).

The development and classification of viruses is one of the main challenges for virologists. By a superior classification, virologists are able to organize the growing number of viruses and their genes detailing genetic and molecular information.

2.3.4 Antiviral potential of medicinal plants

2.3.4.1 Pharmaceutical value and antiviral potential of medicinal plants

According to studies, the antiviral property of plants is distributed throughout the plant kingdom, from higher plants to algae and lichens (Table 2.5) (Mukhtar *et al.*, 2008; Abonyi *et al*., 2009).

Table 2.5 Partial list of human viruses inhibited by medicinal plants (Mukhtar *et al*., 2008)

Virus	Medicinal plant used
Herpes simplex virus (HSV)	*Carissa edulis* Vahl. *Phyllanthus urinaria* L.
Influenza virus	*Geranium sanguineum* L. Elderberry extract
Hepatitis B virus	*Boehmeria nivea* L. *Polygonum cuspiatum* Sieb. & Zucc
Hepatitis C virus (HCV)	*Saxifraga melanocentra* Engl. & Irmsch.
Poliovirus	*Guazuma ulmifolia* Lam. *Stryphnodendron adstrigents*
Viral haemorrhagic septicaemia virus (VHSV)	*Olea europaea* L.
Severe acute respiratory syndrome-associate coronavirus (SARS-CoV)	*Lycoris radiate*
Human immunodeficiency virus (HIV)	*Phyllanthus amarus* Schum. & Thonn. Olive leaf extract (OLE)
Vesicular stomatitis virus (VSV)	*Trichilia glabra* L.
Human adenovirı (type 1)	Black soybean extract
Dengue virus (type 2)	*Azadirachta indica* Juss. (Neem)

Recognition of the biological properties of natural products has encouraged the current focus for the search for new drugs as antibiotics, anticancer agents, antiviral agents, insecticides and herbicides. The use of traditional herbal medicine and natural products is widespread in the daily existence of the African society (Sparg *et al.*, 2002).

Recently, a number of studies have explored immunostimulatory properties of plant extracts having antiviral properties. According to Webster *et al.* (2006), the root extract of the medicinal plant *Hercleum maximum* Bartr. stimulated Inter-leukin 6 (IL-6) production in the macrophage activation assay, confirming antiviral association with immunostimulatory properties. Two common folk medicinal plants in Taiwan, *Plantago major* Linn. and *P. asiatica* Linn., exhibited lymphocyte proliferation and secretion of interferon-gamma (IFN-γ) at low concentrations (Chiang *et al.*, 2003). According to the report of Bessong *et al.* (2005), the methanol extracts of the root of *Bridelia micrantha* Baill., *Combretum molle* R.Br.ex G.Don., *Mucuna coriacea* Baker., *Peltophorum africanum* Sond. showed strong activity against HIV-1 RT (Bessong *et al.*, 2005).

Gebre-Mariam *et al.* (2006) assessed the usage of some Ethiopian medicinal plants for treatment of dermatological disorders. According to this result, the extracts of *Acokanthera schimperi* (A.Dc.) Schweinf. and *Euclea schimperi* (A.Dc.) Dandy. inhibited the virus-induced cytopathic effect of Coxsackie virus B3 (CVB3), influenza virus and HSV-1. The extract of *Plumbago zeylanica* Linn. inhibited CVB3; while HSV-1 was inhibited by *Inula conferiflora* A. Rich. (Gebre-Mariam *et al.*, 2006).

According to Webster *et al*. (2006), *Hercleum maximum* Bartr. root extract stimulated Inter-leukin 6 (IL-6) production in the macrophage activation assay, which confirmed antiviral association with immunostimulatory properties of the *H. maximum* root extract. The study of Klos *et al*. (2009) showed the ethanolic extracts of *Leonotis leonurus* (L.)R.Br. and *Bulbine alooides* (L.)Willd. have great anti-HIV potential through the inhibition of HIV-1PR.

The screening of anti-AIDS agents from folk medicinal plants of the Iberian Peninsula showed that the extracts of *Tuberaria lignose* (Sweet) Samp. and *Sanguisorba minor magnolii* Scop. have anti-HIV activity in an *in vitro* MTT cell proliferation assay. The aqueous extracts of these plants exhibited inhibitory effects against HIV-1 induced infections in MT-2 cells at concentrations ranging from 12.5 µg/ml to 50 µg/ml. These two plants didn't exhibit cytotoxicity at any of the concentrations (Bedoya *et al.*, 2001).

Australian scientists investigated the extracts of 40 different plant species used by Aborigines for antiviral activity (Semple *et al.*, 1998). Several different types of viruses were used in this experiment: DNA virus, Human Cyto-Megalo Virus (HCMV), RNA virus,

Ross River Virus (RRV) and Human Poliovirus (Polio) - type 1. Six of the extracts showed activity against the different viruses.

The aerial part of *Pterocaulon sphacelatum* F.Muell. and the root of *Dianella longifolia* var. *grandis* were the most active extracts against polio, which inhibited the poliovirus at concentrations of 52 and 250 µg/ml, respectively. The most active extracts against the HCMV virus were *Euphorbia australis* Boiss. and *Scaevola spinescens* R.Br.. The extracts of *Eremophila latrobei* subsp. *glabra* and *Pittosporum phylliraeoides* var. *microcarpa* demonstrated antiviral activity against RRV (Semple *et al.*, 1998).

The studies of Chiang *et al*. (2002) showed activity of aqueous extracts and pure compounds of *Plantago major* Linn. against the Herpes Simplex Virus (HSV)-1,-2 and adenoviruses- 3, -8, 11. The aqueous extract of *P. major* exhibited a slight anti-herpes virus activity but in contrast, the pure compounds of five different classes of chemicals found in extracts of this plant have strong antiviral activity against HSV-1 (EC_{50}=15.3 µg/ml). The pure compounds isolated from *P. major*, which exhibited anti-HSV activity belong to the phenolic compounds family, especially caffeic acid.

The investigation on the fractionated plant extracts showing anti-HSV activity demonstrated that there are two major anti-HSV activity compounds from the ethyl acetate fraction, moronic acid and betulonic acid belonging to the triterpenes. The moronic acid was found to be the major anti-HSV compound. The EC_{50} of moronic acid and betulonic acid for inhibiting the wild-type HSV-1 was 3.9 and 2.6 µ/g/ml, respectively (Hassan Khan *et al.*, 2005).

2.3.4.2 Antiphytoviral potential of medicinal plants

As mentioned before plant viruses are responsible for a wide range of plant diseases and increase the serious economic damages of crops all around the world. So, investigating the acute treatment or preventing virus infection is an important issue for scientists. The study on the activity of plant extracts and isolated compounds against the plant viruses can establish the antiviral potential of medicinal plants.

An investigation by Shen *et al.* (2008) was done to investigate the antiviral activity of *Brucea javanica* (L.) Merr against the Tobacco Mosaic Virus (TMV). The obtained result demonstrated that *B. javanica* may have potential to inhibit the TMV (Shen *et al.*, 2008). According to a study, garlic cloves bulb (*Allium sativum* cv. Balady) extract have potential to reduce the local lesions produced by Potato Virus Y (PVY) on *Chenopodium amaranticolor* Coste & Reyn. (Mohamed, 2010). The antiphytoviral assay of the *Plectranthus tenuiflorus* (Vatke) Agnew crude extract on different types of plant viruses like Tobacco Necrosis Virus (TNV), Tobacco Mosaic Virus (TMV) and Tomato Spotted Wilt Virus (TSWV) was done by Othman and Shoman (2004). The result of this study showed that the *P. tenuiflorus* extract reduced the infectivity of above viruses.

Infection with Tomato Yellow Leaf Curl Geminivirus (TYLCV) causes major loss of tomato production. Using water extract of khella seed (*Ammi visnaga* L.); black cumin (*Nigella sativa* L.) and garlic bulb (*Allium sativum* L.) eliminated TYLCV (El-Dougdoug *et al.*, 2007).

2.4 Conclusion

Plants have been used as folk remedies and ethnobotanical literature has described the usage of plant extracts, infusions and powders for centuries for diseases now known to be of viral origin. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant, which are known by their active substances.

There is an increasing need for the search of new compounds with antibacterial and antiviral activity as the treatment for infectious diseases. Ethnopharmacology provides an alternative approach for the discovery of antimicrobial agents, namely the study of medicinal plants with a history of traditional use as a potential source of substances with significant pharmacological and biological activities.

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A number of compounds extracted from various species of higher plants have shown antibacterial and antiviral activity. A number of plant extracts which contain cardiac glycosides reported in traditional medicine to have antibacterial properties were studied and were also screened for antiviral activity. It is possible that the investigation of the cardiac glycosides may provide useful leads to the development of new and effective antibacterial and antiviral treatments.

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Chapter 3

Antibacterial activity of plants containing cardiac glycosides

3.1 Introduction

There is a clinical requirement for more active alternative medicines for treating serious Gram-positive and Gram-negative bacterial infections. Furthermore, the rapid emergence of bacterial infections, which are resistant to many general antibiotics establish the necessity of new therapeutic agents. Antimicrobial resistance are classified in different groups such as microbiological resistance, clinical resistance, cross-resistance and parallel or co-resistance (Shah, 2005).

Medicinal plants are the richest bio resource of drugs for traditional systems and folk medicine, modern medicines, pharmaceuticals, nutrition and food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs. According to an assessment by the World Health Organization (WHO) more than two thirds of the world`s plant species are estimated to have medicinal value (WHO, 2011). Between 25-50% of modern medicines are derived from plants. The WHO estimates that nearly 80% of the world population in developing countries depend on traditional medicinal plants for their health care needs (WHO, 2011). Higher plants have been shown to be a potential source for new antimicrobial agents (Mitscher *et al*., 1987).

Plants have two different types of metabolites namely primary metabolites and secondary metabolites. The primary metabolites have essential functions such as growth and development (Bourgaud *et al.*, 2001; Hoffman, 2003). Over the last 50 years, investigations on the plant secondary metabolites have been increased. These molecules are known to play a major role in the adaptation of plants to their environment, but also represent an important source of active pharmaceuticals. According to Bourgaud *et al.* (2001), these molecules have been described as being phytoalexin agents and have antibiotic, antifungal and antiviral activity. In addition they can be toxic for other plants or exhibit allelopathy activity. Plant secondary metabolites are usually classified according to their specific biosynthetic pathways. The major class of these compounds are terpenes (Zwenger & Basu, 2008). Modifications on the triterpenes lead to derivate the cardiac glycoside compounds (Torssell,

1997). During the past decades several therapeutic effects of cardiac glycosides are described. The beneficial effects of cardiac glycosides in the treatment of heart failure are well known (Newman *et al.*, 2008). Furthermore, according to Newman *et al.* (2008), there are several other biological activities such as an emetics, diuretics, a treatment for leprosy, malaria, ringworm and indigestion. Some investigations also illustrated the antibacterial and antiviral activity of isolated cardiac glycosides from natural resources (Akhtar *et al.*, 1992; Filho *et al.*, 2005; Su *et al.*, 2008). Anticancer properties and potential applications for treatment of cystic fibroses are the novel therapeutic functions of cardiac glycosides (Prassas & Diamandis, 2008).

In this study, the leaves of *Gomphocarpus fruticosus*, *Nerium oleander*; and *Cotyledon orbiculata*; the fruits and leaves of *Strophanthus speciosus*; the bulbs of *Bowiea volubilis* and *Merwilla plumbea*, which all contain cardiac glycosides, were subjected to extraction and tested to estimate their antibacterial activity. Some of these plants are used in traditional medicines as treatments for various ailments such as sores, warts, and cancer. Anti-inflammatory, antiseptic and treatment for heart congestive failure are the other therapeutic potentials of these selected plants (Stafford *et al.*, 2005; Aremu *et al.*, 2010). There are some reports about the antibacterial potential of the selected plants. *Merwilla plumbea* has previously been reported to have anti-inflammatory, antischistosomal, anthelmintic and even weak antibacterial activity (Sparg *et al*., 2002). The study of Sparg *et al.* (2002) showed that the ethanolic extract of *M. plumbea* has slight activity against the tested Gram-positive and Gram-negative bacteria. According to the report of Huq *et al.* (1999), a type of cardenolide isolated from the roots of *N. oleander* demonstrated antibacterial activity against *Bacillus subtilis*, *B. cereus*, *Escherichia coli* and *Pseudomonas aeruginosa.* In an investigation on the antibacterial activity of some African medicinal plants, the Gram-negative bacterium, *P. aeruginosa*, was significantly inhibited (MIC = 31 µg/ml) by the n-hexane and methanol extracts of *G. fruticosus* fruit extracts (Madureira *et al*., 2012). The objective of this study was to investigate the antibacterial properties of selected plants containing cardiac glycosides.

3.2 Material and methods

3.2.1 Plant collection and extraction

According to Handa *et al.* (2008), pharmaceutically the definition of extraction is the separation of medicinally active fractions of plant or animal tissues by using selective solvents in standard extraction procedures.

Collected plants were chosen according to the literature based on the presence of cardiac glycosides, their availability and popularity. Freshly collected *N. oleander* and *C. orbiculata* leaves; and fruit and leaves of *S. speciosus* were obtained from the Botanical Garden of the University of Pretoria. Leaves of *G. fruticosus* were collected from Faerie Glen Nature Reserve, Pretoria. Fresh bulbs of *B. volubilis* and *M. plumbea* were collected from the Agricultural Research Council, Vegetable and Ornamental Plant Institute (ARC Roodeplaat-VOPI), Pretoria. A representative of each plant was collected and herbarium voucher specimens were deposited in the H.G.W.J. Schweickerdt Herbarium (PRU) of the University of Pretoria (Table 3.1).

Table 3.1 Information of plant species selected for the study

Family/species	Plant material used	Time of collection	Voucher specimen number
Apocynaceae			
Nerium oleander	Leaves	December/January	117912
Strophanthus speciosus	Leaves, fruits	December/January	117913
Asclepiadaceae			
Gomphocarpus fruticosus	Leaves	December/January	117914
Crassulaceae			
Cotyledon orbiculata	Leaves	December/January	117911
Hyacinthaceae			
Bowiea volubilis	Bulbs	March	117910
Merwilla plumbea	Bulbs	March	117909

All leaf, fruit and bulb samples were extensively washed with tap water to remove dust and all other undesired materials. All washed materials except *C. orbiculata*, *B. volubilis* and *M. plumbea* were dried at room temperature for two to three weeks. Leaves of *C. orbiculata* and bulbs of *B. volubilis* and *M. plumbea* were freeze dried with a VIRTIS BTK-Bench Top K Manifold freeze dryer (Figure 3.1).

Figure 3.1: Freeze-dryer used to dry *C. orbiculata*, *B. volubilis* and *M. plumbea* plant material.

All dried material was ground to a fine powder using a Janke and Kunkel (IKA Labortechnik, Germany) grinder. According to the literature, cardiac glycosides are more soluble in an alcoholic solvent and ethanol extracts extracting more phytochemical compounds than other solvents (Molin, 1986; Ragavendran *et al.*, 2012). Therefore 20 g of all seven ground plant material (air dried of leaves of *G. fruticosus*, *N. oleander*; and *C. orbiculata*; the fruits and leaves of *S. speciosus* and the freeze dried bulbs of *B. volubilis* and *M. plumbea*) and also two fresh bulbs of *B. volubilis* and *M. plumbea* were soaked in ethanol (150 ml) on the shaker for 24 hours. The extracts were then filtered with vacuum filtration. This procedure was repeated twice. Filtered extracts were then concentrated by rotary vacuum evaporation (Büchi R Rotavapor R-200 and Büchi Heating bath B-491). The final weights of the various crude extracts were weighed and percentage yield of all plant extracts was calculated (Table 3.2). All plant extracts were kept in the glass vials in the cold room at 4^{o}C until further use.

Table 3.2 Percentage yield of plant extracts

Plant name	% Yield
Bowiea volubilis (fresh & dry bulb)	2.5%
Cotyledon orbiculata (leaf)	1.79%
Gomphocarpus fruticosus (leaf)	15.39%
Nerium oleander (leaf)	13.32%
Merwilla plumbea (fresh & dry bulb)	5.3%
Strophanthus speciosus (leaf)	10.74%
Strophanthus speciosus (fruit)	7.8%

3.2.2 Bacteria subculture preparation

The pathogenic bacteria used for this study were obtained from the Agricultural Research Council (ARC), Roodeplaat, Pretoria. The Gram-negative bacteria were: *Escherichia coli* (ATCC 25922) and *Klebsiella pneumonia* (ATCC 4352) and the Gram-positive bacteria were: *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212). Stock solution subcultures of bacteria were made by transferring 50 ml (Mueller-Hinton) nutrient broth (Oxoid) (Bouillon, pH 7.3) in sterilized conical flasks and transferring bacteria with a sterilized inoculation loop into the prepared broth and incubating them overnight at 37^{o}C on an electric shaker. Subcultures for the experiment were then further prepared from these stock solutions by preparing 50 ml of nutrient broth in new sterilized conical flasks. A volume of 0.5 ml of the bacteria was then transferred from stock solutions into these flasks with a sterile pipette. The flasks were again put on an electric shaker in the incubator for 24 hours in the dark at 37^{o}C.

3.2.3 Direct bioautography

Direct bioautography is a method to localize antibacterial activity on a chromatogram. Bioautography is a microbial detection method hyphenated with planar chromatography techniques (Choma & Grzelak, 2010). It is based mainly on antimicrobial or antifungal

properties of analyzed substances. This method combines the simplicity of a microbiological method with high sensitivity and specificity of an analytical method (Choma & Grzelak, 2010). Bioautography assays were performed with *E. coli* (Gram-negative bacteria) and *E. faecalis* (Gram-positive bacteria). Bacterial suspensions were diluted with fresh nutrient broth to furnish 0.5 McFarland standards at 540 nm; this is equivalent to approximately10^8 cfu/ml bacterial suspension.

Developed spotted on the thin layer chromatography (TLC) (silica gel G60 F254, Merck) plates with all nine extracts (leaves of *G. fruticosus*, *N. oleander*; and *C. orbiculata*; the fruits and leaves of *S. speciosus*; the fresh and dry bulbs of *B. volubilis* and *M. plumbea*) were dried for removing of the ethyl acetate(10):methanol(1.35):water(1) (specific cardiac glycoside solvent system) and then sprayed with an overnight grown culture of *E. coli* and *E. faecalis*. The plates were incubated for 24 hours at 37^{o}C and then sprayed with an aqueous solution of 2 mg/ml Iodonitrotetrazolium chloride (INT). The areas of inhibition were compared with the related spots on the reference TLC plate. The growth inhibition areas, whitish to yellowish coloured, were compared with the reference of the related spots on the TLC plate revealed with INT reagent. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a purple-colored formazan product by biologically active organisms. In the microtitre plate dilution antibacterial assay where bacterial growth was inhibited, the solution in the well remained clear after incubation with INT (Eloff, 1998). The action of the INT in the direct bioautography test and microtitre plate is similar. In the direct bioautography, after adding the INT on the infected TLC plate with bacteria, the colour of the TLC plate did not change where the bacteria growth was inhibited by the extract. The colour of the rest of the TLC plate turned to the purple where the bacteria grew.

3.2.4 Antibacterial activity assay

3.2.4.1 McFarland standard preparation

McFarland standards provide laboratory guidance for the standardization of the numbers of bacteria colonies for susceptibility testing or procedures requiring a standardization of the inoculums. It is used as a reference to adjust the turbidity of bacterial suspensions (Lennette, 1985). The concentration of all bacteria used for this study matched with the 0.5 McFarland standards (1 x 10^8 colony forming unit per millilitre) (Zaidan *et al.*, 2005; Kumar *et al.*, 2006; Rigano *et al.*, 2007; Ghosh *et al.*, 2008). The 0.5 McFarland standard is formulated

by adding 9.95 ml 1% sulphuric acid to 0.05 ml 1% barium chloride. The turbidity of this mixture is equal to the 0.5 McFarland standard. The absorbance is read using a spectrophotometer at 540 nm.

3.2.4.2 Microtitre plate dilution method

In this study the microtitre plate dilution method according to Eloff (1998) was used. All four bacterial strains (*E. coli*, *K. pneumonia*, *S. aureus* and *E. faecalis*) were compared with the McFarland standard. A serial microdilution assay with INT added as growth indicator was used to determine the minimum inhibitory concentration (MIC) values for the plants extracts.

All the plant extracts were made up to a stock solution of 20 mg/ml dissolved in 10% dimethyl sulphoxide (DMSO) and broth. To all the wells of the plates, 100 µl of nutrient broth was added using a multi-channel pipette with sterilized tips. To the first three wells of row A, 100 µl of the stock solution (20 mg/ml) of extract 1 was added, 100 µl of the stock solution of the extracts 2 and 3 were added, respectively, to the wells 4-6 and 7-9 of row A. To well 10 of row A, 100 µl of the nutrient extract was added, as a medium/sterility control. To well 11 of row A, 100 µl of the antibacterial agent, Tetracycline (0.2 mg/ml), was added as the positive drug control. A series of dilutions were then made by taking 100 µl of the wells in the first row (row A) and transferring it to the second row of wells (row B), where it was mixed and then 100 µl of row B then again transferred to row C and this method of creating doubling dilutions was carried until row H, where 100 µl was taken out of row H's wells and discarded to keep the volumes in all wells equal.

The optical density of all the bacterial cultures (Gram-negative: *E. coli* and *K. pneumonia* and the Gram-positive bacteria: *S. aureus* and *E. faecalis*) prepared according to 3.2.2 was determined. The turbidity of all bacteria cultures was compared to 0.5 McFarland standard at 540 nm. To all the wells in the plate, except column 10 (sterility control), 100 µl of regulated optical density of bacteria cultures were added. The plates were then covered and incubated at 37^{o}C for 24 hours. After 24 hours, 40 µl of INT (0.2 mg/ml) as an indicator of bacterial growth was added to all the wells except for columns 3, 6 and 9 (these wells served as colour controls). The MIC of the different extracts against the tested bacteria was determined according to the colour changes of the INT. The MIC values were recorded as

the lowest concentration of the extract that didn't turn purple in the presence of INT and completely inhibited bacterial growth. This experiment was repeated twice.

3.3 Results and discussion

3.3.1 Direct bioautography

The direct bioautography method carried out in this study is outlined as follows: (1) preparation and application of extracts on TLC; (2) preparation and application of the bacterial inoculums (*E. coli* and *E. faecalis*) to TLC plates; (3) incubation; and (4) growth detection by colorimetric assay (INT) and measurement of growth inhibition areas. The results of the bioautography showed activity of the fruit extract of *S. speciosus* and the bulb extracts of *B. volubilis* against *E. faecalis* (Gram-positive bacteria) (Figure 3.2). *Bowiea volubilis* was more successful to inhibit the bacteria than the *S. speciosus* fruit extract which is comparable with the results of the microdilution method. None of plant extracts were successful to inhibit the Gram-negative bacteria (*E. coli*) because the whole plate turned to the pink colour after spraying the INT (Figure 3.2).

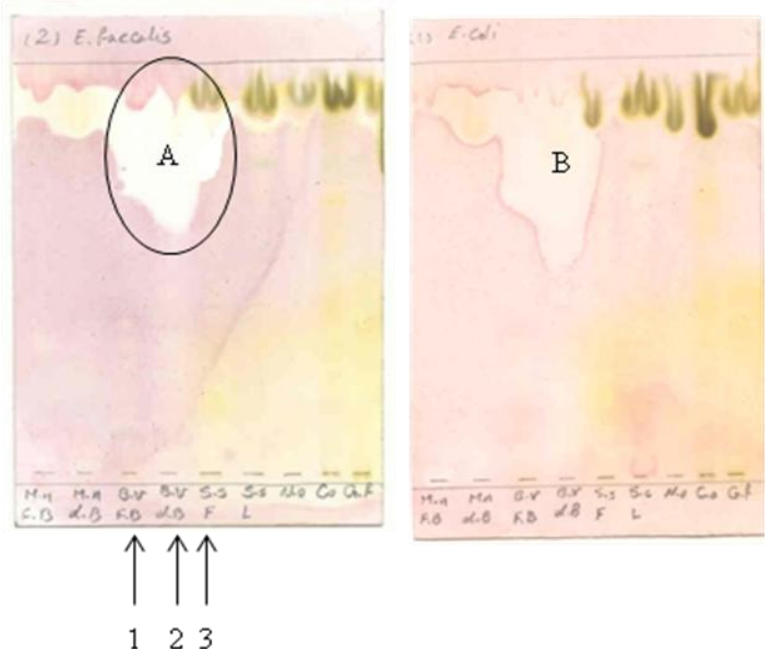

Figure 3.2: A) Chromatogram indicating the zone of bacterial inhibition by plant extracts sprayed with *E. faecalis*. B) TLC of plant extracts sprayed with *E. coli*. 1,2) *B. volubilis* extracts (fresh and dry bulb) 3) *S. speciosus* fruit extract.

The bioautography assay may represent a useful tool for purification of antibacterial substances if tests are performed through the use of chromatograms. Bioautography allows easy localization of activity even in complex substances or compounds as that derived from plant extracts or natural products (Hamburger & Cordell, 1987). Developed chromatogram comparison under identical conditions and visualized with the use of suitable reagent can provide useful information about nature of active compounds.

An advantage of the bioautographic method is the possibility of using mobile phases containing solvents of low volatility, which is completely removed before carrying out tests (Valgas *et al*., 2007). However, too acid or too alkali solvents remain on the TLC plate after a long drying time, inhibiting possible bacterial growth (Hamburger & Cordell, 1987).

3.3.2 Antibacterial activity assay

The MIC values of the ethanolic plant extracts are presented in Table 3.3. Most of the plant extracts tested showed some levels of antibacterial activity. According to Van Vuuren (2008), extracts having activities where MIC values are below 8 mg/ml are considered to possess some antimicrobial activity and natural products with MIC values below 1 mg/ml are considered noteworthy. According to the results, Gram-positive bacteria (*S. aureus* and *E. faecalis*) were more susceptible to the extracts tested than the Gram-negative bacteria (*E. coli* and *K. pneumonia*).

According to the study of Van Vuuren (2008), all tested extracts showed some levels of activity against both Gram-negative and Gram-positive bacteria with the MIC < 8 mg/ml. When comparing all tested plant extracts, *C. orbiculata* extract exhibited the best MIC value to inhibit the *S. aureus* and *E. faecalis* at 1.25 mg/ml. *Bowiea volubilis* dry bulb extract was the most successful extract to inhibit *K. pneumonia* with the MIC value of 2.5 mg/ml. With regards to the inhibition of *E. coli*, three plant extracts; *B. volubilis* (dry and fresh bulb) and *C. orbiculata*; were more successful than the other extracts with 2.5 mg/ml MIC value (Table 3.3).

Tetracycline was used as the positive control. The positive control demonstrated the high antibacterial inhibition activity against all four types of tested bacteria with the MIC < 1 mg/ml. Both Gram-positive bacteria (*S. aureus* and *E. faecalis*) were more susceptible to the

positive control than the Gram-negative ones. The most susceptible bacteria to the Tetracycline was *E. faecalis* with 0.039 MIC value and *K. pneumonia* was the most resistant bacteria to the Tetracycline with the 0.625 MIC value. Figure 3.3 shows a graphical presentation of the microtitre plate dilution, the position of extracts inhibition zones, tetracycline activity and sterility control.

Table 3.3 Minimum inhibitory concentration of South African plants containing cardiac glycosides against Gram-positive and Gram-negative bacteria (MIC recorded in mg/ml)

Plant extracts	*S. aureus* (MIC mg/ml)	*E. faecalis* (MIC mg/ml)	*K. pneumonia* (MIC mg/ml)	*E. coli* (MIC mg/ml)
***B. volubilis* (fresh)**	2.5	2.5	5	2.5
***B. volubilis* (dry)**	2.5	2.5	2.5	2.5
C. orbiculata	**1.25**	**1.25**	5	2.5
G. fruticosus	>5	5	>5	>5
***M. plumbea* (fresh)**	2.5	2.5	>5	5
***M. plumbea* (dry)**	5	>5	>5	>5
N. oleander	5	5	>5	5
***S. speciosus* (leaf)**	>5	>5	>5	5
***S. speciosus* (fruit)**	>5	>5	>5	5
Tetracycline	0.156	0.039	0.625	0.312

According to the results of this experiment, *G. fruticosus* only demonstrated slight activity with MIC values of 5 mg/ml against *E. faecalis* and it was not active against the other three types of bacteria. These outcomes are supported by antibacterial activity studies of McGaw *et al.* (2000) on South African medicinal plants. McGaw and co-workers tested several South African medicinal plants against four different types of bacteria (*Bacillus subtilis, E.*

coli, K. pneumonia and *S. aureus*). The results showed that the ethanolic extract of *G. fruticosus* (leaf) was not active against any of the tested bacteria. Only the leaf water extract of this plant showed activity against *E. coli* with a MIC value 6.25 mg/ml (McGaw *et al.*, 2000).

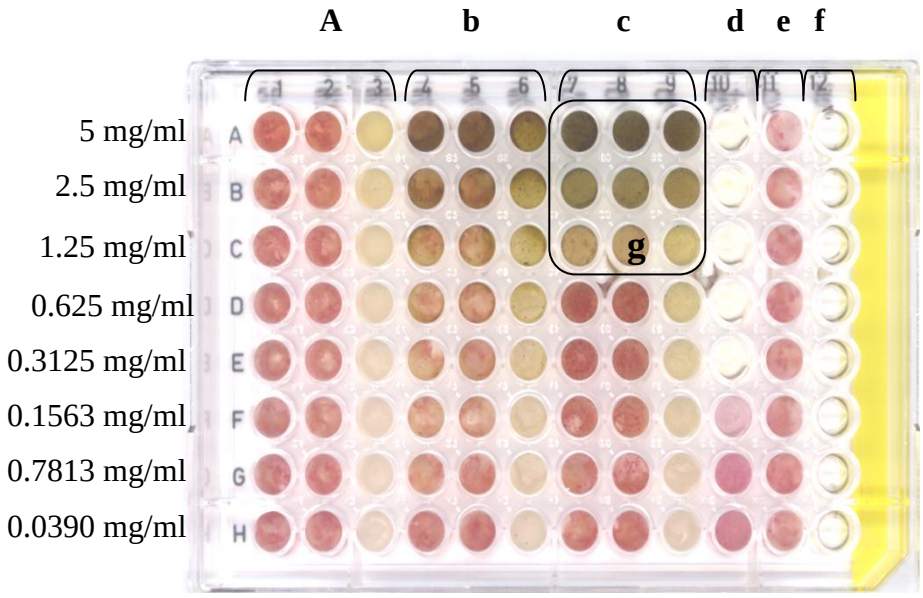

Figure 3.3: The graphical presentation of the microtitre plate test. [a,b,c: three different extracts in triplicate, d: tetracycline (positive control), e: DMSO (solvent control), f: broth (sterility control), g: inhibition zone].

Another study was done on the phytochemical analysis and antibacterial activity of *N. oleander* by Bhuvaeshwari and co-workers (2007). The antibacterial screening disc diffusion method was used to determine the inhibitory potential of *N. oleander* leaf extract with different solvents for extraction. Positive results were established by the presence of clear zones of inhibition around active extracts. The results obtained were compared with that of the zone of inhibition produced by standard antibiotic discs. Out of the seven bacterial species analysed for antibacterial effect, *S. aureus*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* showed better zones of inhibition (10, 9, 7 mm, respectively). From the extracts tested, the ethanolic extract gave better results. This showed that the natural products from the leaves of *N. oleander* have certain active principles responsible for antibacterial activity (Bhuvaeshwari *et al.*, 2007). The *N. oleander* extract examined in this assay exhibited inhibitory activity against all types of bacteria except *K. pneumonia*, but the MIC value obtained (5 mg/ml) was not noteworthy.

An investigation was done by Street *et al.*, (2009) on the effect of cadmium (Cd) uptake and accumulation on the plant growth and biological activity of *M. plumbea* with an increase in the Cd concentration. Antibacterial activity of *M. plumbea* bulb extracts at the zero concentration of Cd, which means the normal bulb, illustrated a MIC value of 6.25 mg/ml against all four types of bacteria (*B. subtilis, S. aureus, K. pneumonia* and *E. coli*). It is interesting to know that with increasing the concentration of Cd, the inhibitory activity of *M. plumbea* bulb extracts against the tested pathogen had increased (Street *et al.*, 2009).

The screening of antibacterial activity of *M. plumbea* was done by Sparg *et al.* (2002). Poor antibacterial activity against both Gram-positive and Gram-negative bacteria was shown with *M. plumbea* extracts. Results of Sparg *et al*. (2002) study showed that the *n*-hexane extract did not have any inhibitory activity (> 12.5 mg/ml) against the four tested bacteria. Poor MIC inhibitory activity was observed for aqueous (*E. coli, K. pneumonia and S. aureus*: 6.3 mg/ml and *B. subtilis*: 12.3 mg/ml) and dichloromethane extracts (*K. pneumonia, S. aureus* and *B. subtilis*: 6.3 mg/ml and *E. coli*: 3.1 mg/ml) of *M. plumbea*. The ethanolic extract demonstrated higher inhibitory activity. There were no differences between the activity against the Gram-positive and Gram-negative bacteria. The ethanolic extract of *M. plumbea* inhibited all four tested bacteria at the MIC value of 1.6 mg/ml (Sparg *et al.*, 2002). The MIC obtained against *S. aureus* and *E. faecalis* was 2.5 mg/ml by the fresh bulb in the present study though the other types of bacteria were not inhibited by the bulb extract of *M. plumbea.*

In an interesting investigation by Stafford *et al*. (2005) on the effect of storage on the chemical composition and biological activity of eight different South African medicinal plants, ethanol and water extracts of fresh and stored (90 days, 1 year and 5 year) *B. volubilis* and *M. plumbea* bulbs were tested against four bacterial pathogens. These extracts were tested initially at 12.5 mg/ml in 96-well microtitre plates. According to the results of this test, the stored ethanol extracts (90 days) of both extracts showed the highest activity against *B. subtilis, S. aureus, K. pneumonia* and *E. coli*. The MIC concentration of fresh extract of *B. volubilis* bulb against *B. subtilis* was 6.25 mg/ml and against the other three bacteria was 3.13 mg/ml. The 90 days stored *B. volubilis* bulb extract inhibited *B. subtilis, S. aureus, K. pneumonia* at 3.13 mg/ml and *E. coli* at 1.56 mg/ml, respectively. The MIC value of the fresh extract of *M. plumbea* against all tested bacteria was 6.25 mg/ml. The 90 days

stored *M. plumbea* bulb extract inhibited the pathogen bacteria at 3.13 mg/ml (Stafford *et al*., 2005). These results pointed out that the dried fresh material and the dried stored material produce more active compounds than the fresh material in the ethanolic extracts. It may be as a result of breaking and degradation of the cell membranes of the plant during the drying process which can release more compounds during extraction (Stafford *et al*., 2005).

Sparg *et al*. (2005) examined the effect of the harvest season of cultivation and plant age on the pharmacological activity of *M. plumbea* bulbs. According to the result of this study, the different harvest seasons had no significant effect against the test bacteria. The increasing age and maturity of the bulbs showed a significant effect on the tested bacterial pathogens. The study of Sparg *et al*. (2005) confirms that the different harvested seasons of the *M. plumbea* bulb would not decrease or increase the antibacterial inhibitory result considerably.

An antibacterial and antifungal assay of traditional South African medicinal plants used against venereal disease was done by Buwa & Van Staden (2006). In this experiment three different solvents (water, ethanol and ethyl acetate) were used for plant extraction. One of these medicinal plants, which was used in the present study, was the *B. volubilis* bulb. The MIC recorded of this screening demonstrated that the ethyl acetate extract of *B. volubilis* bulb did not exhibit activity against the bacteria tested (*B. subtilis, S. aureus, K. pneumonia* and *E. coli*). The MIC value of the water extract against four types of bacteria was > 12.5 mg/ml. The ethanolic extract of *B. volubilis* exhibited the highest activity against all bacteria tested with a MIC of 3.125 mg/ml (Buwa & Van Staden, 2006).

When comparing all the previous results on the antibacterial activity of bulb extracts and the present experiment outcome illustrates that although *M. plumbea* was not active against all types of bacteria and just showed activity against *S. aureus*, the MIC value was near or even better than the previous tests. The reason that no inhibition was observed against *E. faecalis, K. pneumonia* and *E. coli* can be attributed to the stock solution concentration. The extracts tested in this experiment were tested initially at 5 mg/ml, which is lower than the initial concentration of the previous reported tests. As mentioned before, the MIC value of *M. plumbea* against all tested bacteria was 6.25 mg/ml which is higher than the concentration of the extract in the first row of 96-well in this study. Just the stored bulb extract decreased the MIC value of the *M. plumbea* bulb. In the past studies the MIC concentration of *B. volubilis*

fresh bulb extracts was more than 3.13 mg/ml. In comparison to the literature, the obtained results of *B. volubilis* bulb extracts, which was tested in this study showed more inhibitory activity against the bacteria with a MIC value of 2.5 mg/ml.

Aremu *et al.* (2010) evaluated the *in vitro* pharmacological activity and phytochemistry of ten South African medicinal plants used as anthelmintic treatments. Different solvents (petroleum ether, dichloromethane, ethanol and water) were used for making extracts of selected plants. In this experiment also the ethanol extracts showed more activity in general against all pathogens. The MIC value obtained for the *C. orbiculata* leaf extract against *B. subtilis, S. aureus,* and *E. coli* bacteria was 1.56 mg/ml and against the *K. pneumonia* was 3.13 mg/ml (Aremu *et al.*, 2010).

The comparison of the Aremu *et al.* (2010) MIC value of the *C. orbiculata* extract (1.56 mg/ml) and the present study (1.26 mg/ml) shows almost the same MIC value of this plant extract against the Gram-positive bacteria in both studies. Also these results confirm that the Gram-positive bacteria were more inhibited by the *C. orbiculata* extract. These types of bacteria are more susceptible than the Gram-negative bacteria in the presence of this extract. The MIC value of the Gram-negative bacteria was higher (2.5 and 5 mg/ml) than the investigation done by Aremu *et al.* (2010). The reason may be due to the types of bacterial strain or natural variation between plants, time of harvesting, maturity etc.

It seems there is limited research about the antimicrobial activity of *S. speciosus* extracts. The results of this study showed that the *S. speciosus* fruit extract was more active than the leaf extract though its inhibition is not very significant.

According to obtained result of this study, the most susceptible bacteria were Gram-positive and the most resistant *K. pneumonia*. Various studies have already shown that Gram-positive bacteria are more susceptible towards plant extracts as compared to Gram-negative bacteria (Lin *et al.*, 1999; Parekh & Chanda, 2006). The lipopolysaccharides present on the outer membrane of Gram-negative bacteria makes them impermeable to most antibacterial compounds (Clements *et al*., 2002). This can explain the low number of active plant extracts against *E. coli* and *K. pneumoniae*. The observed trend is in agreement with previous research findings by various workers (Lin *et al*., 1999; Tshikalange *et al*., 2005).

3.4 Conclusion

Antimicrobial resistance to antibiotics is emerging as a serious health issue and alternatives to treat infectious diseases in the future need to be developed. Many plants and their isolated compounds have been screened for their possible antimicrobial activity. Anthocyanins, dihydrochalcones, flavones, isoflavones and flavonoids have been reported to possess antimicrobial activity (Rajendran, 2011). In the antibacterial test not only the potential of inhibition of microorganism is important but also some elements such as the storage period and condition of plants, the drying process of plant materials which can affect the chemical composition of the material, the size of the inoculants, the nature of the culture medium, the concentration of the test compound, the pH of the medium, the temperature and time of incubation are the factors which are involved in testing the antimicrobial activity of the plant extract and compounds (Rajendran, 2011). In general, ethanolic extracts show higher inhibitory activity against most bacterial strains (Sparg *et al*., 2002; Stafford *et al.*, 2005).

According to literature, antibacterial extracts seem to become more active during storage and in some cases half concentration of plant extracts from the fresh material was required from the stored material to inhibit bacterial growth. According to the study of Van Vuuren (2008), the plants selected for this study had some levels of antibacterial activity, but comparing their MIC values they did not show noteworthy activity. In the present study the Gram-positive bacteria were more susceptible than the Gram-negative ones.

In conclusion, according to the results presented and the previous tests, it seems that plants containing cardiac glycosides are not very successful in inhibiting the bacterial strains, although some of them like *N. oleander, C. orbiculata* and *B. volubilis* demonstrated antibacterial activity. It could be that the inhibitory activity against bacteria of these plant extracts are not linked to the presence of cardiac glycosides and rather other types of compounds are responsible for their inhibitory activity. It can also be mentioned that further work must be carried out on some of the plants like *S. speciosus* because there is a lack of information on the potential of its biological properties.

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Chapter 4

Cytotoxicity and anti-HSV-2 activity of plants containing cardiac glycosides

4.1 The Herpes Simplex Virus

A virus, which is smaller than a bacterium, invades living cells and uses their chemical machinery to keep itself alive and to replicate itself. It may reproduce directly or with mutation and its ability to mutate is responsible for the virus to change slightly in each infected person making treatment difficult (Wagner *et al.*, 2008).

Herpes Simplex Virus types 1 and 2 (HSV-1 and HSV-2) are clinically significant human pathogens. Both types of this virus are alpha herpes viruses with double stranded DNA surrounded by an icosahedral capsid and a lipidic envelope, which is formed by various glycol-proteins (Dodson *et al.*, 2007; Bertol *et al.*, 2011). The process of HSV infection begins with attachment to a cellular receptor, followed by entry into the cytoplasm via one of a number of host cell-type-dependent pathways such as transferring of the viral nucleocapsid to the nucleus on the cellular microtubule, or uncoating at the nuclear pore to insert the viral DNA and trans-activators into the nucleus (Dodson *et al.*, 2007; Bertol *et al.*, 2011).

According to Khan *et al*. (2005), after establishing the membrane-bound enzymes, HSV is able to reactivate. Primary symptoms of HSV infection are “flu-like” syndromes, with fever, headache, malaise, diffuse myalgias, followed by local symptoms consisting of genital itching, tenderness, lesions, and painful papules over genital regions and ulceration (Khan *et al*., 2005). Among HSV-related pathologies, genital herpes is an important sexually transmitted disease (STD) commonly caused by HSV-2, but in some rare cases it is also caused by HSV-1. In immunocompromised patients and neonates, HSV infections can cause serious systemic illnesses. Herpes Simplex Virus-2 infection can be a risk factor for the transmission of Human Immunodeficiency Virus (HIV). Herpes Simplex Virus is able to produce several optical diseases; such as an immunopathological disease, Herpetic Stromal Keratitis (HSK), which is one of the leading causes of blindness in the western world (Khan *et al.*, 2005).

There are some effective antiherpes drugs, such as acyclovir, ganciclovir, valaciclovir, penciclovir and famciclovir (a prodrug of penciclovir) (Khan *et al.*, 2005). But drug resistant strains of HSV frequently develop following therapeutic treatment. Resistance to acyclovir and related nucleoside analogues can occur following mutation in either HSV thymidine kinase or DNA polymerase. The other serious problem of the use of anti-HSV drugs is that because these types of medicines are very expensive, those patients with frequent attack may not be able to have the funds for the cost of long-term treatment (Khan *et al.*, 2005).

Therefore because of the participation of HSV in some previously mentioned serious diseases and to the problems related to drug resistance, the exploration of novel anti-HSV agents exhibiting different mechanisms of action is essential.

Medicinal plants have been used for the treatment of many infectious diseases for a long time. At present, there is an increasing emphasis on investigating and determining evidence from medicinal plants to treat the infectious disorder. According to literature, there are large numbers of synthetic and plant-derived anti-HSV drugs that have been investigated. For example, a triterpenoid component of *Glycyrrhiza glabra* L. (liquorice root) containing glycyrrhetinic acid has been found to improve the resistance of thermally injured mice to opportunistic infection of HSV-1. Furthermore it was reported that a compound from broccoli (*Brassica oleracea* L.) inhibits HSV (Khan *et al.*, 2005).

Bourne *et al.* (1999) examined 19 plant derived antimicrobial compounds *in vitro* by plaque reduction assay, to determine their anti-HSV-2 activity. Four compounds, carrageenan lambda type IV, cineole, curcumin and eugenol provided significant protection ($P < 0.05$) in a mouse model of intravaginal HSV-2 infection. There are interesting results from *Plantago major* L. for treating several diseases. The antiviral activity of aqueous extracts and pure compounds of *P. major* has been examined and the results illustrated that the aqueous extract of *P. major* exhibited only a slight antiherpes virus activity. However the pure compounds showed potent antiviral activity. Among them, caffeic acid had strongest activity against HSV-1 with an EC_{50} of 15.3 µg/ml (Khan *et al.*, 2005).

According to Khan *et al*. (2005), extracts of *Hypericum mysorense* B. Heyne, *Hypericum hookerianum* Wight and Arn. and *Usnea complanta* (Mill. Arg) Mot. showed significant antiviral activity at a non toxic concentration to the cells. Other examples of plant extracts,

which demonstrated antiviral activity against the HSV, are the extracts from *Scaevole sericea* (Gaertn.) Roxb., *Psychotria hawaiiensis* (A. Gray.), *Pipturus albidus* (Hook. & Arn.) A. Gray and *Eugenia malaccensis* Linn. which showed antiviral activity against HSV-1 and -2 (Khan *et al.*, 2005).

In the present study the ability of the selected plant extracts (leaves of *Gomphocarpus fruticosus* and *Cotyledon orbiculata*; the fruits and leaves of *Strophanthus speciosus*; the bulbs of *Bowiea volubilis* and *Merwilla plumbea*) were examined for their anti-HSV-2 activity. Promising antiviral activity of the cardiac glycoside was demonstrated against viral pathogens such as anti-HSV-1/-2 and HIV, although literature regarding the anti-HSV-2 potential of the selected plant extracts in the literature was only found for *N. oleander* (Su *et al*., 2008; Bertol *et al*., 2011; Singh *et al.*, 2012). According to Van Wyk *et al.*, (2005), all the selected plants are known to contain cardiac glycosides. Therefore, because of the presence of cardiac glycosides in the selected plants, they were examined for anti-HSV-2 activity.

4.1.1 Cytotoxicity

Cytotoxicity is an important factor in understanding the mechanisms of action of chemicals on cells and tissues. Cytotoxicity and use of cell culture systems play an important role in toxicological assessment of new natural products, which they assume to have biological activity such as anticancer and antiviral activity, especially at the early stages of development. Using cytotoxicity assays in a project can result in saving time, money and prevent experimentation on animals. In other words, the development of *in vitro* cytotoxicity assays has been driven by the need to rapidly evaluate the potential toxicity of large numbers of compounds, to limit animal experimentation whenever possible, and to carry out tests with small quantities of compound (Putnam *et al.*, 2002).

There are several methods of colorimetric cytotoxicity assays with different endpoints; such as MTT (3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide), XTT (sodium 2, 3, -bis (2- methoxy-4- nitro-5- sulfophenyl)-5- [(phenylamino)- carbonyl]- 2H-tetrazolium inner salt), Resazurin and LDH (lactate dehydrogenase). MTT and XTT measure the activity of living cells by mitochondrial dehydrogenases. The XTT assay differs from the MTT assay by producing orange formazan crystals, which are soluble in aqueous solution.

The Resazurin assay can determine metabolic activity of living cells and LDH assesses the membrane integrity via direct damage to the cell plasma membrane (Putnam *et al.*, 2002). According to Berridge *et al*. (2005), the XTT cell proliferation assay was first described in 1988 by Scudiero *et al*. as an effective method to measure cell growth and drug sensitivity in tumour cell lines. Tetrazolium salts have been widely used as detection reagents for many years in histochemical localization studies and cell biology assays (Berridge *et al*., 2005).

The tetrazolium dye, XTT can be effectively used in cell based assays to measure cell growth, cytotoxicity, and apoptosis assays. XTT is a colourless or slightly yellow compound that it is reduced to a soluble, brightly coloured orange derivative by a mix of cellular effectors. This colour change is accomplished by breaking apart the positively-charged quaternary tetrazole ring. The sensitivity of an XTT assay is greatly improved by the usage of an intermediate electron carrier, PMS (N-methyl dibenzopyrazine methyl sulphate). PMS helps drive XTT reduction and the formation of its formazan derivative (Berridge *et al*., 2005). Formazan production is indicative of the number of viable cells therefore an increase or decrease in cellular viability results in a change in the amount of formazan formed, including the degree of cytotoxicity caused by the test compound (Sigma-Aldrich, 2010). The mechanism of action of the XTT assay is by measuring the activity of living cells by assessing the activity of mitochondrial dehydrogenases. Mitochondrial oxidoreductases are thought to contribute substantially to the XTT response with their reductants being transferred to the plasma membrane (Figure 4.1).

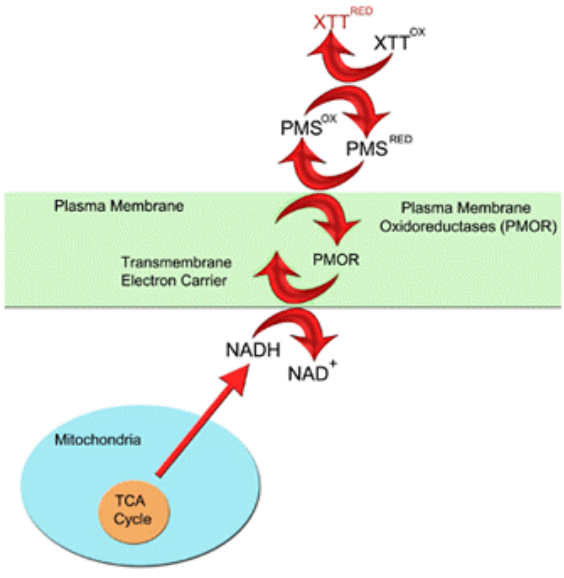
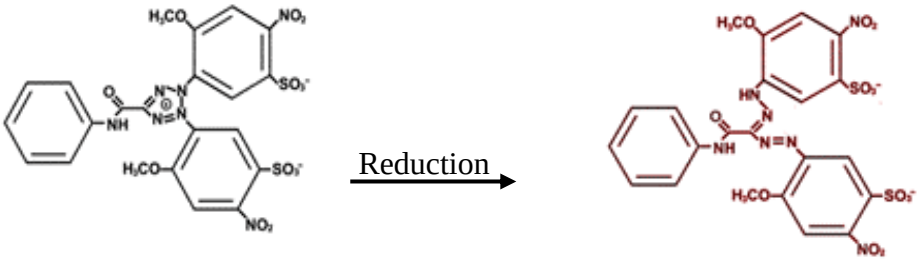


Figure 4.1: The colorimetric reduction of XTT by cellular enzymes (ATCC, 2012).

In this experiment mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring by producing orange formazan crystals, which are soluble in aqueous solutions (Figure 4.2) (Sigma-Aldrich, 2010). The existence of cardiac glycosides in some plant species often indicates toxicity. The aim of this study was to determine the cytotoxicity and antiviral activity of the selected plants extracts.



XTT Formazan Derivative

Figur 4.2: The reduction of XTT to the coloured formazan derivative (ATCC, 2011).

4.2 Materials and methods

4.2.1 Plant extracts, cells and viruses

- **Plant extracts**

  All nine plants extracts were used for the general cytotoxicity and anti-HSV-2 tests. Extracts were made according to the extraction procedure as described in Chapter 3.

- **Cells and virus**

  The Human Embryonic Kidney (HEK 293) cell line (CRL-1573) was obtained from American Type Culture Collection (ATCC) (The Global Bioresource Centre). The Vero cell line (Kidney epithelial cells extracted from an African Green Monkey) was obtained from the Centre of Vaccine and Immunology at the NICD (National Institute for Communicable Diseases). The cytotoxicity test was done at the cell culture laboratory, Department of Plant Science, University of Pretoria. The HSV-2 virus was a clinical isolate. The anti-HSV-2 test was performed at the Department of Virology, National Health Laboratory Service, University of KwaZulu-Natal.

4.2.2 Cytotoxicity

4.2.2.1 Screening assay

The toxicity screening of plant extracts was conducted based on the XTT sodium salt method. The cytotoxicity of the ethanol extracts were measured by the XTT colourimetric assay based on the bio reduction of the XTT salt using the Cell Proliferation Kit II (Roche Diagnostics). This method was described by Zheng *et al.* (2001).

The Vero cells were seeded (100 µl) onto a 96-well microtitre plate at the concentration of 1.0 x 10^5 cells/ml. The extracts were each prepared to a stock solution of 20 mg/ml. Two concentrations were used per sample; 100 µg/ml and 50 µg/ml; in triplicate. Nine samples were tested in each 96-well plate. Dimethyl sulfoxide (2%) served as a negative control. Actinomycin D with concentrations ranging between 0.05 and 0.025 µg/ml was used as the positive control. After the 72 h incubation period the XTT reagent (50 µl) was added to all the wells and the tray was then further incubated for 3 h to allow XTT formazan production. The absorbance of the colour complex was read at 490 nm with a reference wavelength set at 690 nm using a BIO-TEK Power-Wave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa).

4.2.2.2 *In vitro* cytotoxicity assay: Determination of IC_{50}

In order to evaluate the cytotoxicity effect of the nine ethanol extracts, the XTT colourimetric assay was performed on the HEK 293 cell line. This assay is based on the ability of living cells to reduce the yellow water-soluble XTT into an insoluble orange formazan product due to the amount of mitochondrial succinate dehydrogenase enzyme that is present in the cells. The reduction can only take place in viable cells (Berridge *et al.* 1996).

The HEK 293 cells were seeded (100 µl) onto a 96-well microtitre plate at the concentration of 1.0 x 10^5 cells/ml. The plate was then incubated for 24 h at 37^{o}C and 5% CO_2 to allow the cells to attach to the bottom of the wells. The extracts were each prepared to a stock solution of 20 mg/ml and added to the microtitre plate. Serial dilution concentrations were made from 400 µg/ml to 3.125 µg/ml. The microtitre plate was incubated for a further 72 h. Dimethyl sulfoxide (2%) (sample concentration of 400 µg/ml) was used as a negative

control. Actinomycin D with concentrations ranging between 0.05 µg/ml to 39 x 10^{-5} µg/ml was used as the positive control.

After the 72 h incubation period the XTT reagent (50 µl) was added to all the wells and the tray was then further incubated for 3 h to allow XTT formazan production. After the incubation the absorbance of the colour complex was read at 490 nm with a reference wavelength set at 690 nm using a BIO-TEK Power-Wave XS multi-well plate reader.

The assay was performed in triplicate to calculate 50% inhibitory concentration (IC_{50}) of the cell population for each extract. The results were analysed using the GraphPad Prism 4 statistical program (Version 4 Graph Pad Software, San Diego, CA, USA) (Chiang *et al*., 2003).

4.2.3 Anti-HSV-2 activity of selected plant extracts

The antiviral activity of the selected plant extracts against the HSV-2 virus was evaluated by the XTT method. The trypsin treated Vero cell line were seeded onto 96-well plates with a concentration of 1.0 x 10^5 cells/ml and a volume of 70 µl per well. After incubation at 37^{o}C with 5% CO_2 for 6 h, 20 µl of the test virus was added and incubated for another 2 h. Different concentrations of the extracts were then added to culture wells in triplicate (Chiang *et al*., 2003). The experiment was started with 2.5 mg/ml (highest concentration) and diluted with 10 fold dilutions on a logarithmic scale i.e. 1/10, $1/10^2$, $1/10^3$, $1/10^4$, $1/10^5$, 1.10^6, $1/10^7$.

The maximum concentration of dimethyl sulphoxide (DMSO) (0.1%) was used as a negative control. Actinomycin D was used as a positive control. After incubation at 37^{o}C with 5% CO_2 for 3 days, a mixture of 0.1 ml phenazin methosulphate (PMS; electron-coupling reagent) and XTT 1 mg/ml was added to each well with a volume of 50 µl. The trays were further incubated for 2 h to allow XTT formazan production. The absorbance was determined with the ELISA reader at a test wavelength of 450 nm and a reference wavelength of 690 nm.

Viral inhibition rate was calculated as $(A_{tv} - A_{cv}) / (A_{cd} - A_{cv})$ x 100%. A_{tv} indicates the absorbance of the test compounds with virus infected cells. A_{cv} and A_{cd} indicate the

absorbance of the virus control and the absorbance of the cell control, respectively. The antiviral concentration of 50% effectiveness (EC_{50}) was defined as the concentration which achieved 50% inhibition of virus-induced cytopathic effects (Chiang *et al*., 2003).

4.3 Results and discussion

4.3.1 Screening assay

The screening test does not show the exact IC_{50} values. This test was done to demonstrate whether the tested extracts were toxic or not and/or to give an idea about the ranges of cytotoxicity of the plant extracts for further investigation. The result of this assay illustrated that all plant extracts were toxic to the Vero cell line. The IC_{50} values of all tested extracts, except the *M. plumbea* (fresh bulb), was below 50 µg/ml. *Merwilla plumbea* (fresh bulb) extract showed less toxicity than the other extracts with an IC_{50} between 50 µg/ml to 100 µg/ml (Table 4.1). For reaching the accurate IC_{50} values of all extracts the *in vitro* cytotoxicity test was conducted.

Table 4.1 Screening test of selected plant extracts against the Vero cell line

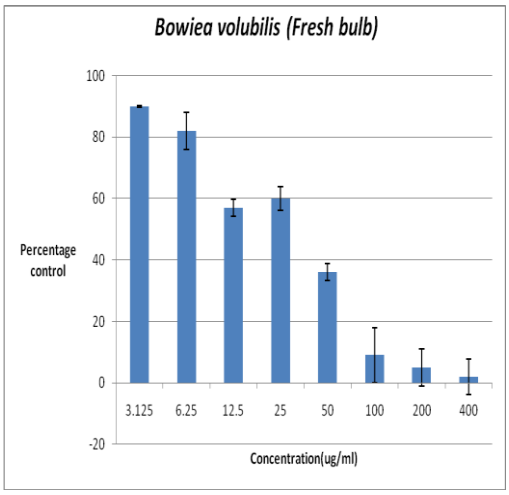
Plant extracts	Vero cell IC_{50} (µg/ml)
***B. volubilis* (fresh)**	<50
***B. volubilis* (dry)**	<50
C. orbiculata	<50
G. fruticosus	<50
***M. plumbea* (fresh)**	50<x<100
***M. plumbea* (dry)**	<50
N. oleander	<50
***S. speciosus* (leaf)**	<50
***S. speciosus* (fruit)**	<50

4.3.2 *In vitro* cytotoxicity: Determination of IC_{50}

Before conducting the antiviral activity test, the cytotoxicity of the extracts on the HEK 293 and Vero cell lines was studied. An antiviral drug should be active against the virus without inducing significant toxic effect on the host cell (Gebre-Mariam *et al.*, 2006). A change in colour is observed after an incubation period of 2 hours due to the addition of the XTT reagent.

According to Raghunandan *et al.* (2011), the orange formazan product is spectrophotometrically quantified using an ELISA plate reader at a wavelength of 490 nm. A low absorbency value indicates a decrease in the rate of cell proliferation and a reduced amount of viable cells. On the contrary, a high absorbency value indicates more viable cells are present and there is an increase in cell proliferation. According to Atjanasuppat *et al.* (2009), the cytotoxicity activity research of extracts is categorized by IC_{50} into four groups, i.e., $x \leq 20$ µg/ml: highly toxic, $20 < x < 100$ µg/ml: quite toxic, $100 < x < 1000$ µg/ml: slightly toxic, and $x > 1000$ µg/ml: non toxic. The cytotoxicity activity of the nine plant extracts are shown in Figure 4.3 and Table 4.2.

In this study, the IC_{50} values illustrated that all the plant extracts were toxic against the Vero and HEK 293 cell line. However the *M. plumbea* fresh bulb extract was less toxic than the rest of extracts. There are numerous reports about the *in vitro* cytotoxicity of the plants containing cardiac glycosides. In an investigation on the antiviral activities of medicinal plants of southern Nepal, *Carissa carandas* L. belonging to the Apocynaceae family, showed the lowest concentration of extract to produce a cytotoxic effect on the Vero cell line (25 µg/ml IC_{50} value) and had cytotoxic effects. This plant species is known to be poisonous due to containing cardiac glycosides, which could be the source of cytotoxicity (Taylor *et al.*, 1996). Sparg *et al.* (2002) tested aqueous extracts of *M. plumbea* for assessing cytotoxicity using the tetrazolium salt reduction (MTT) assay. Secondary Vervet Monkey Kidney cells (VK) were treated with *M. plumbea* extracts from 3.9 mg/ml up to 1000 mg/ml. The results showed this extract to be extremely cytotoxic to VK cells. Optical examination of the monolayer of VK cells showed morphological changes and cell death when treating with this extract. There was severe cytotoxicity at all concentrations of the extract tested (Sparg *et al.*, 2002).



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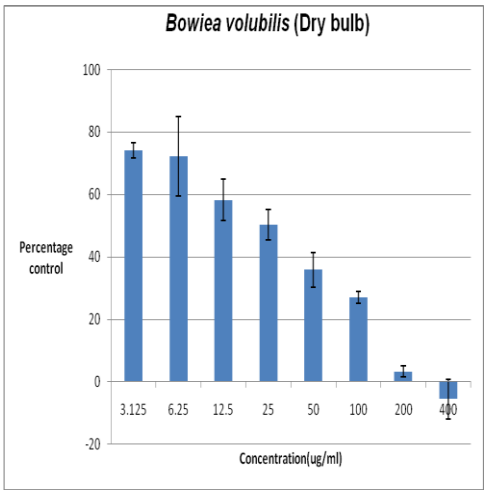

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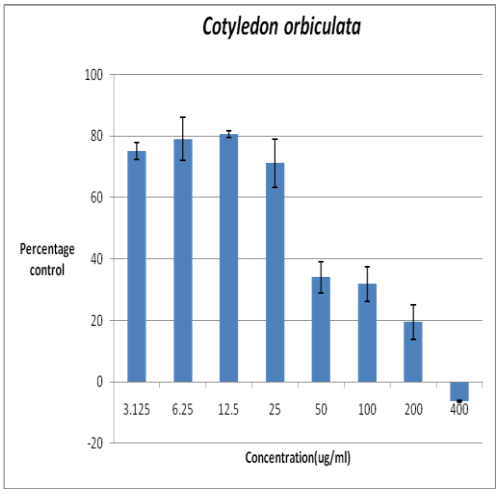

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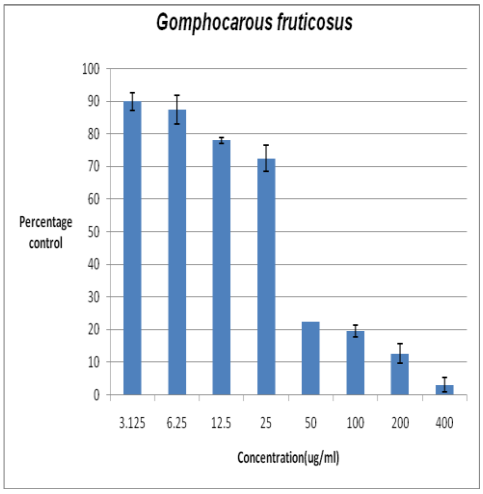

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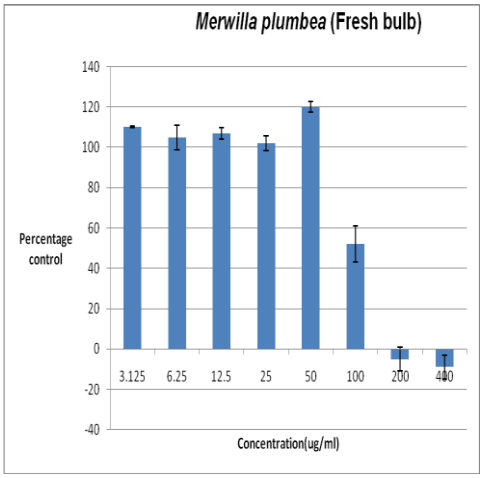

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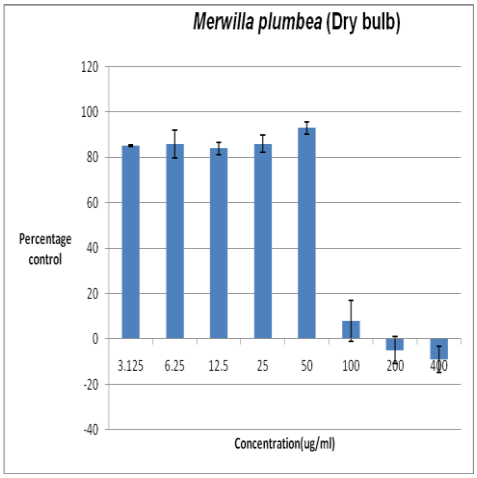

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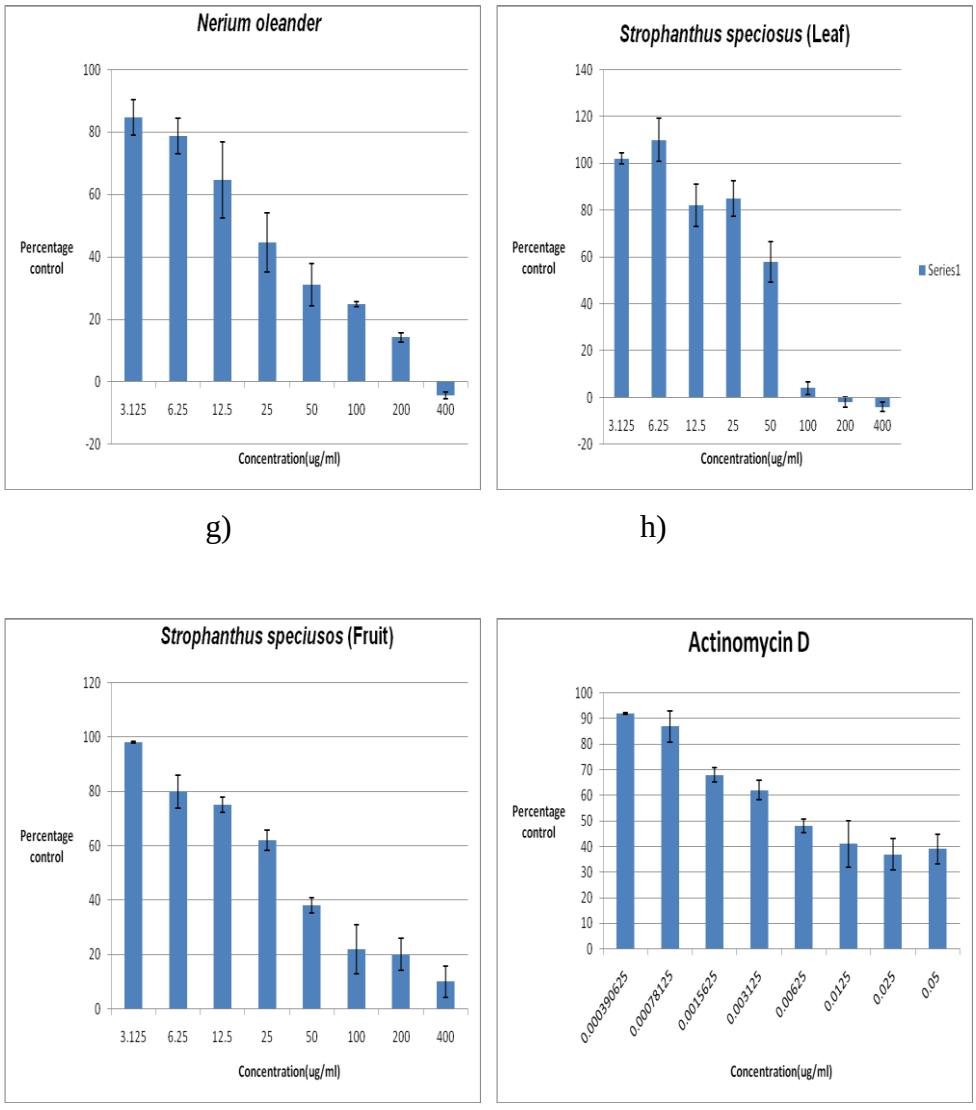

g) h)

i) j)

Figure 4.3: Dose response curve of cytotoxicity results of plant extracts against HEK 293; a) *Bowiea volubilis* (fresh bulb); b) *Bowiea volubilis* (dry bulb); c) *Cotyledon orbiculata*; d) *Gomphocarpus fruticosus*; e) *Merwilla plumbea* (fresh bulb); f) *Merwilla plumbea* (dry bulb); g) *Nerium oleander*; h) *Strophanthus speciosus* (fruit); i) *Strophanthus speciosus* (leaf); j) Actinomycin D.

The poisoning effect of oleander (*N. oleander*) has been reported in man and animals. All parts of the plant, including the leaves, flowers, and twigs, are poisonous and contain cardiac toxins such as oleandrin, neriin, folinerin, digitoxigenin, and nerigoside. In the inhibition of Na^+/K^+-ATPase catalytic activity assay, the 50% inhibition concentration (IC_{50}) values (µmol/L) of these compounds were 0.22, 0.62, 1.23, and 2.69 µmol/L, respectively (Jortani *et al.*, 1996).

Siddiqui *et al.* (2012) reported two cytotoxic isolated compounds from *N. oleander* leaves, cis-karenin and trans-karenin, with ED_{50} value of 15.0 and 7.0 µg/ml, respectively. Another significant cytotoxic compound with ED_{50} <0.1 µg/ml for the KB (oral carcinoma) cell line was identified as oleandrin.

Table 4.2 The cytotoxic activity of plant extracts against HEK 293 cells

Plant extracts	HEK 293 IC_{50} (µg/ml) ± SD
B. volubilis (fresh)	28.83±0.6
B. volubilis (dry)	23.34±0.58
C. orbiculata	44.63±1.9
G. fruticosus	48.46±1.24
M. plumbea (fresh)	97.06±31.04
M. plumbea (dry)	48.06± 8.4
N. oleander	24.89±0.44
S. speciosus (leaf)	57.95±7.57
S. speciosus (fruit)	56.15±2.24
Actinomycin D	0.007353

4.3.3 Anti-HSV-2 assay

The anti-HSV-2 investigation result was obtained from a laboratory facility in the Department of Virology, University of KwaZulu-Natal where the antiviral activity of the plant extracts was determined. Due to the extensive concentration dilution in a 10 fold reduction series on a logarithmic scale (from 2.5 mg/ml to 25 x 10^{-7} mg/ml) no results were obtained. It is possible that the virus inhibitory activity (EC_{50} value) and cytotoxic activity (CC_{50} or IC_{50} value) of the plant extracts were so close, and because of the broad range

between each dilution, the activity of the plant extracts was not observed. According to the literature, the cytotoxicity of plants containing cardiac glycosides have been recognized but there is a lack of evidence and information on the antiviral investigation especially anti-HSV-2 activity of the plants extracts tested in this study. Most of the studies have been done on the *N. oleander* extract and it needs further investigation on the antiviral properties of the plants containing cardiac glycoside however some of the investigations are able to support the anti-HSV property of cardiac glycosides (Su *et al*., 2008).

An aqueous extract of *N. oleander*; namely Anvirzel, was evaluated on HIV infection of human peripheral blood mononuclear cells. The anti-cancer property of oleandrin, the principle cardiac glycoside in Anvirzel, has been detected but its efficacy against HIV is unknown. Treatment with oleandrin significantly reduced the infectivity of virus produced from infected cells without any change in the total amount of virus produced. This is in contrast to treatment with azidothymidine (AZT); the common treatment for patients with HIV and HIV-Related Complex; a potent inhibitor of HIV replication that has been shown to significantly reduce virus production. Relative to untreated cultures, virus in cultures treated with oleandrin had significantly reduced expression of the envelope protein and suggesting a novel mechanism of action of infectivity. These results exhibited the potential value of the *N. oleander* aqueous extract, containing oleandrin as a novel candidate with anti-HIV therapeutic properties (Singh *et al*., 2012).

In an antiviral investigation on the medicinal plants of southern Nepal, the methanol extract of *C. carandas* was the most active, showing activity against HSV, Sindbis virus and Poliovirus at a concentration of 12 µg/ml. The result of the cytotoxicity on the Vero cell line showed that *C. carandas* had the lowest concentration (25 µg/ml) and was the most toxic plant extract. The chemical analysis of this plant extract showed the presence of cardiac glycosides, which can be the source of cytotoxicity. The extract of *Streblus asper* Loureiro exhibited significant antiviral activity. This plant extract also contain cardiac glycosides (cardenolides) (Taylor *et al*., 1996). According to literature the cardiac glycosides have an antiviral therapeutic activity (Su *et al*., 2008; Bertol *et al*. 2011).

Van Vuuren (2008) revealed that a significant MIC value for an extract is below 8 mg/ml, which is considered as an antimicrobial agent and pure compounds with MIC value lower

than 1 mg/ml are considered noteworthy. According to these MIC values, the interaction of the different types of compounds in an extract can decrease the inhibitory activity of the extract comparing to just individual compound. Therefore, the presence of various types of compounds other than those of the cardiac glycosides in the plant extract may have had a combined inhibitory effect on the antiviral activity of the cardiac glycosides.

4.4 Conclusion

Herpes Simplex Virus infection is a major contagion in immunosuppressed persons. Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) is a serious disease where infections due to HSV have risen significantly. The development of resistant strains of HSV to the available drugs such as acyclovir, mainly among immunocompromised patients, makes this situation problematic. There is thus an urgent need to identify and develop new alternative agents for management of HSV infections (Tolo *et al.*, 2005).

No evidence could be found in the literature of the selected plant extracts for cytotoxicity on the HEK 293 cell line. Most of the cytotoxicity investigations were done on the Vero cell line. Cytotoxicity assays showed a high toxic effect of the tested extracts against the HEK 293 cell line except in the case of *M. plumbea* fresh bulb extract, which showed a moderately toxic effect on the cell line. The screening assay on the Vero cell line and *in vitro* cytotoxicity assay on the HEK 293 cell line results were similar and exhibited almost the same toxicity of the plants tested in this study.

Because of the high cytotoxic activity of plant extracts and extensive concentration dilutions, no anti-HSV-2 activity was obtained. As no results were obtained for the anti-HSV-2 experiment we can theorise that a low SI value would most likely be obtained based on the high cytotoxicity values and expected (but not obtained) HSV-2 inhibition values.

Therefore, for the further investigation to discover the possible anti-HSV-2 activity of plants containing cardiac glycosides it would be suggested to decrease the range of dilution to start at 150 µg/ml with 8 dilutions ending at 0.58 µg/ml. Furthermore the experiment should be conducted with an isolated cardiac glycoside compound to ensure that the activity of the cardiac glycoside is not affected by the presence of other compounds in the extract.

4.5 References

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Chapter 5

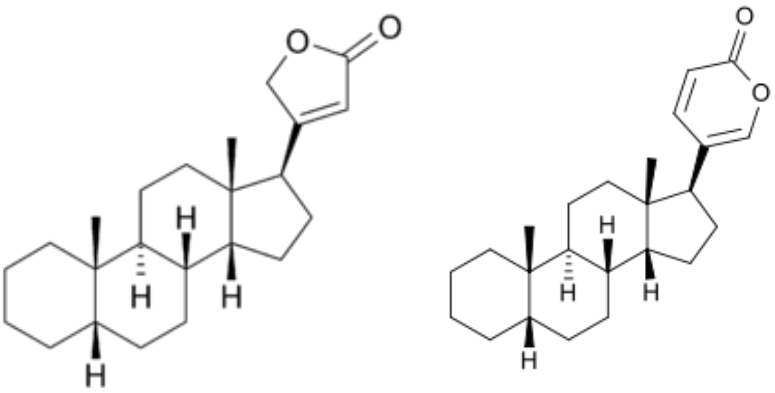
Isolation and identification of compounds from *Strophanthus speciosus* fruit extract and antibacterial and cytotoxicity activity of an isolated cardiac glycoside

5.1 Introduction

South Africa, which contains about 4000 species of plants with medicinal values, has one of the richest and diverse flora in the world. Since prehistoric till the modern time, plant extracts have been used to treat a wide variety of ailments. The organic compounds isolated from natural sources are known as natural products or secondary metabolites (Herbert, 1994). Plant secondary metabolites, based on their biosynthetic origin, can be derived into three main groups: the terpenoids, the alkaloids (nitrogen-containing compounds) and the phenolic compounds (Taiz & Zeiger, 2006). Plant-derived products contain a great diversity of phytochemicals such as phenolic acids, flavonoids, saponins, cardiac glycosides and other small compounds. These compounds possess numerous health-related effects such as antimutagenic, anticarcinogenic, antibacterial and antiviral activities (Cowan, 1999).

Modification of triterpenes leads to the derivation of the cardiac glycoside compounds which are one of the subgroups of steroids. Cardiac glycosides are divided into two different major groups; namely the cardenolides and bufadienolides (Figure 5.1). These types of compounds are produced by a variety of plant and animal species and include compounds such as oleandrin, cymarin, digitoxin, digoxin and strophanthidin. These compounds share the common features of a steroidal aglycone linked at the 3β-OH group to one or more sugar moieties (Shi *et al*., 2010).

Although some of cardiac glycosides are used as treatment for congestive heart failure disorder, they are highly toxic to humans and animals (Filigenzi *et al.*, 2004). These compounds such as digoxin, digitoxin and ouabain are able to inhibit the Na^+K^+ATPase and are widely used in the treatment of heart failure. Recently, other important biological activities of these compounds such as anticancer and antiviral activity have been described but the toxicity of these compounds limit their extensive use (Karkare *et al*., 2007; Su *et al*., 2008).



Cardenolide **Bufadienolide**

Figure 5.1: Structure of two classes of cardiac glycosides: cardenolides and bufadienolides.

As far as identification of bioactive anti Herpes Simplex Virus (HSV) molecules and structure-activity relationship studies are concerned, several reports of pure compounds from natural products demonstrating promising anti-HSV activities are noted. These bioactive compounds are polyphenolics, glycosides, terpenes (monoterpenes, diterpenes and triterpenes), polysaccharide, polyketides and pheophorbides (Khan *et al.*, 2005). Bertol *et al.* (2011) screened 65 cardenolide compounds obtained from plants for anti HSV-1 and HSV-2 activity. Among them a cardenolide isolated from *Digitalis latana* Ehrh. showed lower IC_{50} against HSV-1 and -2.

The bioautography results in Chapter 3 indicated that the fruit extract of *Strophanthus speciosus* against *Enterococcus faecalis* (Gram-positive) bacteria. This resulted in the isolation of the compounds from the *S. speciosus* fruit extract. In this study chromatographic methods were used to identify secondary metabolites from *S. speciosus* fruit extracts with special focus on cardiac glycosides. Furthermore, studies were done to evaluate the antibacterial and cytotoxic activity of the isolated cardiac glycoside.

5.2 Material and methods

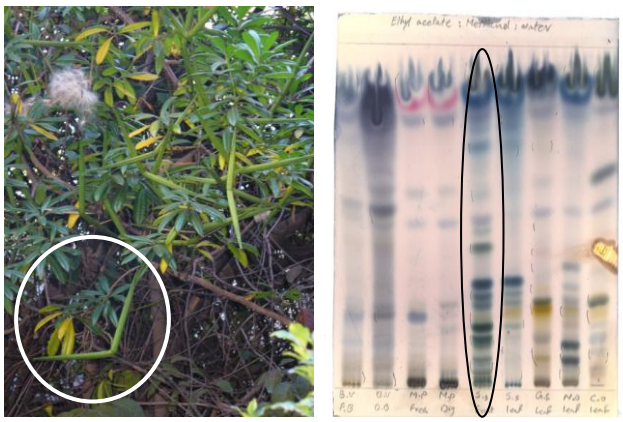
5.2.1 Collection and extraction of *S. speciosus* fruit

Strophanthus speciosus fruit were collected from the Botanical Garden of the University of Pretoria during December and January. These fruits were washed and left to completely dry in the room temperature. The dried material was ground to a fine powder using a Janke and

Kunkel (IKA Labortechnik, Germany) grinder. According to Eloff (1998), ethanol, acetone, and methanol are the well known organic solvents to extract bioactive compounds. Ethanol is the most commonly used organic solvent by herbal medicine companies because the finished products can be safely used internally by consumers of herbal extracts (Wendakoon *et al.*, 2011). Therefore, dried fruits of *S. speciosus* (250 g) were extracted twice with 100% ethanol, each time with 2 L of ethanol for 48 hours. The total ethanolic extracts were combined and evaporated under reduced pressure on a rotary evaporator (Büchi R Rotavapor R- 200 and Büchi Heating bath B- 491). The extract was stored in the cold room until used.

5.2.2 Thin layer chromatography

A thin layer chromatography (TLC) plate was performed on all nine extracts of the six selected plants (the leaves of *Gomphocarpus fruticosus*, *Nerium oleander*; and *Cotyledon orbiculata*; the fruits and leaves of *Strophanthus speciosus*; the dried and fresh bulbs of *Bowiea volubilis* and *Merwilla plumbea*) using a specific cardiac glycoside solvent system: ethyl acetate(10):methanol(1.35):water(1).



A B

Figure 5.2: A) *S. speciosus* fruit. B) TLC plate showing separated bands of the *S. speciosus* fruit extract.

The tests showed more variation of compounds in the fruit extract of *S. speciosus* (Figure 5.2). According to this result and also the results of the bioautography of the plant extracts in Chapter 3, *B. volubilis* and *S. speciosus* were candidates for the isolation of potential antimicrobial compounds. According to Raimonda *et al*. (2009), *B. volubilis* is indicated as a vulnerable taxon (Status: VU A2ad), which means that this taxon is facing a high risk of

extinction in the wild. The population of *B. volubilis* has been rapidly reduced in relation to its life history. *Strophanthus speciosus* is indicated as least concern (LC) in the category of the red list of South African plants (Raimonda *et al*., 2009). With this in mind and also considering the percentage yield of these plant extracts (fresh and dry bulb of *B. volubilis*: 2.5% and fruit of *S. speciosus*: 7.8%) obtained in Chapter 3, Section 3.2.1, the fruit extract of *S. speciosus* was selected for compound isolation.

5.2.3 Extraction and fractionation of *S. speciosus* fruit extract

The total concentrated extract (50 g) was subjected to fractionation on a silica gel column (10 cm x 70 cm) (Figure 5.3), using hexane/ethyl acetate mixtures of increasing polarity (0 to 100%) and ethyl acetate/methanol (MeOH) mixtures also of increasing polarity. A total of 100 fractions were collected.

Figure 5.3: First silica gel column for concentrated *S. speciosus* fruit extract.

The fractions with similar TLC profiles were combined and lead to 26 major fractions. The TLC of the 26 pooled fractions was developed with ethyl acetate (10): methanol (1.35):water (1) as eluent and then observed under UV (245 and 366 nm) after development and also dipped in vanillin (acidic vanillin: 0.34% vanillin in 3.5% sulphuric acid in ethanol) and heated to detect compounds, which do not absorb UV light (Figure 5.4).

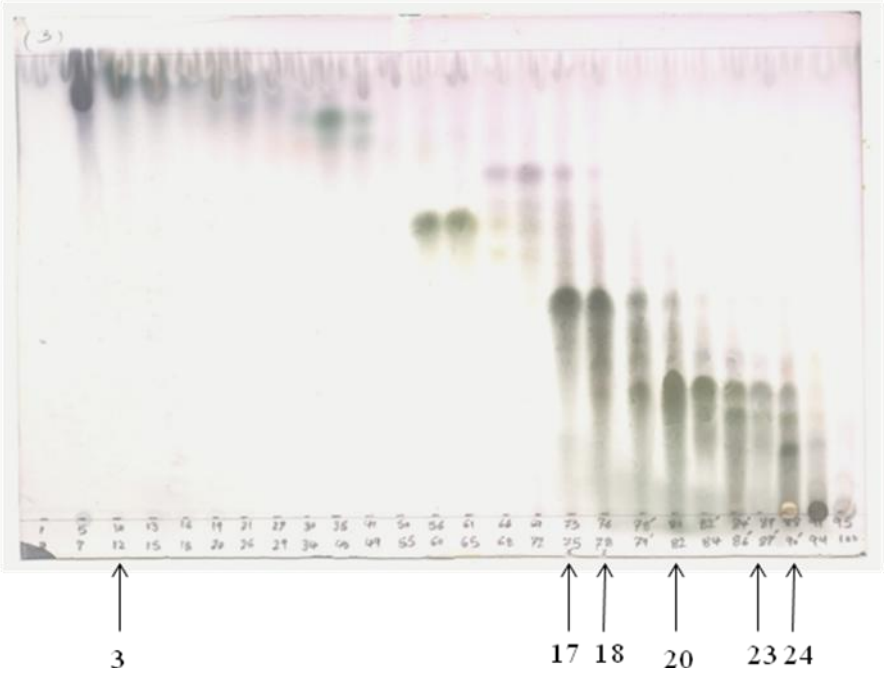


Figure 5.4: The TLC result of 26 major fractions.

To investigate the pure compound of major fraction 3 (0.34 g), a silica gel column was utilized. Hexane: ethyl acetate (8:2) was used to run the column. This solvent system was also used to develop the TLC plate. Twenty nine sub fractions were collected, spotted on TLC plates and developed in hexane: ethyl acetate (9:1) solvent, yielding pure compound **A** (0.34 g).

Major fraction 23 was evaporated after which ethyl acetate was added and the fraction re-dissolved and left in the flask until white crystals were formed (0.177 g). Fraction 24 was separated on a silica gel column eluted with 100% dichloromethane (DCM) and then DCM:MeOH (99:1) with increasing polarity. Fifty sub fractions were collected and spotted on the TLC plates and developed in ethyl acetate(10):methanol (1.35):water(1). Due to evaporation of sub-divisions of fraction 24, three sub fractions, which had similar TLC profiles, were combined and evaporated until white crystals were formed (250 mg) (Figure 5.5). The purification of these two major fractions led to the isolation of pure compound **B**.

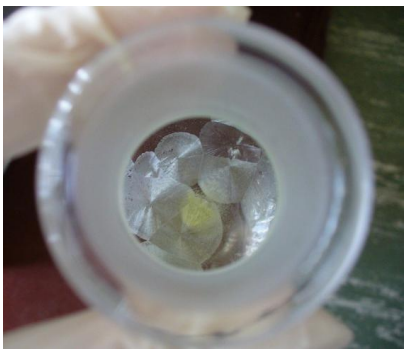


Figure 5.5: Crystals produced from fraction number 24.

Based on the result of the initial TLC outline of the 26 major fractions and the availability of extensive amounts of them; combined fractions 17-18 (1 g) and 20 (1.1 g) were separately subjected to the second silica column chromatography. The combined fraction 17 and 18 were subjected to silica gel column chromatography, using DCM(97):MeOH(3) as solvent. The percentage of methanol was increased to 15% and finally 100% methanol was used. One hundred and fifty sub fractions (50 ml) were collected. These fractions were also combined from the results of the TLC leading to 20 sub fractions. The pooled sub fractions 4 and 5 had the highest mass and thus were chosen for separation on the sephadex column (Sigma-Aldrich, South Africa). The compounds were further separated by 100% methanol, which yielded a pure compound. Separation of the compounds in fraction 20 was done by the silica gel column chromatography using 500 ml of DCM:MeOH (5%) and DCM:MeOH (10%). Ethyl acetate(10):methanol(1.35):water(1) was used for developing the spotted fractions on the TLC plate. After combining the same fractions and evaporating them, the crystallized fraction was subjected to the Nuclear Magnetic Resonance (NMR) identification. Purification of these three major fractions led to isolate pure compound **C**.

Nuclear magnetic resonance spectroscopy and High Resolution Mass Spectrometry (HRMS) were used to elucidate the structures of the compounds. Figure 5.6 represents the schematic presentation of the isolation steps of the ethanolic extract of the fruit extract of *S. speciosus*.

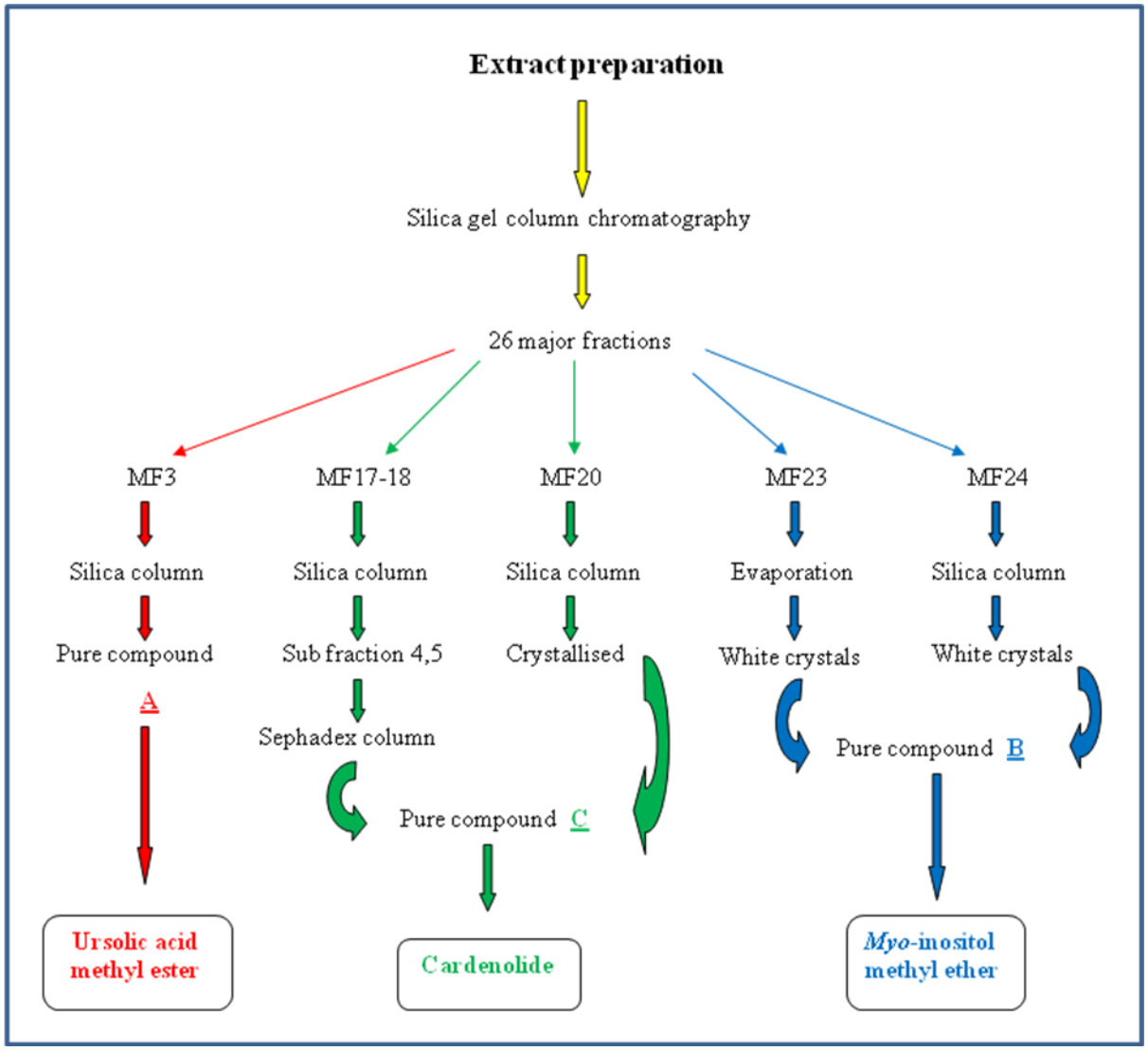


Figure 5.6: The schematic presentation of the isolation steps of the ethanolic extract of the fruit extract of *S. speciosus*. (MF: Major Fraction)

5.2.4 Antibacterial activity of the isolated cardiac glycoside

The microtitre plate dilution method of Eloff (1998) was used to determine the antibacterial activity of the isolated cardiac glycoside against two Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and two Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumonia*) with the 0.5 McFarland standards. The methodology used is as previously described in Chapter 3 in section 3.2.2 and 3.2.4; however the stock solution was 5 mg /ml.

5.2.5 Cytotoxic activity of the isolated cardiac glycoside

5.2.5.1 Screening assay

The screening assay cannot give the exact IC_{50} value of the tested samples. This test was done to determine the range of the cytotoxicity value of the sample for further testing. The procedure of this assay was described in Chapter 4 in Section 4.2.2.1. The Vero cell line was used in this assay.

5.2.5.2 *In vitro* cytotoxicity: Determining the IC_{50} value

The cytotoxicity of the isolated cardiac glycoside was measured by the XTT (Sodium 3`-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate) colourimetric assay based on the bio reduction of the XTT salt using the Cell Proliferation Kit II. The assay is based on the ability of metabolic active cells to reduce the tetrazolium salt XTT to orange coloured compounds of formazan. The dye formed is water soluble and the dye intensity can be read at a certain wavelength with a spectrophotometer. The XTT measures the activity of lining cells by assessing the activity via mitochondrial dehydrogenases (Siddiqui *et al.*, 2012). The amount of 1 mg/ml of compound **C** was prepared as stock solution of 100 µg/ml and added to the microtitre plate. The pure compound was serially diluted and concentrations ranged from 100-0.781 µg/ml. The cell line tested in this experiment was HEK 293. The procedure of the cytotoxicity assay is described in Chapter 4 in Section 4.2.2.2.

5.3 Results and discussion

5.3.1 Identification of isolated compounds

5.3.1.1 Compound A: fraction 3; sub fraction 14-20

Pure compound **A** was identified from the major fraction 3; sub fraction 14-20, as a triterpene. According to the ^{1}H and ^{13}C NMR data (Table 5.1), it is an ursolic acid methyl ester (Figure 5.6 and 5.7).

Ursolic acid (UA) is a triterpene compound which exists abundantly in the plant kingdom. They have been reported to exhibit anti-HIV inhibition and cytotoxicity to tumour cell lines (Ma *et al.*, 2005).

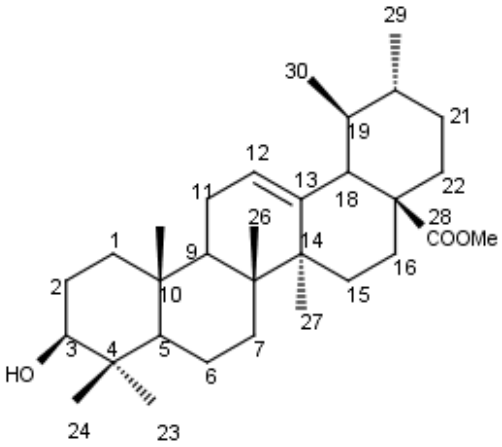


Figure 5.7: The structure of the ursolic acid methyl ester isolated from the *S. speciosus* fruit extract.

According to Liu (1995) and Kondo *et al*. (2011), medicinal plants containing ursolic acid as an active ingredient are *Calluna vulgaris* L., *Eribotrya japonica* Lindl., *Eucalyptus hybrid, Glechoma hederaceae* L., *Melaleuca leucaderaceae* L., *Melaleuca leucadendron* L., *Ocimum sanctum* L., *Rosmarinus officinalis* L., *Pyrola rotundifolia, Psychotria serpens* L., *Sambucus chinensis* Lindl., *Solanum incanum* L., *Tripterospermum taiwanense* and *Vaccinium macrocarpon* Aiton.

The chemical shifts of ^{13}C and ^{1}H NMR data of the isolated ursolic acid (**A**) compares well with the studies of Seebacher *et al*. (2003) and Fu *et al*. (2013). Comparing the carbon chemical shifts showed the similarity of the C_1 to C_{18} of ursolic acid (**A)** isolated from *S. speciosus* fruit extract with compound No 6 isolated by Fu *et al*. (2013) (Table 5.1). The C_{19} to C_{30} chemical shifts of the isolated compound **A** and compound No 2 are more similar (Table 5.1). Ursolic acid isolated (No 6) by Fu *et al*. (2013) was tested against the Hepatitis C Virus (HCV). The compounds A and B showed slight inhibition of HCV infection.

No evidence was found where ursolic acid methyl ester was isolated from the *S. speciosus* fruit extract previously.

Table 5.1 ^{1}H and ^{13}C NMR data of the isolated ursolic acid methyl ester (A) from *S. speciosus* fruit extract and Ursolic acid tested (No 2 and No 6) by Fu *et al*. (2013)

Carbon position	**A** δ_C	No 2 δ_C	No 6 δ_C	**A** δ_H	No 2 δ_H	No 6 δ_H
1	38.2	38.0	80.3	-	-	3.79 (dd)
2	39.1	28.6	39.6	-	1.88 (o)	2.36 (o), 2.31 (o)
3	76.3	78.4	76.0	-	3.51(dd)	3.62 (dd)
4	39.0	39.6	39.9	-	-	-
5	54.8	53.5	54.2	0.84	1.09 (d)	0.85 (d)
6	30.2	30.9	18.9		2.21 (m), 1.81 (m)	1.56 (o), 1.66 (o)
7	32.6	73.7	34.1	4.1 (br s) or 1.35 (o)	4.30 (dd) -	1.35 (o), 1.55 (o)
8	41.6	44.7	41.0	-	-	-
9	48.7	49.0	49.2	-	1.67 (m)	2.04 (dd)
10	39.1	39.6	44.1	-	-	-
11	23.0	24.2	28.1	3.20 (m)	2.03 (m)	3.26 (td), 2.50 (m)
12	125.1	126.9	129.0	-	5.69 (br d)	5.69 (t)
13	137.7	139.6	137.7	-	-	-
14	47.1	46.3	42.5	-	-	-
15	29.2	33.1	29.1	-	2.84 (m), 2.28 (m)	2.31 (o)
16	27.5	27.1	27.1	-	2.29 (m)	1.82 (o), 2.21 (o)
17	49.1	49.8	51.9	-	-	-
18	54.9	54.9	53.4	3.13 (m)	2.86 (d)	3.10 (d)
19	39.1	39.4	42.3	-	1.86 (m)	1.96 (td)
20	48.3	48.3	51.1	1.35 (m)	1.37 (m)	2.22 (m)
21	*	71.0	210.3	-	3.87 (td)	-
22	47.3	47.3	52.0	-	2.70 (dd), 2.25 (m)	3.04 (d), 2.76 (d)
23	29.2	29.1	29.2	1.26 (s)	1.26 (s)	1.27 (s)
24	17.8	17.9	16.8	1.01 (o)	1.06 (s)	1.09 (s)
25	16.3	16.3	12.4	1.26 (m), or 1.01(m) 1.35 (o)	0.99 (s)	1.25 (s)
26	15.1	10.9	18.2	1.26 (o)	1.37 (s)	1.15 (s)
27	23.8	24.1	24.5	-	1.49 (s)	1.22 (s)
28	180.0	179.7	177.9	-	-	-
29	20.0	18.2	19.0	1.42 (o)	1.16 (d)	1.09 (d)
30	16.6	16.8	13.2		1.43 (d)	1.18 (d)

m: multiplicity; s: singlet; d: doublet; o: overlap; t: triplet.

5.3.1.2 Compound B: fraction 23 and 24

The compound was identified as the known *myo*-inositol compound. The NMR data established the structure of the compound **B** as *myo*-inositol-methyl-ether (Figure 5.6). Inositols are polyols of cyclohexane with the empirical formula $C_6H_{12}O_6$ and *myo*-inositol is a six carbon cyclic polyalcohol (Campbell *et al.*, 2011; Rebecca *et al*., 2012).

Inositol metabolism is essential for the development of plants, animals and some microorganisms. The essential role of inositol in many cellular processes including membrane formation, cell wall biogenesis, stress response and signal transduction have been well documented (Lackey *et al*., 2003).

The other important functions of *myo*-inositol are that it functions as an element required in the formation of lecithin, which protects cells from oxidation and an important factor in the building of cell membranes. Inositol also has a metabolic effect in preventing too much fat to be stored in the liver, therefore it is called a lipotropic compound (Rebecca *et al*., 2012). The functions of *myo*-inositol in humans have been linked to bipolar disorder, production of L-chiro-inositol and D-chiro-inositol in insulin action, multiple sclerosis, Alzheimer`s disease and regulation of the sorbitol pathway in diabetic patients (Rebecca *et al*., 2012).

The ^{13}C NMR spectrum of this compound gave rise to four prominent signals. These signals are comparable with the *myo*-inositol isolated by Rebecca *et al*. (2012) in the zone of 75.15, 73.20, 72.98 and 71.93 ppm with relative intensities of 1:2:1:2. This data is similar to carbons C5-C-1-C-3-C-4-C-6 (Figure 5.8).

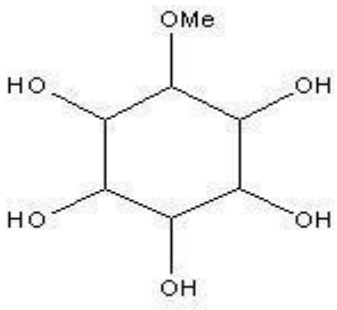


Figure 5.8: *Myo*-inositol-methyl ether isolated from the *S. speciosus* fruit extract.

In the plant kingdom, inositols have very important roles in general plant growth, seed storage, nitrogen fixation and protection of plants during stress (Campbell *et al.*, 2011). *Myo*-inositol is a pioneer to biosynthesis of many compounds involved in phosphorus storage, signal transduction, stress, protection, hormonal homeostasis and cell wall biosynthesis in plants (Rebecca *et al*., 2012).

According to Sureshan *et al.* (2009), among the nine theoretically possible inositols, six isomers, namely *myo*-, D-chiro-, L-chiro-, neo-, muco-, and scyllo-inositols are known to occur in nature, myo-inositol being the most abundant. *Myo*-inositol is synthesized from glucose. One or more methyl ethers of these inositols have been isolated from plants and these methyl inositols have important functions in plant biology. The methylation is a modification of inositols found extensively in plants. Methyl ethers of all naturally occurring inositols have been isolated and are presumed to have important physiological functions in plants (Sureshan *et al.*, 2009).

The D-Glucose-6-Phosphate (G-6-P) converts to L-*myo*-inositol-1-phosphate (I-1-P) by the enzyme L-*myo*-inositol-1-phosphate synthase and is subsequently dephosphorylated by inositol monophosphatase to *myo*-inositol (Lohia *et al*., 1999; Loewus & Murthy, 2000). Chhetri *et al*. (2006) reported the distribution of free *myo*-inositol in vegetative and reproductive structures of different pteridophytic species in some plant families such as: Gleicheniaceae, Dryopteridaceae, Equisetaceae, Lycopodiaceae, Polypodiaceae, Hypolepidaceae and Selaginellaceae.

Myo-inositol has been reported to be found in citrus fruits, bees, grains and nuts such as *Phaseolus vulgaris* L. (common bean), *Prunus domestica* L. (plum), *Cucumis melo* L. (melon), *Citrus sinensis* (L.) Osbeck (orange), *Pisum sativum* L. (pea), *Prunus dulcis* (Mill.) D.A.Webb (almond), *Glycine max* (L.) Merr. (soybean) and *Hylocereus polyrhizus* (Weber) Britton & Rose (dragon fruit) (Rebecca *et al*., 2012; Campbell *et al.*, 2011). No evidence could be found in the literature with regards to the isolation of a *myo*-inositol from *S. speciosus*.

5.3.1.3 Compound C: fraction 17-18 and 20

Column chromatography of the major fraction 17,18 and 20 of *S. speciosus* fruits ethanolic extract resulted in isolation of a cardenolide glycoside (**C**) (Figure 5.6). The analysis of this compound proved that it is cardenolide with a large molecular mass compound with an aglycone and two sugar moieties, which is responsible for the striped effect on the TLC plate. According to the ^{1}H and ^{13}C NMR and HMRS data, the chemical structure in Figure 5.9 was suggested. The ^{13}C NMR showed 38 carbon signals and ^{1}H NMR data demonstrated 58 hydrogens. The NMR data and high mass spectrometry established $C_{38}H_{58}O_{15}$ as the molecular formula of compound. The molecular weight of this compound is 776 g/mol.

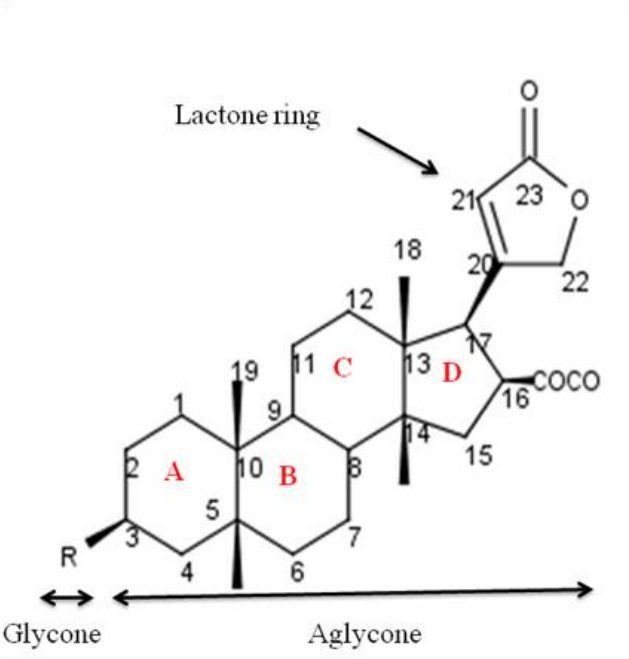


Figure 5.9: The chemical structure of the isolated cardiac glycoside from the *S. speciosus* fruit extract.

The studies of the isolated compound from *S. speciosus* fruit extract is comparable with a cardenolide isolated from the rhizomes of *Tupistra chinensis* Bak. by Pan *et al*. (2012). The comparison of this ^{13}C NMR data with those of Pan *et al*. (2012) suggests a strong similarity between the aglycone parts of the two compounds (Table 5.2).

Comparing the analysis of the isolated cardenolide NMR data and the cardenolide isolated from *T. chinensis* rhizome confirmed the chemical structure of the aglycone portion of this molecule. The chemical shifts of the carbons of the lactone and rings B, C and D match perfectly (Table 5.2), confirming thus the presence of an acetate group on C_{16}. However,

slight differences have been determined for some carbons positioned on ring A (C_1, C_3, C_5 and C_{19}). It could be due to the presence of a different glycone and/or an additional substitution on that ring. Further chemical experiments and analysis such as high resolution NMR analysis and Heteronuclear Multiple Bond Correlation (HMBC) is required to identify the aglycone portion and X-ray crystallography, which is the method for determining the molecular conformations of biological macromolecules, is needed to confirm the A ring chemical structure.

Table 5.2 ^{1}H and ^{13}C NMR data of the isolated cardenolide compounds from *S. speciosus* fruit extract (^{1}H: 200 MHz, ^{13}C: 50 MHz) and *T. chinensis* rhizome (^{1}H: 500 MHz, ^{13}C: 125 MHz) (Pan *et al*., 2012)

Carbon Position	*S. speciosus* **C**	*T. chinensis*	*S. speciosus* **C**	*T. chinensis*
	δ_C	δ_C	δ_H (*J* in Hz)	δ_H (*J* in Hz)
1	31.1	31.5	1.51 (m)	1.52 (m)
2	27.3	27.4	1.61 (m)	1.62 (m)
3	74.9	73.5	3.90 (br s)	3.95 (br s)
4	**30.8**	**30.8**	1.82 (m)	1.84 (m), 1.64 (m)
5	37.2	38.1	1.64 (m)	1.66 (m)
6	27.6	27.7	1.94, *	1.90 (m), 1.26 (m)
7	22.1	22.2	1.81, *	1.80 (m), 1.19 (m)
8	42.5	42.7	1.61 (m)	1.61 (m)
9	**36.6**	**36.6**	1.71 (m)	1.71 (m)
10	36.0	36.2		
11	**21.9**	**22.0**	1.41, *	1.44 (m), 1.23 (m)
12	**39.9**	**39.9**	1.51, *	1.54 (m), 1.42 (m)
13	**51.3**	**51.4**		
14	**84.8**	**84.6**		
15	**41.3**	**41.3**	2.78 (m), 1.79 (m)	2.78 (m), 1.79 (m)
16	**75.8**	**75.9**	5.45 (m)	5.46 (m)
17	**57.2**	**57.4**	3.28 (d)	3.27 (d)
18	**16.4**	**16.4**	0. 92 (s)	0.92 (s)
19	24.0	24.3	0.94 (s)	0.94 (s)
20	**171.5**	**171.6**		
21	**77.4** or **77.6**	**77.5**	5.03 (m)	5.02 (dd), 4.96 (dd)
22	**121.6**	**121.7**	5.98 (s)	5.98 (s)
23	**176.6**	**176.7**		
Ac	**172.2**, **21.0**	**172.1**, **20.9**	1.94 (s)	1.93 (s)

m: multiplet; s: singlet, d: doublet, br s: broad singlet

5.3.2 Antibacterial assay

The cardiac glycoside (cardenolide) isolated from *S. speciosus* fruit extract did not show any antibacterial activity against the two Gram-positive bacteria (*S. aureus* and *E. faecalis*) and two Gram-negative bacteria (*E. coli* and *K. pneumonia*) (Figure 5.10).

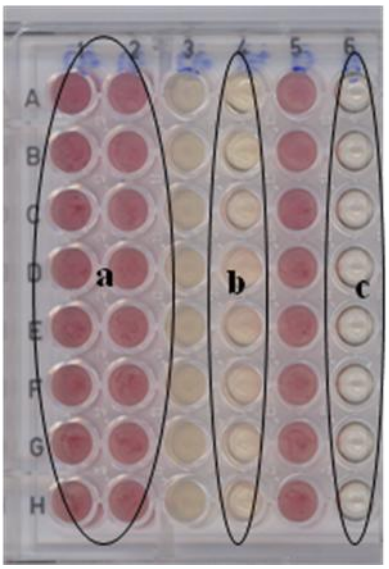


Figure 5.10: a: Microtitre plate showing no inhibition of the cardiac glycoside isolated against the tested bacteria, b: Positive control, c: Sterility control.

Several studies have been reported earlier on the antibacterial activity of plant extracts containing cardiac glycosides like *N. oleander* (Bhuvaeshwari *et al*., 2007). But little is known about the antibacterial activity of only the cardiac glycoside compounds in the extracts. It seems that the inhibition activity of these plants is not because of the presence of cardiac glycoside and the existence of other types of compounds is responsible for their inhibitory activity.

5.3.3 Cytotoxicity activity

5.3.3.1 Screening assay

The toxicity screening assay on the Vero cell line of the cardiac glycoside isolated in this study showed that estimation of the IC_{50} value of the pure compound was < 25 µg/ml. This test, in general, does not show the exact IC_{50} values.

5.3.3.2 *In vitro* cytotoxicity: Determining the IC_{50} value

One of the major concerns about the clinical usage of cardiac glycoside compounds is their toxicity. Therefore, the disadvantage of side effects and toxicity of these compounds may limit their future usage in treating infection, especially viral contagions, as compared to the safety profile of the recognized and common medicine like acyclovir. Further studies will be required to determine the potency and therapeutic values of these types of compounds for treating viral infections in consideration of their cytotoxicity. Cytotoxicity assays are essential for the initial phases of antiviral drug development because they define the concentrations to be used, to avoid cell damage and selectivity for the virus *in vitro*. The assessment of cytotoxicity is usually performed by cell viability assays, such as the dye uptake by nonviable cells, or by alterations in cell functions (Eisenbrand *et al*., 2002). The exact cytotoxicity of the isolated cardiac glycoside compound in the HEK 293 cell line was determined by the XTT assay. The concentration that showed 50% cytotoxic effect in HEK 293 (IC_{50}) of this assay measured at 4.62 µg/ml (Figure 5.11).

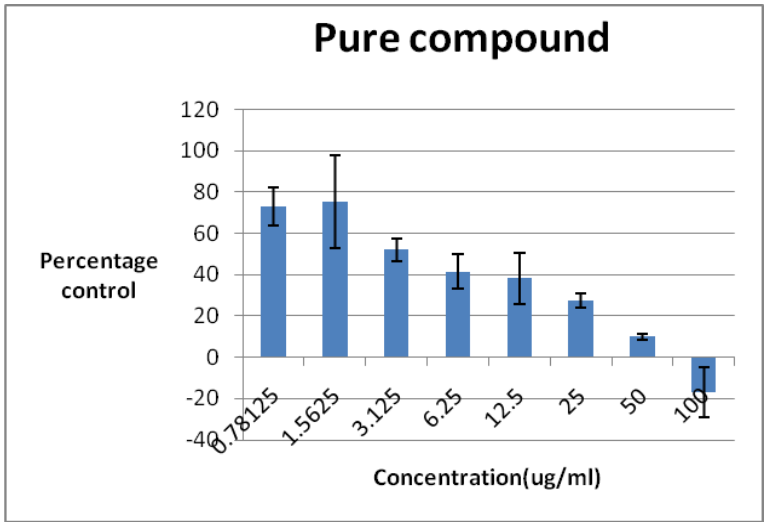


Figure 5.11: Dose response curve of cytotoxicity results of isolated cardenolide against HEK 293.

Actinomycin D was used as positive control. The result of this anticancer drug was 0.00735 µg/ml. According to Roy *et al*. (2005), the cardenolides isolated from the *Asclepias curassavica* L. (Milkweed) belonging to the Asclepiadaceae, manifested toxicity against four cancer cell lines (IC_{50} 0.01 to 2.0 µg/ml).

Although the isolated compound was not tested against the HSV-2 virus but it is intresting to know about the relation between the cytotoxicity and anti-HSV-2 of the cardiac glycosides in other literatures. The literature review on the antiviral activity and particularly anti-HSV activity of cardiac glycosides revealed the anti-HSV property of these compounds (Bertol *et al.*, 2011). To investigate the anti-HSV of the cardenolide isolated, the EC_{50} value (EC_{50} is the concentration that inhibited 50% of HSV replication in HEK 293 cell line) and selectivity index (SI) value (IC_{50}/EC_{50}) should be determined. The SI value is determined as the ratio of IC_{50} or CC_{50} to EC_{50}. It is desirable to have a high therapeutic index giving maximum antiviral activity with minimal cell toxicity.

According to the literature, triterpenes demonstrate several biological activities including antiviral activity because of the naturally occurring sugar conjugates (Khan *et al.*, 2005). According to Su *et al.* (2008), the analysis of the anti-HSV-2 activity of digitoxin and its analogues such as; digoxin, ouabain octahydrate and G-strophanthin; which exhibited antiviral activity, was revealed to contain a glycone (sugar) in their structures. The presence of a glycone (sugar) as well as the various forms of them in a cardiac glycoside compounds may lead to various activities as well as altering the therapeutic role of the compound.

There are different types of mechanisms of action of HSV inhibition such as inhibiting and/or reducing the viral DNA synthesis, inhibiting capsid protein synthesis of virus, affecting a late event in the life cycle of virus synthesis or inhibition of viral binding and entry into the host cell (Khan *et al.*, 2005). According to the Khan *et al.* (2005), two triterpene anti-HSV compounds, namely moronic acid and betulonic acid, were identified from the ethyl acetate extract of *Rhus javanica* Linne which demonstrated oral therapeutic activity in mice.

Su *et al.* (2008) investigated the anti-HSV activity and cell viability of digitoxin and its analogues and its mechanisms of actions. The results showed that the digitoxin inhibited both HSV-1 and HSV-2 with the IC_{50} of 8.154 µg/ml and EC_{50} of 3.8247×10^{-2}. The SI of digitoxin to HSV-1 and HSV-2 were both 213.2. They also examined the anti-HSV-1 and HSV-2 effects of several structure analogues of digitoxin which among them, digoxin, ouabain octahydrate and G-strophanthin showed comparable anti-HSV activity (EC_{50}

between 2.0448 x 10^{-3} and 1.015235 x 10^{-1} µg/ml or between < 0.05 and 0.13 µM) and cytotoxicity (IC_{50} between 7.934 and 52.7661 µg/ml or between 10.21 and 15.11 µM). In presence of these compounds, the plaque size was significantly reduced (Su *et al*., 2008). They concluded that the presence of digitoxin and its analogues in anti-HSV activity inhibited the early stage of HSV replication and blocked the virus release.

Brazilian scientists conducted the anti-HSV-1 and anti-HSV-2 assay screening several cardenolide derivatives obtained from different sources. The tested cardenolide showed antiviral activity via inhibition of viral proteins synthesis (ICP27, U_L42, gB, gD), the blockage of virus release and the reduction of viral cell to cell spread. Glucoevatromonpside, one of the cardenolides isolated from a Brazilian cultivar of *Digitalis lanata* Ehrh., illustrated synergistic antiviral effects with acyclovir and anti-Na^+K^+-ATPase activity, which it was suggested that cellular electrochemical gradient alterations might be involved in the mechanism of viral inhibition (Bertol *et al.*, 2011).

Hartley *et al*. (2006) described the ionic contra viral therapy (ICVT) of the cardiac glycoside (digoxin) to treatment of DNA virus infections. This compound inhibited the *in vitro* replication of the DNA viruses such as HSV. Antiviral activity of digoxin was influenced by extracellular K^+. It is interesting that the antiviral effect of digoxin was increased when it was combined with furosemide (a loop diuretic used in the treatment of congestive heart failure and edema) (Hartley *et al*., 2006).

5.4 Conclusion

The chromatographic investigation on the *S. speciosus* fruit extract led to the isolation of three different compounds namely, ursolic acid methyl ester, *myo*-inositol and a cardenolide type of cardiac glycoside. The specific structure of the isolated cardenolide was not identified and further analysis is needed such as high resolution NMR analysis and HMBC to identify the aglycone portion and X-ray crystallography, which will aid in determining the molecular conformation. Due to the complexity of the isolated large cardenolide compound, specifically the glycone portion, it requires advanced structural analysis to determine the complete and accurate structure, which may be a novel compound.

This compound was subjected to antibacterial and cytotoxicity assays. The isolated cardiac glycoside was not successful in inhibiting any strain of bacterial pathogens tested. No literature could be found regarding the antibacterial activity of cardenolides.

The results of the present cytotoxicity test compared well with literature values especially with Bertol *et al.* (2011) with the IC_{50} value of 4.62 µg/ml. This experiment for the first time tested the compound on the HEK 293 cell line. Further studies will be required to explore the possible antiviral activity and its mechanism of the isolated cardiac glycoside. More investigations must be done to demonstrate the importance and potential role of the glycone in anti-HSV activity of cardiac glycosides.

Strophanthus speciosus fruit extracts are known to contain the cardenolide compounds (Van Wyk *et al*., 2005), but no evidence was found in the literature of ursolic acid and *myo*-inositol compounds being isolated from *S. speciosus* fruits before. Therefore this study represents the first report of the isolation of these two compounds from *S. speciosus* fruit extract.

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Chapter 6

Antiphytoviral activity of plants containing cardiac glycosides

6.1 Introduction to Potato Virus Y (PVY)

Plant viruses are obligate parasites consisting of a nucleus, nucleic acid (DNA or RNA), replication occurs and the coat protein is synthesized in the host cell. Plants do not have an immune system as animals, and the virus is absolutely dependent on its host (Fan *et al.*, 2011). Plant viruses and virus diseases have been studied for more than 100 years and much attention has been given to their control. It has been difficult to find a solution to solve the plant virus infection because of the lack of any effective means of curing virus infected plants. Chemotherapy, thermotherapy and meristem-tip therapy can be successful, but they cannot be used on a large scale (Thresh, 2003). Consequently, controlling plant viruses and developing plant virus inhibitors are tremendously complicated (Fan *et al.*, 2011).

Potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world after rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.), with a yield of 315 million tons in 2006. Potato is one of the most important vegetable crops in South Africa with a total production of 2,195,400 tonnes in 2011 (FAOSTAT, 2013). In South Africa, potato prices on the major fresh produce markets increased significantly by an average of R3 354 per ton in 2009 (Trends in the Agriculture Sector, 2010).

Bos (1982) provided a detailed review of losses caused by plant virus infection and the economic consequences arising from these losses. The economic effects that result from virus epidemics in stands of cultivated plants include loss of revenue, increased production costs, shortages in supply and greater unreliability of production. In spite of the developing human knowledge about plant viruses, and virus elimination programs, there has been a recent increase in the PVY infection rate of potatoes in South Africa (Visser & Bellstedt, 2009). According to the study by Khan *et al.* (2003), about 37 viruses naturally infect cultivated potatoes. This is attributed to lack of host control, the use of infected propagation material in cultivation, incorrect irrigation and farming practices. Global warming may be contributing to an increase in host number, with associated increase in viral distribution. Viral mutation and recombination or the importations of new cultivars or propagation

material into the country are some of the factors contributing to an increase in virus infections (Visser & Bellstedt, 2009). Potato Virus Y is one of the most economically important potato virus and causes severe yield losses (10-80%) to potato yield in all potato growing regions in the world (Valkonen, 2007).

Potato Virus Y genus *Potyvirus*, belonging to the largest plant virus family, Potyviridae, is one of the most common viruses that infect a wide range of plant species, primarily from the family Solanaceae. This virus has a worldwide distribution and is one of the most economically important viruses of potato. Different species of aphids are the elements of transmission of this virus (Tribodet *et al.*, 2005; Barker *et al.*, 2009).

According to the symptoms and reactions, which are illustrated in potato and tobacco (*Nicotiana tabacum* L.), PVY has been classified into different strains. The first recognized strain of these type viruses was PVY^{C}, which commonly induces hypersensitive reactions including the formation of mild mosaic patterns or stipple streak (Visser & Bellstedt, 2009). Potato Virus Y^{O} is a common strain which has been known since 1931 and it is commonly found wherever potatoes are grown. The strain isolated from this type of virus causes mild tuber damage and mild to severe mosaic leaf drop, which is different from leaf necrosis. The stipple streak strain PVY^{C} produces hypersensitive reactions or a systemic mosaic in potato and PVY^{O}-like symptoms in tobacco (Carnegie & Van de Haar, 2004; Visser & Bellstedt, 2009). During the 1950s to the 1970s an epidemic of a new strain of a virus was reported as tobacco veinal necrosis (PVY^{N}), in European and South American countries, which was associated with high yield loss. The symptom of infection by this strain is frequently less and produces mild and severe mosaic symptoms or even sometimes there are no symptoms and damage to the tubers. In tobacco, the virus produces severe veinal necrosis and leaf death. This strain nowadays is distributed all around the world. Both PVY^{O} and PVY^{N} are aphid transmissible (Carnegie & Van de Haar, 2004; Visser & Bellstedt, 2009). Moreover there are several relatively new strains, such as $PVY^{N}W$, which is a recombinant of PVY^{N} and PVY^{O}. This new strain infects plants without producing disease symptoms on the growing plant. Potato Virus Y^{NTN}, a new subgroup of PVY^{N} was described for the first time in the 1950s, and is able to produce superficial necrotic ring spots on infected tubers. These symptoms led to being called Potato Tuber Necrotic Ring Spot Disease (PTNRD), which

has a larger economic impact than infection by the other strains of PVY (Carnegie & Van de Haar, 2004; Visser & Bellstedt, 2009).

Fortunately the host range of PVY is limited mainly to members of the same family as the potato, the Solanaceae (Blanchard *et al.*, 2008). These include tomatoes (*Solanum lycopersicum* L.), tobacco and some weeds. The symptoms of the PVY^{N} and PVY^{NTN} are shown in Figure 6.1.

A B C

Figure 6.1: A) Necrotic symptoms on leaves of potato (upper row) and tobacco (lower row) caused by PVY^{N}. B, C) Necrotic symptoms on potato tubers of the variety Nicola caused by PVY^{NTN} (Burrow & Zitter, 2005).

Enzyme linked immunosorbent assay (ELISA) has been used to detect plant viruses since the 1970s (Voller *et al*., 1976). ELISA utilizes the ability of antibodies produced in animals to recognize the antigen proteins (Clark & Adams, 1977) and these bind to the solid phase called the polystyrene microtitre plates (Ehlers & Paul, 1984). Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) was conducted to examine and confirm the presence of the PVY^{N} virus from the source of virus. DAS-ELISA is a very sensitive plant virus identification technique. There are some advantages of using the DAS-ELISA test. The virus particles are concentrated from extracts by coating antibodies, and inhibitory components of extracts are removed by rinsing before addition of the detecting antibody and enzyme substrate (Khan *et al.*, 2003). This method has higher sensitivity than the other serological methods for the identification of most viruses, especially those that are found only in low concentrations in infected plants such as Potato Leaf Roll Virus (PLRV) and Potato Virus A (PVA). Lower requirements of antiserum than other serological methods are the other benefit of this method (CIP Training Manual, 1999).

6. 2 Antiphytoviral activity of plant extracts

Many chemicals cause human disease. Arsenic and skin cancer, asbestos and decrements of IQ (Intelligence Quotient) are examples of the effects of chemicals on humans (Carpenter *et al.*, 2002). Many synthetic chemicals are used in the control of plant diseases and seed biodeterioration during storage, but due to their residual toxicity and non-biodegradation during storage, carcinogenicity and teratogenicity on non-target organisms and pollution of the environment (soil and ground water) are consequences (Pimentel & Levitan, 1986). These disadvantages of the synthetic chemicals led the scientists to investigate the new non-toxic treatments to control plant viral diseases.

Plants provide mankind with various secondary metabolites with biological activity such as insecticidal, fungicidal and antiviral activity. Plant secondary metabolites, as a rich source of antimicrobial agents, has been extensively applied all around the world as traditional medicine to treat viral diseases. Therefore, direct selection of antiphytoviral compounds from plants can be used to identify new potent antiviral agents. Due to the advantages of plant extracts as antipathogen agents over chemical pesticides such as environment compatibility, new specific mechanisms of action against the viruses and also variety and rich resources; secondary metabolites of plant have been considered as a new treatment to manage plant pathogens (Zhang *et al.*, 2007).

Medicinal plants have been widely used to treat a variety of infections. Many crude extracts and compounds from plants have been screened to determine their antiviral activity against human and plant viruses (Zhang *et al.*, 2007). The treatment of viral diseases with natural products from plant species is also a broadly investigated area.

According to Yan *et al.* (2010), eight compounds namely brusatol, bruceine B, bruceoside B, yadanzioside I, yadanzioside L, bruceine D, yadanziolide A, and an aglycone of yadanziolide D isolated from the seeds of *Brucea javanica* (L.) Merr. exhibited potent anti-Tobacco Mosaic Virus (TMV) activity. These compounds showed strong antiviral activities, with IC_{50} values in the range of 3.42 to 5.66 μM.

Shen and co-workers (2008) suggested that bruceine-D from *B. javanica* may have the potential as a natural viricide, or a lead compound as a new antiviral treatment. The

compound exhibited significant inhibitory activity against the infection and replication of TMV, PVY and Cucumber Mosaic Virus (CMV) (Shen *et al.*, 2008). Mohamed (2010) screened the antiviral properties of garlic cloves and onion bulb juice against PVY *in vitro* and *in vivo*. Both extracts reduced the *in vitro* and *in vivo* infectivity of PVY. However, the garlic extract was more effective in reducing the local lesions produced by PVY on *C. amaranticolor* as a local lesion host (Mohamed, 2010).

In recent years, triterpenes and triterpenoid glycosides have demonstrated novel activity against plant viruses (Zhang *et al.*, 2007). Cardiac glycosides are a type of triterpenoids which are derived by modification of the triterpenes. The plants selected for this study, namely *Gomphocarpus fruticosus, Nerium oleander, Cotyledon orbiculata, Strophanthus speciosus, Bowiea volubilis* and *Merwilla plumbea* all contain cardiac glycosides (Van Wyk *et al.*, 2005). Some of the selected plants have been used as a traditional herbal medicine for the treatment of some diseases for instance cancer and treatment for heart congestive failure, and are known to be a promising resource of natural products with medicinal properties (Aremu *et al.*, 2010). Recently, interesting investigations have been done on the antiviral activity of the cardiac glycosides against the human virus such as Herpes Simplex Virus 1 and 2 (HSV-1 and HSV-2) as well as Human Immunodeficiency Virus (HIV) and demonstrated the antiviral potential of these interesting compounds (Prinsloo *et al*., 2007; Bertol *et al*., 2011). However, little is known about the antiviral activity of selected plants against plant viruses. The objective of the study was to identify the active antiphytoviral extracts of the selected South African plants containing cardiac glycosides and evaluate the inhibitory activity of the isolated cardiac glycoside compound (Chapter 5, Section 5.3.1.3) against the PVY^N plant virus.

6.3 Materials and methods

6.3.1 Sources of inoculum (virus), host plants, soil medium and plant extracts

- **Inoculation (Virus)**

  Potato virus Y^N (Isolate: NN333B 28 149: Mooi Rivier, Kwazulu-Natal, SA) was obtained from the Agricultural Research Council (ARC), Vegetable and Ornamental Plant Institute (VOPI), Roodeplaat, Pretoria, South Africa. The virus was propagated

and maintained in tobacco plants. Potato virus Y^N infected *in vitro* plantlets were also used as sources of inoculum for PVY^N. *In vitro* tobacco plantlets were taken from the test tubes and hardened off by closing the plants with a small cup for 3-4 weeks to allow them to get used to the greenhouse environment. They were then given another month after hardening off to grow before the actual trial was planted to ensure the availability of enough source of virus material.

- **Host plants**

  Nicotiana tabacum (cultivar: Samsun NN) were grown in an insect proof glasshouse at ±23°C day and ±18°C night temperatures, with a 16:8 h light: dark photoperiod. Irrigation was scheduled three times in a week and soluble nutrient fertilization (Multifeed P, Plaaskem, (Pty), LTD) supplied on a weekly basis. Seedlings were transplanted into 20 cm plastic pots two to three weeks post emergence. Seedlings were tested prior to virus inoculation by DAS-ELISA to ensure that they were free from PVY^N or other potato viruses and (Tobacco Mosaic Virus) TMV. These plants were used as a systemic and local lesion host of PVY^N virus for *in vivo* and *in vitro* experiments. Plants were monitored and sprayed for insect pests, such as aphids and whiteflies, when required, to prevent cross contamination by different viruses.

- **Soil medium**

  Pots were filled with pasteurized potting soil supplied from the Just Nature Company, Onderstepoort, South Africa. The soil was pasteurised to ensure that the mixture was free from soil-borne pathogens. Supplementary soluble nutrient fertilization (Multifeed P, Plaaskem, (Pty), LTD) was given to the plants on a weekly basis to avoid situations where symptoms are mistaken as nutrient deficiencies.

- **Plant extracts**

  All nine plants extracts (the leaves of *G. fruticosus, N. oleander, C. orbiculata*; the fruits and leaves of *S. speciosus*; the bulbs of *B. volubilis* and *M. plumbea*) and the cardiac glycoside compound isolated from *S. speciosus* fruit extract (described in Chapter 5) were used for the phytotoxicity and antiphytoviral tests. Plant extracts were made according to the extraction procedure as described in Chapter 3, Section

3.2.1. The procedure of cardiac glycoside isolation was described in Chapter 5, Section 5.2.3 and 5.3.1.3.

6.3.2 Phytotoxicity test

To investigate the possibility of the toxic effects of the extracts and compound on *N. tabacum* plants, extracts were applied to the leaf surface at a low concentration (20 mg/ml) and at a high concentration (50 mg/ml). The extracts and compound were dissolved in 10% dimethyl sulfoxide (DMSO). Distilled water was then added to the dissolved extracts and compound to yield the required concentrations. At three leaf stage, plants were mechanically rubbed with the extracts/compound over the upper and the under surface of the leaves. Two replicates were used for each extract/compound. The trial was planted in a randomized block design (Figure 6.2).

Figure 6.2: A randomized block design of the *N. tabacum* plants for the phytotoxicity test.

Plants were maintained in the glasshouse at ±23^{o}C day and ±18^{o}C night temperatures. Plants were evaluated by observing mechanical damage and local lesions on the leaves. The leaf symptoms of the phytotoxicity test were observed and scored using a scoring rate of 0-3 [0: no damage (0%), 1: mild damage (10%), 2: severe symptoms (40%) and 3: very severe symptoms (80%)]. Results were recorded after 2 hours, 24 hours and 7 days.

6.3.3 Detection of PVY^N with DAS-ELISA in sources of inoculum

Leaves from the bottom, middle and top parts of the plants were collected to be used in the DAS-ELISA test. The antisera for the detection of PVY^N was sourced from Bioreba (Avt. No.112712 Biorebal, Switzerland). Polystyrene microtitre plates (F96 cert Maxisorp, Nalge Nunc International, Nunc ^{TM}, Denmark) were coated with 100 µl of anti PVY^N IgG (Avt. No.112712 Bioreba), diluted at 1:1000 in coating buffer (0.05M sodium carbonate bicarbonate buffer, pH 9.6). The plates were incubated for 4 hr at 37°C or overnight at 4°C. After incubating, plates were washed in PBS-Tween thrice for 3 minutes.

Potato leaves, positive controls (leaves of *N. tabacum*) and leaves of healthy plants of *N. tabacum* as negative controls, were used to conduct virus assays. Leaves were homogenised using a mortar and pestle, after adding six to 10 ml of sample/conjugate buffer consisting of 0.02M phosphate buffered saline, pH 7.4, containing 0.05% Tween-20 (PBS-Tween), 2% polyvinylpyrrolidone (PVP) and 2% egg albumin. One hundred microliter sample extracts were added in duplicate, and incubated for 4 hr at 37°C or overnight at 4°C. After incubating, plates were washed in PBS-Tween three times for 3 min. The third step for DAS-ELISA was carried out by adding PVY^N antibody conjugated to alkaline phosphatase at a dilution of 1:1000, in sample conjugate buffer. One hundred microliters was dispensed in duplicate wells of the ELISA plates. Figure 6.3 shows the procedure of the DAS-ELISA test.

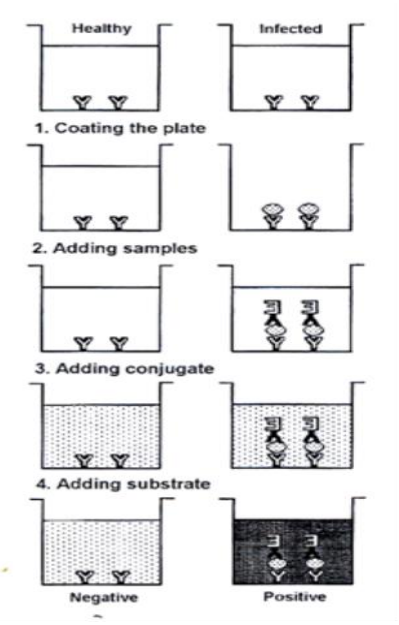

Figure 6.3: Steps in the DAS-ELISA test (CIP Training Manual, 1999).

Figure 6.4 and 6.5 represent how the ELISA test was conducted. All plates were incubated for 3 to 4 hours at 37°C or overnight at 4°C. The positive or negative reaction of the sample was determined by adding 100 µl of 4-nitrophenyl phosphate disodium salt hexahydrate (Fluka Biochemiko), diluted at 1mg/ml in 10% diethanolamine buffer, pH 9.8 to each well. After one hour of incubation in substrate solution at room temperature, readings were taken at 405 nm using a Flow Titertek Multiskan Plus ELISA plate reader (Labsystems, Finland).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Bank	Blank
B	Blank	S 1	S 8				S 1	S 8				
C	Blank	S 2	S 9				S 2	S 9				
D	Blank	S 3	S 10				S 3	S10				
E	Blank	S 4	NC				S 4	NC				
F	Blank	S 5	PC				S 5	PC				
G	Blank	S 6					S 6					
H	Blank	S 7					S 7					

Figure 6.4: Schematic representation of the ELISA plate and added samples. (S: sample, NC: negative control, PC: positive control).

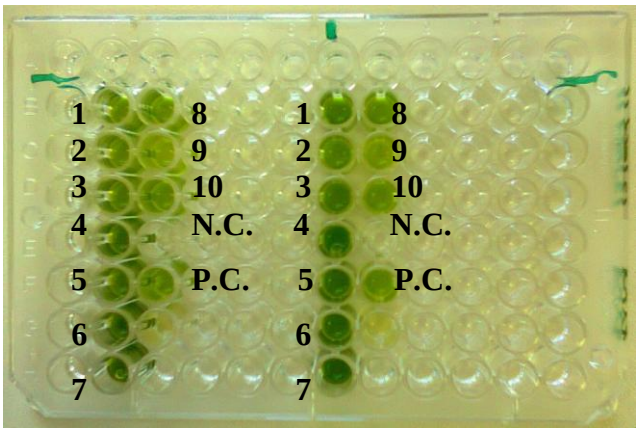

Figure 6.5: ELISA plate with samples that were used as sources of PVY^N virus and negative and positive controls. (Number 1-10: plant samples, N.C.: negative control, P.C.: positive control).

6.3.4 Antiphytoviral activity

The aim of this experiment was to determine potential inhibition of the extracts or isolated cardenolide on PVY^N *in vivo* and *in vitro* on *N. tabacum*.

6.3.4.1 *In vivo*

The experiment consisted of three treatments at the leaf stage of tobacco plants: 1) plants sprayed with the extracts and compound the day before they were infected with the virus, 2) plants infected with the virus but not treated with the extracts and compound (positive control) and 3) plants not infected with a virus and not treated with extracts and compound (negative control). The experiment was designed in three blocks with each block containing 60 *N. tabacum* plants. The 60 plants of each replicate were placed randomly in their respective block. The day before inoculation with PVY^N, extracts and compound were sprayed on the plants at concentrations of 20 mg/ml and 50 mg/ml to determine the more effective concentration to control the virus which is less toxic to the plants.

The sap was made by grinding 20 g (± 100 leaves) of virus infected material in a chilled mortar and pestle then pressed through a cheese cloth material to remove excess leaf material. The sap was then mixed with 200 ml 0.01M phosphate buffer (0.01M sodium phosphate pH 7.4: 0.89 g Na_2HPO_4 + 0.426 g KH_2PO_4) and 0.05 g of Celite (diatomaceous earth) which was used as an abrasive (wounding agent) to allow the virus to enter the plant. Inoculation was achieved by rubbing ground liquid sap containing PVY^N over the *N. tabacum* leaf surface with fingers while supporting the leaf with the other hand. The inoculation was done the day after the plants were treated with extracts and compound. After inoculation, the plants were sprayed with water to remove the Celite and to make ensure that they were not burnt by the excess Celite. Inoculated plants were maintained in the greenhouse with approximately 25°C day and 18°C night temperatures. Insect pests were monitored and controlled by spraying with recommended insecticides such as Hunter (Imidocloprid) for aphids and whiteflies and Profenofos for red spider mites, as recommended by the manufacturers. Plants were irrigated twice a day (morning and evening).

The first evaluation of the extracts and compound efficacy against the virus infection was conducted two weeks after inoculation. Leaf symptoms were observed and recorded using a

scoring rate of 0-5, where 0 represented no symptoms, 1-2 represented mild symptoms, 3 represented moderate symptoms, 4 represented severe to very severe symptoms and 5 represented very severe symptoms.

To calculate the percentage virus symptoms severity, based on the symptoms observed at sampling points, the scoring rates (0-5) were translated to percentages and these were as follows: 0 was equivalent to 0%, 1-equivalent to 20%, 2-equivalent to 40%, 3-equivalent to 60, 4-equivalent to 80% and 5-equivalent to 100%. After four weeks (30 days) of inoculation, leaf symptoms were checked again, and recorded as final observation.

6.3.4.2 *In vitro*

Infection by PVY^N of the inoculated plants was confirmed by the DAS-ELISA method and this was conducted as described in Section 6.3.3. ELISA readings were taken and used to determine if there were any differences in the virus concentrations, correlating it with symptoms noted for each treatment after four weeks.

6.3.5 Statistical analysis

All information and data were analysed using statistical software GenStat 64-bit Release 14.1 (PC/Windows 7) Copyright 2011, VSN International Ltd. Data processing system and XLstat (Ad-ons on Microsoft Excell 2010) was used for the trial-plan.

6.4 Results and discussion

6.4.1 Phytotoxicity test

Plant phytotoxicity may occur when chemicals that are employed as plant protection products for pest, bacterial or viral infection cause temporary or long lasting damage to plants. The estimation of the possible damages of a plant protection product to a crop or plant product is a necessary element in its efficacy evaluation. Symptoms of phytotoxicity can be observed on the crop emergence or during its growth or maybe at harvest. The phytotoxicity symptoms can affect the whole plant or any part of the plant such as the roots, shoots, leaves, flowers, and fruits (Phytotoxicity assessment, 2007).

Phytotoxicity can result in different symptoms. Modifications in the development cycle is a type of phytotoxicity symptom which can result in inhibition or delay in emergence or growth and modifications, particularly delay in flowering, fruiting and maturing, etc., or non appearance of certain organs. The other type of the symptoms of plant toxicity is thinning, which can result in the loss of whole plants, by failure to emerge or to grow after transplanting, or by disappearance of plants after emergence (Phytotoxicity assessment 2007). The toxic effect of the chemicals or plant protection products can change or discolour the whole plant or parts thereof and can result in chlorosis, whitening, change in intensity (lighter or darker), browning or reddening. The discolouration may be localized in spots, internal or external. The other type of phytotoxic effect is necrosis, which is the local death of tissues or organs, generally appearing first as a discolouration. Necrotic spots on leaves sometimes disappear and result in perforations on the leaves. Deformations are any morphological modification of the plant or part of it including roots, which can make digress from the normal type. It includes curling, rolling, stunting or elongation and change in size or volume (Phytotoxicity assessment 2007).

In this study, the tobacco plants were examined with different treatments of plant extracts to observe their possible phytotoxicity effect. Figure 6.6 illustrates that the most phytotoxic plant extract in this study is *M. plumbea* (bulb) especially at the high concentration (50 mg/ml) and the other extracts have a mild toxic effect on the tobacco leaves.

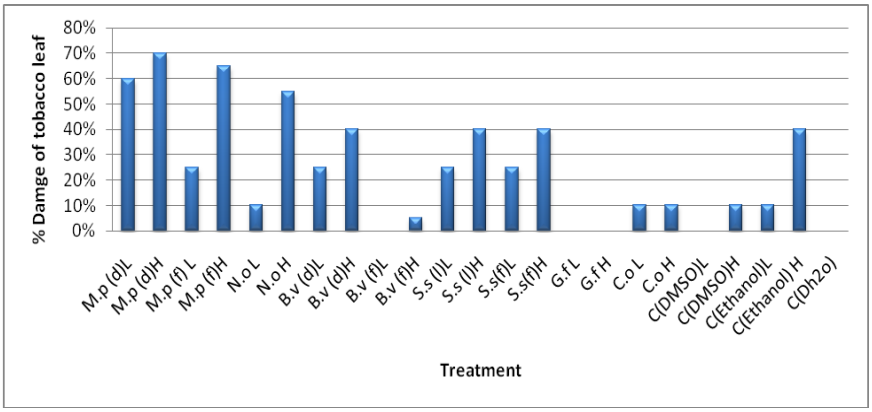

Figure 6.6: Percentage of the leaf damage after treatment with selected plant extracts. (L: low concentration (20 mg/ml), H: high concentration (50 mg/ml), M. p (d): *M. plumbea* (dry bulb), M. p (f): *M. plumbea* (fresh bulb), N. o: *N. oleander*, B. v (d): *B. volubilis* (dry bulb), B. v (f): *B. volubilis* (fresh bulb), S. s (l): *S. speciosus* (leaf), S.s (f): *S. speciosus* (fruit), G. f: *G. fruticosus*, C. o: *C. orbiculata*, C: control).

After two hours of inspection, leaf damage was experienced with *M. plumbea, B. volubilis* and *N. oleander* at 50 mg/ml at the areas where the extracts were rubbed (Figure 6.7).

Figure 6.7: Damaged areas of *N. tabacum* leaves treated with *M. plumbea* at 20 mg/ml when the damage was observed 2 hours after the treatment.

Second observation of the probable symptoms was done after 24 hours post extract application (Figure 6.8). Most of the extracts showed some leaf damage especially *M. plumbea, B. volubilis* and *N. oleander* at 20 mg/ml and 50 mg/ml. The damaged areas noted during the first observation had not spread further when comparing the results with the second observation. The damages were mainly on the regions where the leaves were rubbed with the extracts. The last monitoring took place after 7 days. There was no increase in damage on plants in comparison to the second and third observation (Figure 6.9).

Figure 6.8: Damaged areas of *N. tabacum* leaves treated with *M. plumbea* at 20 mg/ml when the damage was observed 24 hours after the treatment.

Figure 6.9: Damaged areas on *N. tabacum* leaves treated with *M. plumbea* at 20 (left) and 50 mg/ml (right) observed 7 days after the treatment.

The solvent control (5% DMSO) did not show any toxicity to the plant. However, a high dose of 10% DMSO showed slight damage on the *N. tabacum* leaves. *Nicotiana tabacum* plants treated with distilled H_2O as the control were not affected and showed no sign of damage.

According to the literature, most of the phytotoxic and allelopathic activity tests have been done on the Apocynaceae family, especially *N. oleander*. These experiments illustrated different allelopathic activity of *N. oleander* (Uludag *et al.*, 2006). The water extract of *N. oleander* did not show any undesirable effect on maize growth, even its residue increased maize yield (Uygur & Iskenderoglu, 1995). On the other hand, the aqueous extract of *N. oleander* inhibited germination of weeds such as *Digitaria sanguinalis* (L.) Scop, and *Lolium multiflorum* Lam.. A pot study showed that *N. oleander* promoted the growth of maize, cotton (*Gossypium herbacium L.*) and soybean (*Glycine max* L. Merril) (Uludag *et al.*, 2006).

The aqueous and methanol extracts of *N. oleander* leaves showed inhibitory effect on the seed germination and early growth of *Parthenium hysterophorus* L.. Methanol extracts of a white flowered variety illustrated higher inhibition compared to that of the pink flowered variety. On the other hand *N. oleander* extracts tested on seed germination and early growth of crop plants, had no effect on wheat, Ragi (*Eleusina coracan* L.) and Green gram (*Vigna*

radiate L.) even at 1:3 dilution of the stock preparation (Amruth Kumar *et al.*, 2011). In the present experiment, *N. oleander* especially at the lower concentration did not have a toxic effect on the *N. tabacum* plants. Germination inhibition assays on two milkweed species belonging to the Apocynaceae family, *Asclepias syriaca* L. and *Asclepias speciosa* Torr., indicated no inhibition of wheat, maize, and soybean or hemp seed (*Cannabis sativa* L.) germination (Harry-O`kuru *et al*., 1999). The examined *G. fruticosus* leaves extract which also belongs to the Apocynaceae family, did not exhibit phytotoxic effects on the *N. tabacum* plants in the present study (Figure 6.10).

These results illustrated that no extract caused systemic damage to the tobacco plants and the injuries were limited to the area on leaves where treated with the extracts. As a result, the damage on the leaves can be described as mechanical damage causing by rubbing. It can thus be speculated from these results that symptomic damage noted is not due to the plant extracts but due to mechanical damage. There is a lack of information of the phytotoxicity of the selected plant extracts in the literature.

Figure 6.10: At both the concentrations (20 mg/ml and 50 mg/ml) *G. fruticosus* extract did not cause any injury on the leaves of *N. tabacum*.

6.4.2 DAS-ELISA assay

The main objective of this investigation was to detect the PVY^N *in vitro* in *N. tabacum.* The result of the DAS-ELISA test indicated that all of the plants which assumed to be the source of PVY^N were identified as PVY^N positive. As shown in Figure 6.11, all plant samples and also the positive control turned yellow after adding the PNP solution as indicator.

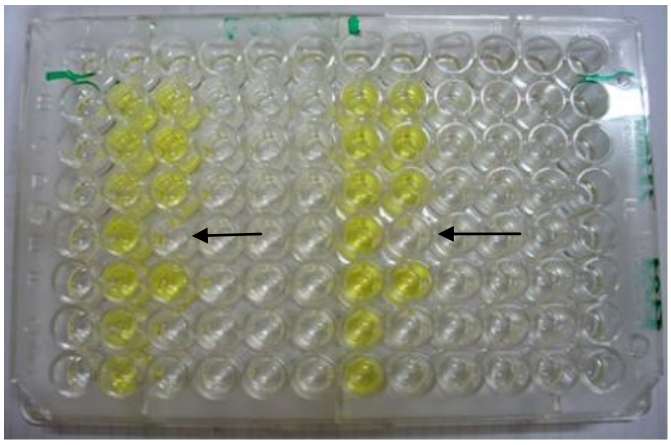

Figure 6.11: DAS-ELISA plate test with the samples of source of PVY^N virus and negative and positive controls after adding PNP solution. Yellow wells indicate the presence of virus and clear wells indicate the absence of virus. The arrows in the figure indicate the clear wells of the negative controls.

As expected, no colour change was observed in the negative control (healthy tobacco plant sample) and it remained colourless with the DAS-ELIS test. A healthy observation is generally below 0.1. Each well with > 0.16 optical density value shows the virus existence. All samples, which were tested to confirm the presence of the PVY^N in *N. tabacum* showed an optical density higher than 0.16 (Figure 6.12).

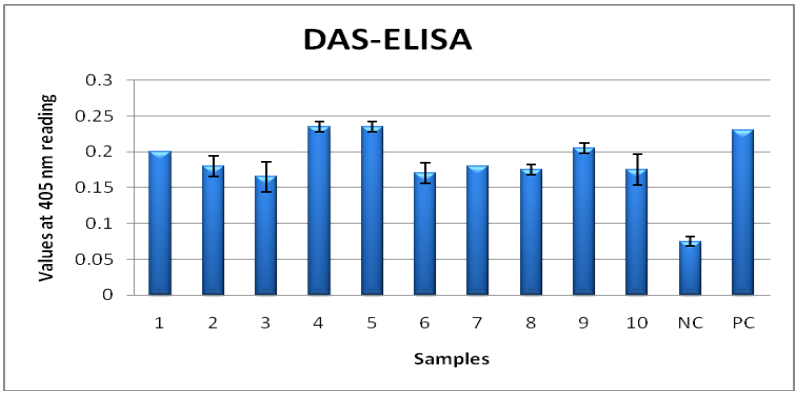

Figure 6.12: Values of the DAS-ELISA reading to detect the PVY^N *in vitro* in *N. tabacum*. (Numbers 1 to 10 plant samples which was tested for confirming the presence of the PVY^N virus from the source of virus, NC: negative control or healthy tobacco plant, PC: positive control).

This proves the presence of PVY^N in *N. tabacum*. The negative control as is expected illustrates the optical density below 0.1, which means it is free of the PVY^N.

6.4.3 Antiphytoviral test

6.4.3.1 PVY^N symptoms on *N. tabacum*

The PVY^N virus exhibited some symptoms such as local lesions, mottling mosaic lesions, necrotic lesions and ring-spot (Figure 6.13).

A B C

Figure 6.13: Ring-spot (A and B) and mottling mosaic lesions, (C) symptoms on leaves of tobacco caused by PVY^N.

Most strains of PVY can infect *N. tabacum* and cause vein clearing followed by leaf mottling mosaic lesions, except the PVY^N strain, which induces severe vein necrosis. Some isolates within the PVY^N strain also cause necrotic tuber ring-spot disease (Xu, 2008).

6.4.3.2 *In vivo*: Effect of extracts and compound on the infectivity of PVY^N

The effect of different concentrations of plants extracts containing cardiac glycosides and the isolated cardiac glycoside from *S. speciosus* fruit extract on PVY^N was evaluated. Symptoms like leaf mottling, mosaic lesions and necrosis were observed in some plants after a month indicating no protection. Analysis of variance (ANOVA) is a technique of decomposing the total variation in data in terms of variation due to identifiable and interesting sources that cause this variation (Gelman, 2006). The accumulate analysis of variance was done to determine the degrees of freedom (d.f.), sum of squares (s.s.), mean of

squares (m.s.), variance ratio (v.r.), and *F* probability (*F* pr.). Figure 6.14 describes the analysis of variance. According to the statistical analysis, those sources of variations which represent $P < 0.05$ have a significant relationship. In this study two sources of variances, namely treatment and the combination of concentration and treatment together have a significant relationship ($P < 0.05$) as shown in Figure 6.14.

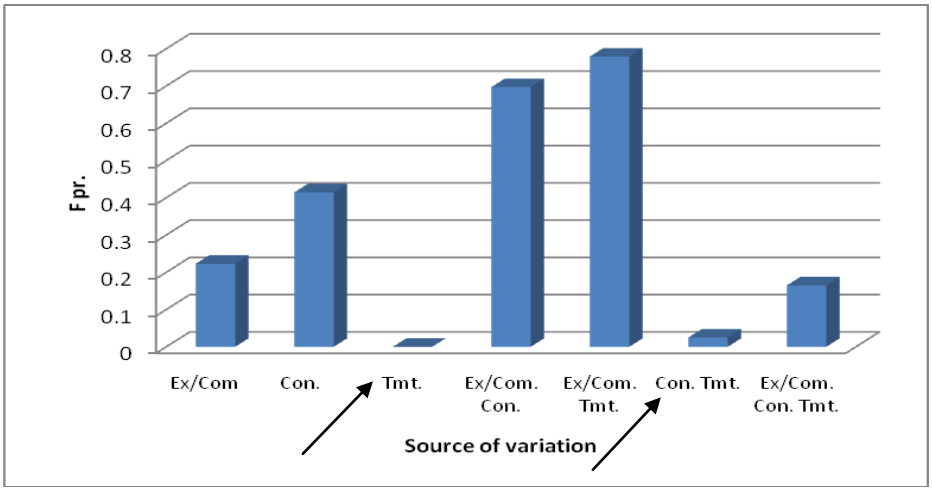

Figure 6.14: Analysis of variance in percentage infectivity test. The interaction is significant between treatment and concentration.treatment. The arrows show the two sources of variances which had significant relationship ($P < 0.05$). (Ex/Com: extract/compound, Con: Concentration, Tmt: treatment).

Figure 6.15 and line graphs of the Figure 6.16 shows that the infection of the plants decreased with the application of the extracts/compound in comparison to the positive control, although the extracts/compound could not completely protect the *N. tabacum* plants against the PVY^N infection in contrast to the negative control. The downward trend of the D1C-TV (treated with extracts and compound one day before infected with PVY^N virus) illustrates that with an increase in concentration of extracts from 20 mg/ml to 50 mg/ml, the percentage infection of the *N. tabacum* plants was decreased (Figure 6.16). High concentration extracts are more effective in reducing the virus infection of *N. tabacum.* The negative control shows extremely low infection of the *N. tabacum* plants. But the positive controls, which were infected with PVY^N, demonstrated the infection is higher than the *N. tabacum* plants which were treated with extracts (Figure 6.16).

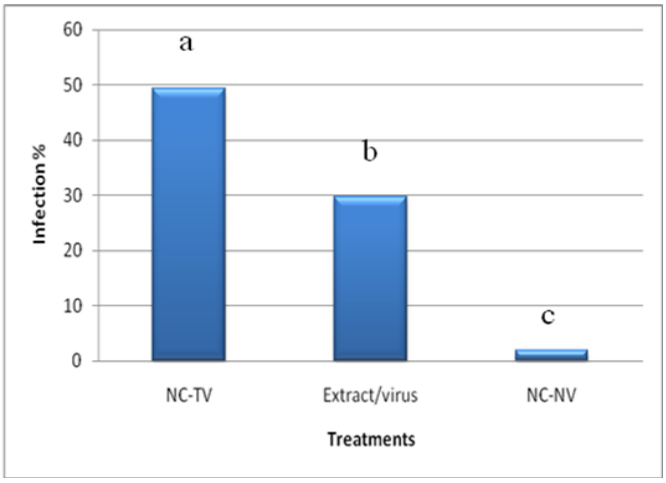

Figure 6.15: Percentage infection of PVY^N of all treatments in the *in vivo* test. (Values of Bars not followed by the same letter are significantly different.) (NC-TV: positive control, Extract/virus: treated with extracts and compound one day before infected with virus, NC-NV: negative control.)

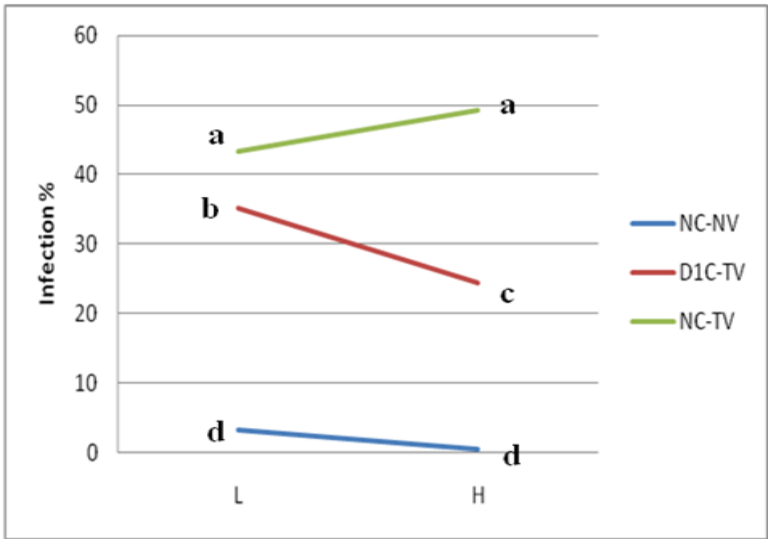

Figure 6.16: Percentage of infection in each treatment in relation to the concentration in the *in vivo* test. Values of Bars not followed by the same letter are significantly different. **(**L: low dosage (20 mg/ml), H: high dosage (50 mg/ml), NC-NV: negative control, D1C-TV: treated with extracts and compound one day before infected with virus, NC-TV: positive control).

Figure 6.17 illustrates the percentage infection on the *N. tabacum* plants in relation to the extracts/compound, treatments and concentration together. According to the Figure 6.17,

there are significant differences between the positive controls and treatment with extract/compound. Of the nine extracts and one compound, six of the extracts [*M. plumbea* (dry bulb), *N. oleander*, *B. volubilis* (fresh bulb), *C. orbiculata*, *M. plumbea* (fresh bulb)] and the compound (cardiac glycoside) were successful in controlling the virus infection in comparison to the positive control although they could not completely stop the infection.

The experiment demonstrates that only the high dosage of the extracts was able to control the virus. The result shows that the *M. plumbea* (dry bulb) is the most active extract although not significantly better than the other five which inhibits the virus.

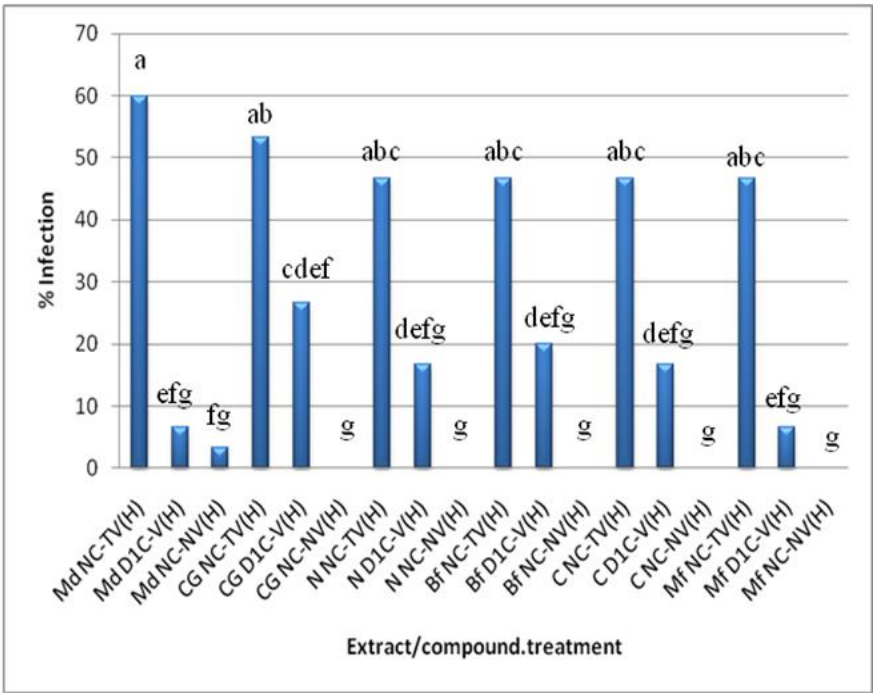

Figure 6.17: Percentage infection of treatments by high concentration (50 mg/ml) of extracts and compound in the *in vitro* test. (Values of Bars not followed by the same letter are significantly different. [H: high concentration (50 mg/ml), Md: *M. plumbea* (dry bulb), CG: cardiac glycoside, N: *N. oleander*, Bf: *B. volubilis* (fresh bulb), C: *C. orbiculata*, Mf: *M. plumbea* (fresh bulb), NC-TV: positive control, D1C-V: applying chemicals one day before infecting with virus, NC-NV: negative control]).

In 2012, Jing *et al*. examined the antiviral potential of the 126 Chinese plants belonging to the 36 different families. Extracts from nine species strongly inhibited both infection and

viral replication. One of these plants was *Rhodiola eurycarpa* (Ford.) S. H. Fu. (Jing *et al*., 2012). *Rhodiola eurycarpa* and *C. orbiculata* which were tested in this study both belong to the *Crassulaceae* family. The result of the present study showed that the *C. orbiculata* had significant activity to decrease the percentage infection and symptom as compared to its positive control (Figure 6.17).

6.4.3.3 *In vitro*: Effect of extracts and compound on systemic PVY^N infection

Although no physical symptoms were observed in some cases after mechanical infection, the presence of viruses were detected through the DAS-ELISA test. This result indicated that PVY^N does not necessarily always produce physical symptoms. It has been shown through the DAS-ELISA test that the antiserum reacted strongly with infected plants. Figure 6.18 shows the analysis of variance in the DAS-ELISA test and the two sources of variances; concentration and treatment; which were significant different ($P < 0.05$). Figure 6.18 describes the analysis of variance and shows evidence of a statistically significant activity of concentration and treatments because these two sources of variance show $P < 0.05$.

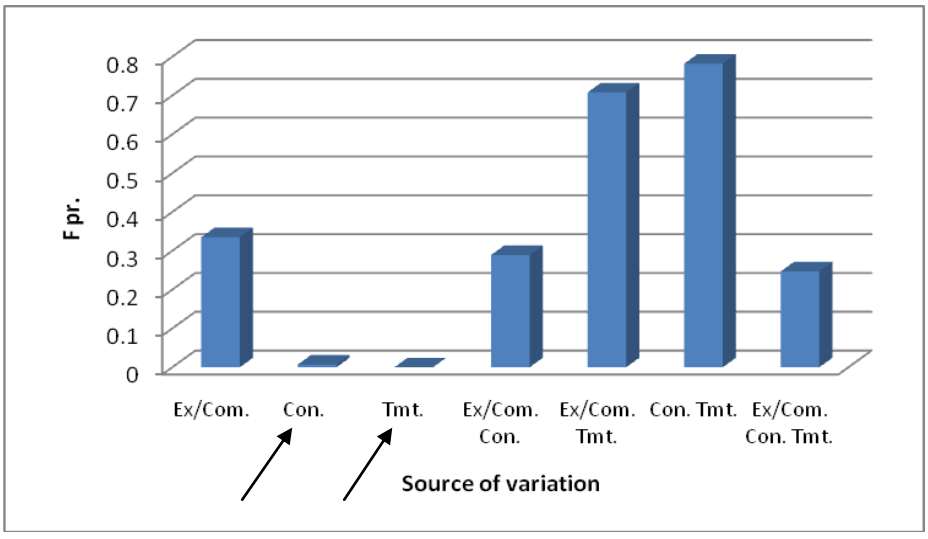

Figure 6.18: Analysis of variance in DAS-ELISA test. The arrows show the two sources of variances with significant relationship ($P < 0.05$). (Con.: concentration, Tmt.: treatment).

The DAS-ELISA value of those treated plants which was near to the negative control value (0.1), illustrated that the virus concentration in treated plants was very low. Moreover, no

visible mosaic symptoms were observed on uninfected leaves (negative control) of treatment plants, while the disease symptoms on the positive control were severe. When comparing the positive and negative control ELISA values, the middle column of Figure 6.19 shows that the extracts were able to decrease virus infection but they could, however, not completely stop the virus infection. It means that some extracts were able to reduce the infection and some of them could not.

The ELISA values identify extracts which were successful to decrease the plant infection. Figure 6.19 illustrates that there are significant differences between each of treatments. ELISA values of < 0.1 demonstrate no infection and shows that the negative control plants were free of virus as expected. The DAS-ELISA value of the positive control was > 0.1, so it demonstrates the presence of the virus in the *N. tabacum* plants.

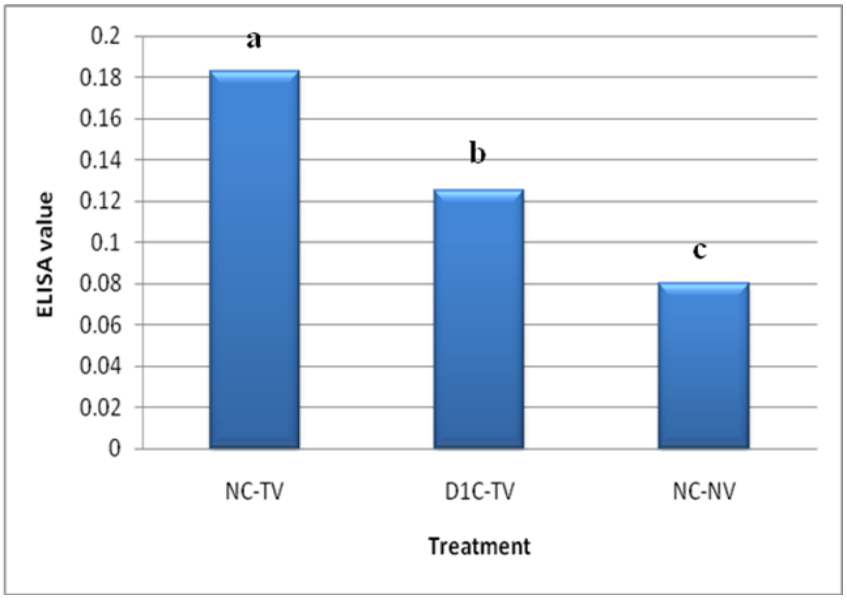

Figure 6.19: The DAS-ELISA value of treatments in *in vivo* test (healthy absorbance < 0.1). Values of Bars not followed by the same letter are significantly different. (NC-NV: negative cotrol, D1C-TV: treated with extracts and compound one day before infected with virus, NC-TV: positive control.)

The interaction of the concentrations and each treatment are shown in Figure 6.20. This graph shows that the low concentration (20 mg/ml) extracts were not as succesful to control the virus infection as the higher concentration (50 mg/ml).

The high concentration of extracts and compound reduced the plant infection and it become closer to the negative DAS-ELISA value reading (0.1). The negative control (NC-NV) values as expected are less than 0.1 and proves that there was no infection. The positive control (NC-TV) demonstrates the DAS-ELISA value more than 0.15, which indicates a high infection of the tobacco plants.

The *M. plumbea* value of the DAS-ELISA reading was very close to the negative control. In the *in vivo* test *M. plumbea* dry bulb extract also illustrated antiviral activity, which demonstrates the antiphytoviral activity potential of this bulbous plant. The results indicate that extracts of *M. plumbea* were active in controlling systemic infection of tobacco plants by PVY^N. The *S. speciosus* (fruits) extract was also able to reduce the infection of tobacco plants by the PVY^N virus (Figure 6.21).

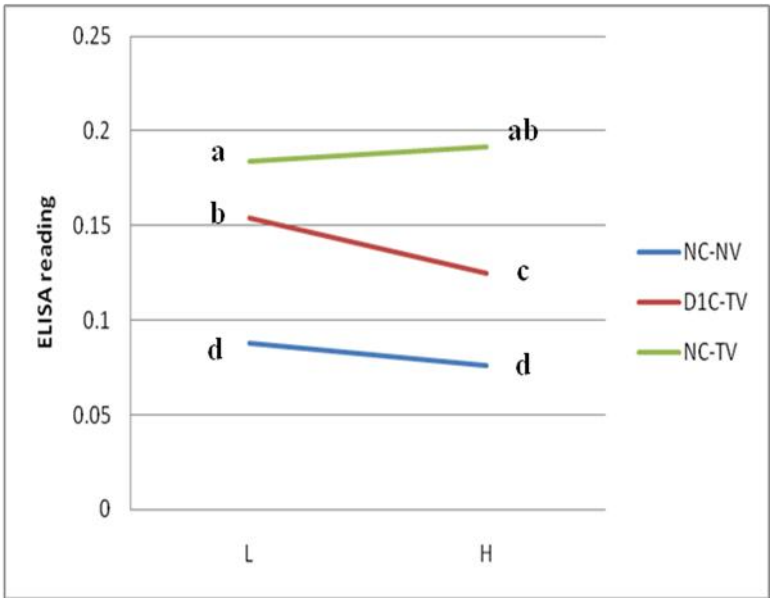

Figure 6.20: ELISA value of infection in dosage–treatment interaction (no infection < 0.1). Values of Bars not followed by the same letter are significantly different. [L: low dosage (20 mg/ml), H: high dosage (50 mg/ml), NC-NV: negative control, D1C-TV: treated with extracts and compound one day before infected with virus, NC-TV: positive control.]

The results of the present study indicate that the tested extracts and compound with regards to controlling the local infection and the symptoms were more active than controlling systemic infection. A similar assumption was reached by Naylor *et al.* (1998) and Othman

and Shoman (2004). Othman and Shoman (2004) indicated that the antiphytoviral activity of *Plectranthus tenuiflorus* (Vatke) Agnew on some important viruses such as Tobacco Necrosis Virus (TNV), Tobacco Mosaic Virus (TMV), and Tomato Spotted Wilt Virus (TSWV) was more active in case of the existence of the symptoms than systemic infection. It can be attributed to several possibilities; such as, the virus replication might be faster than inducing resistance in treated plants, and the antiphytoviral might have an effect on the virus movement more than virus multiplication thereby delaying the onset of systemic symptoms without significant effect on the virus accumulation (Othman & Shoman, 2004).

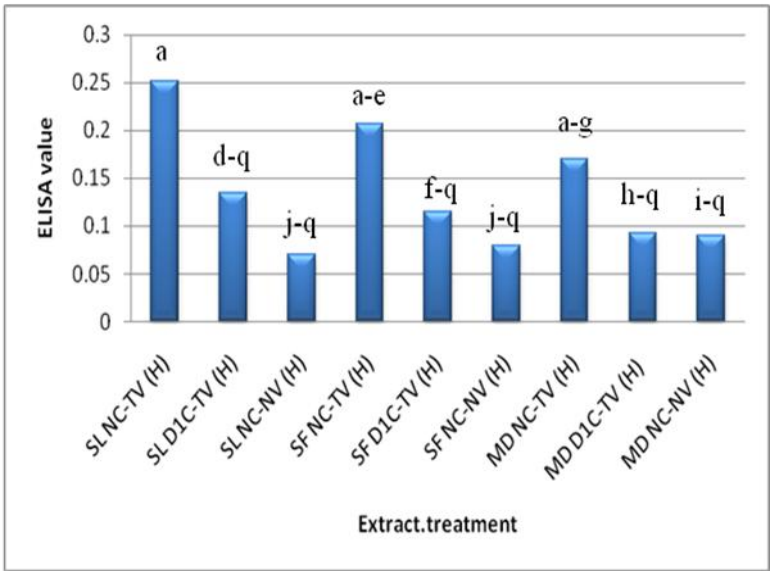

Figure 6.21: ELISA values of the interaction of extract/compond, treatment and high concentration which showed significant difference in the statistic analysis. Values of bars not followed by the same letter are significantly different. [H: high concentration (50 mg/ml), SL : *S. speciosus* (Leaves), SF: *S. speciosus* (Fruits), MD: *M. plumbea* (Dry bulb), NC-TV: positive control, D1C-TV: treated with extracts and compound one day before infected with virus, NC-NV: negative control.].

The analysis of the interaction of all extracts and compound, treatment and concentration shows that of the nine extracts and one compound at two concentrations tested against the PVY^N virus, just three of the extracts demonstrated significant activity against the virus. *Strophanthus speciosus* (leaves and fruits) and *M. plumbea* (dry bulb) extracts exhibited significantly better activity against the PVY^N infection when compared to the control and

other extracts. Figure 6.21 shows those extracts, which were successful to control the virus infection and the significant different activity between the most active extracts and their positive controls.

Mohamed (2010) investigated the antiviral potential of garlic (*Allium sativum* L.) clove extract and onion bulb (*Allium cepa* L.) extract against PVY and found that garlic was more effective in reducing the local lesions produced by PVY on *Chenopodium amaranticolor* Coste & A.Reyn. than the onion extract. The latex of *Ficus nitida* Thunb showed activity against the Bean Yellow Mosaic Virus (BYMV) and Zucchini Yellow Mosaic Virus (ZYMV) (Mahmoud *et al.*, 2010).

The ELISA assay on khella seed (*Ammi visnaga* L.), black cumin (*Nigella sativa* L.) and garlic bulb (*A. sativum*) water extracts against some plant viruses, illustrated that tomato plants sprayed with plant extracts under field conditions were free from Tomato Mosaic Virus (TMV), CMV, Potato Virus X (PVX) and PVY (El-Dougdoug *et al.*, 2007).

Chinese scientists (2009) chose 108 species of plants which showed antiviral capacity in Chinese traditional medicine to screen for antiphytoviral activity. TMV was selected as a model target. Among them, an extract from *Coreopsis drummondii* (D. Don) Torr. and Gray predominantly suppressing the infection and replication of TMV was selected, and a constituent with antiviral activity was isolated from it (Qijian *et al.*, 2009). The compound was identified as 1-phenyl-1,3,5-triheptalkyne. Its activity against TMV was investigated by local lesion and leaf discs assay. The results indicated that the compound showed a significant inhibitory activity against TMV *in vitro*, with 73.5% inhibition against the infection of TMV and 84.3% against replication of TMV at a concentration of 0.2 mg/ml. The anti-TMV activity of this plant extract and isolated compound therefore shows potential as a candidate for development of antiphytoviral drugs (Qijian *et al.*, 2009).

In recent years, many active compounds from plants such as high molecular weight molecules like proteins or polysaccharides and low molecular weight molecules like alkaloids, flavones and terpenes have been isolated. Triterpenoids and triterpenoid glycosides are found in many plant species. According to Zhang *et al.* (2007), on the structure-activity relationship of triterpenes and triterpenoid glycosides against TMV,

triterpenoid glycosides were found to have potential inhibitory activity against TMV replication *in vivo*. The activity was closely related to the molecular structure of the compounds. They discovered that according to the structures of the compounds, with the same main structure, but with transformation of substituent groups, the anti-TMV activity of the compounds will change over a wide range.

The analysis of the relationship between chemical structures and bioactivities demonstrate that the activities of triterpenoids and triterpenoid glycosides are not only determined by the core structure but also by the substituents, which sometimes affect the activity dramatically. According to Zhang and co-workers (2007), the triterpenes of the ursane type, a lactone at the position C_{20} ones of the oleanane triterpenoids, an α-hydroxy at C_{19}, xylosyl at C_3, and glycosyl at C_{28} all decreased the inhibitory activity of triterpenoid glycosides (Zhang *et al.*, 2007). Comparing the Zhang *et al.* (2007) study with the present study illustrates an interesting result. In this study the cardiac glycoside isolated from the *S. speciosus* fruit extract, which belongs to the secondary modified triterpenoids, also showed activity against the PVY^N *in vivo*. Structures of cardiac glycoside compounds have some difference to triterpenoid glycosides, which could possibly be the cause of the difference of their activity.

In a study by Shen *et al.* (2008) the antiviral effect of a compound isolated from *B. javanica* seed on TMV in a systemic host was demonstrated. The compound was sprayed twice; one day before inoculation and one day after inoculation; at the certain concentration. The plants were screened for virus presence by the ELISA technique. The ELISA value of treated plants was near to the negative control, suggesting that the virus concentration in treated plants was very low (Shen *et al.*, 2008). In the present study, the extracts and compound were sprayed just one time (one day before inoculation). It could be possible to investigate more effective activity of the extracts and compound if they were sprayed the day after inoculation also.

6.5 Conclusion

Plant viruses can cause numerous diseases in a wide range of crop plants and causing an estimated $600 billion in annual losses worldwide (Jing *et al.*, 2012). To control viral plant diseases, many approaches have been used, including chemicals, breeding, cross-protection and transgenic plants. However, there are no accurate treatments that can totally inhibit plant

viruses after they have infected plants. As the discovery of plants extracts with antiphytoviral activity, much attention has been given to the selection of natural products with antiviral activity. Many crude extracts from plants have been screened to determine their antiviral activity against plant viruses due to their advantages over chemical pesticides (Jing *et al*., 2012).

Several strategies have been adopted to manage the virus diseases on potato through using insecticides without success especially for viruses transmitted by insects in a non-persistent manner (Sikora *et al.*, 1998). Recently the efforts were directed toward searching for substances more effective including chemicals, biological or natural plant products that can induce resistance in the treated plants against viruses (Kloepper *et al.*, 1992). This idea came from the fact that when a plant is infected by a pathogen, the reaction of the plant toward this pathogen is development of a systemic resistance to subsequent infection (Kessmann *et al.*, 1994; Ryals *et al.*, 1996).

According to Shen *et al.* (2008), many crude extracts from plants have been screened to determine their antiviral activity against plant viruses. Bioassay guided fractionation of some crude extracts showing inhibitory effect has been extensively studied and led to the isolation of many active constituents (Shen *et al.*, 2008).

Comparing both the *in vivo* and *in vitro* experiment results, the higher concentration of *M. plumbea* (50 mg/ml) dry bulb is the most successful extract to control the PVY^N infection and has viricide potential. In both *in vivo* and *in vitro* experiments, the higher concentration of the extracts showed good activity against the PVY^N. The isolated cardiac glycoside from *S. speciosus* fruit extract which inhibited the infectious symptoms of PVY^N, is a cardenolide. But the type of cardiac glycoside of the *M. plumbea* is a bufadienolide type. If the cardiac glycoside of the *M. plumbea* would be the active substituent against the PVY^N, it can be suggested that the bufadienolides are more active against the PVY^N than the cardenolides. However, this requires further investigation. Furthermore, increasing the concentration of the extracts and compound to 100 mg/ml and also applying the extracts and compound twice, before and also after inoculation, will probably be more successful to control the virus and should be investigated further. With this in mind that the *M. plumbea* was the only

extract that showed phytotoxicity effect, the phytotoxicity activity of higher extract concentrations must be considered.

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Chapter 7

General discussion and recommendations for future research

7.1 General discussion

According to Fabricant & Farnsworth (2001), the investigation of fossils revealed human use of plants as medicines can be dated back at least to the Middle Palaeolithic age almost 60,000 years ago. The value of this exploration is more than a significant anthropologic or archaeological fact. This significance is due to the identification of the methodology of medicinal agents. Today according to the World Health organization (WHO), as many as 80% of the world's people have used traditional medicine for their primary healthcare needs. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatment of various diseases (Mahmood & Shah, 2012). The goals of using plants as sources of therapeutic agents are to isolate the bioactive compounds such as digoxin, digitoxin, morphine, taxol, vinblastine and vincristine for direct use as drugs, as well as to produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce the medicinal agents of higher activity and/or lower toxicity (Fabricant & Farnsworth, 2001).

As mentioned, medicinal plants have been traditionally used for different kinds of ailments including infectious diseases. There is an increasing need for substances with antibacterial and especially antiviral activity since the treatment of bacterial and viral infections with the available antiviral drugs often leads to the problem of resistance (Sritharan & Sritharan, 2004). The emergence of resistance to antibiotics in several pathogenic microorganisms in the past two decades has gradually caused the conventional treatments to be less effective (Sritharan & Sritharan, 2004). Bacteria can be resistant to antibacterial medications because of their structure or physiology, or they are able to develop genetic mutations to circumvent the action of the drug (Morley *et al*., 2005). Herpes Simplex Virus (HSV), a member of the Herpesviridae family, known from the ancient Greeks, can cause a range of diseases from the uncomplicated mucocutaneous infection to those which are threatening to human life (Whitley & Roizman, 2001). Since the treating agents available for HSV infections are either low in quality or limited in efficiency, there is a need to search for new and more effective antiviral agents for HSV infections (Khan *et al.*, 2005; Shah, 2005).

Chapter 7

Like humans and animals, plants are also subject to a variety of different diseases. In order to maintain a sufficient food supply for the world`s population, it is necessary to find solutions to combat plant disorders that are capable to destroy crops on a large scale (Bos, 1982). Potato Virus Y^N (PVY^N) is a plant pathogen which causes a major economic disease in most Solanaceous crops such as potato. According to the report of the FAO (2010), the total production of potato was 2,090,210 tonnes in South Africa which is the most important vegetable crop in South Africa. This virus is the most economically important disease problem in seed potatoes in many areas of the world. The virus is responsible for decreases in yield and quality (Gray *et al.*, 2010).

Taking into account all of the damages and consequences to humans and plants due to bacterial and virus infections, investigation into more successful treatments is vital. Recent studies demonstrated the potential of plant extracts and their isolated compounds to prevent bacterial as well as viral infections in humans and also the plant kingdom.

In this study, the ethanolic extracts of selected plants (leaves of *Gomphocarpus fruticosus*, *Nerium oleander*, *Cotyledon orbiculata;* the fruits and leaves of *Strophanthus speciosus;* the bulbs of *Bowiea volubilis* and *Merwilla plumbea*), which are known to contain cardiac glycosides, were investigated for their biological activity. All extracts were tested against four different strains of bacteria, the HSV-2 virus as well as PVY^N. The possible antimicrobial property of an isolated cardiac glycoside was examined against four strains of bacteria and PVY^N.

According to Van Vuuren (2008), extracts with minimum inhibitory concentration (MIC) values lower than 8 mg/ml are considered to possess some antimicrobial activity. In this study all tested extracts illustrated some levels of activity against both the Gram-negative (*Escherichia coli* and *Klebsiella pneumonia*) and Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) bacteria with the MIC < 8 mg/ml. *Cotyledon orbiculata* with a 1.25 mg/ml MIC value showed the best activity against *S. aureus* and *E. faecalis*. *Bowiea volubilis* dry bulb extract was the most successful extract to inhibit *K. pneumonia* with a MIC value of 2.5 mg/ml. Three plant extracts, the dry and fresh bulb of *B. volubilis* and *C. orbiculata*, were the most active to inhibit *E. coli* with a MIC of 2.5 mg/ml when compared to the other extracts.

The investigation of the biological activities of plant extracts must include their toxicity for safety for their future use as a therapeutic agent (Harbell *et al*., 1997). Therefore plant extracts were first assayed for cell toxicity in two susceptible cell lines namely the Human Embryonic Kidney (HEK 293) cell line and the Vero cell line (African Green Monkey kidney). The antiviral activity evaluation was conducted against the HSV-2 on Vero cells. Both cytotoxicity and anti-HSV tests were conducted using the XTT method. The cytotoxicity results on both cell lines tested exhibited highly toxic effects of the plant extracts. The ranges of the IC_{50} values of the plant extracts were from 23.34 µg/ml of *B. volubilis* dry bulb extract to 57.95 µg/ml of *S. speciosus* leaf extract, except for the *M. plumbea* with the lowest toxicity with an IC_{50} value of 97.06 µg/ml. According to Atjanasuppat *et al*. (2009), the 50% inhibition concentration value of the plant extracts with an IC_{50} approximately from 20 µg/ml to 100 µg/ml is quite toxic. Due to the strong toxic effects of the extracts and also because of the extensive concentration dilutions to perform the anti-HSV assays, no anti-HSV activity was observed. Also the unsuccessful result of the anti-HSV experiment can be due to the inhibitory effects of the other compounds present in the extract.

According to the general thin layer chromatography and bioassay results, *S. speciosus* fruit extract was chosen for the isolation of pure compounds with an emphasis on cardiac glycosides. Three pure compounds were isolated; namely ursolic acid methyl ester, *myo*-inositol-methyl ether and an unidentified cardiac glycoside. No evidence could be found in the literature with regards to the isolation of ursolic acid methyl ester and *myo*-inositol compounds from *S. speciosus* fruit. The isolated cardiac glycoside was tested against Gram-negative (*E. coli* and *K. pneumonia*) and Gram-positive (*S. aureus* and *E. faecalis*) bacteria. This compound was not successful in inhibiting any of these bacteria. No evidence could be found in the literature of the inhibitory activity of cardiac glycoside compounds against *S. aureus*, *E. faecalis*, *E. coli* and *K. pneumonia*. When comparing the results of the antibacterial activity of plant extracts and the isolated cardiac glycoside it is speculated that the responsible substance or substances of the plant extracts to inhibit the tested bacterial pathogens is not the isolated cardiac glycoside. The XTT cytotoxicity test of the isolated cardiac glycoside showed extreme toxic effects of this compound against the two tested cell lines (HEK 293 and Vero cell line) with a 4.62 µg/ml IC_{50} value. The IC_{50} value of an extract or compound which is less than 20 µg/ml is extremely cytotoxic (Atjanasuppat *et al*., 2009).

No evidence could be found in the literature of previous cytotoxicity studies of cardiac glycoside compounds on the HEK 293 cell line. According to the literature there is a strong possibility of antiviral activity of the cardiac glycosides.

The ethanolic extracts of all selected plants and isolated cardiac glycoside were subjected to *in vitro* and *in vivo* anti-PVY^N screening. The statistical analysis of variance of the *in vivo* result showed there was a significant relationship between the treatment and the combination of concentration and treatment of the extracts and compound. It indicated that with the increasing concentrations of extracts and isolated cardenolide, the symptoms of PVY^N virus on the tobacco (*Nicotiana tabacum*) plant decreased. The *in vivo* antiphytoviral results showed that the high concentration (50 mg/ml) of *M. plumbea* (fresh and dry bulb), *N. oleander, B. volubilis* (fresh bulb), *C. orbiculata* and isolated cardenolide reduced the PVY^N symptoms on tobacco plants significantly in comparison to the positive control.

The statistical analysis of variance of the *in vitro* test results demonstrated that there was a noteworthy relationship between concentration and treatments. In this test it was also indicated that with the increasing concentrations of extracts and isolated cardenolide, the DAS-ELISA values were decreased. The *in vitro* antiphytoviral result of DAS-ELISA test showed that the high concentration (50 mg/ml) of *S. speciosus* (leaves & fruits), and especially *M. plumbea* (dry bulb) have significant antiphytoviral activity. Considering both the *in vivo* and *in vitro* results, *M. plumbea* has potential antiphytoviral activity against PVY^N. The results illustrated that even though the isolated compound decreased the symptoms of the PVY^N on the tobacco plant, it was not successful in inhibiting the virus inside the plant cells.

The aim of this study was to determine the plant extract with the least cytotoxicity and best bioactivity that would be considered as a new approach to antibacterial and antiviral medication. After conducting this study it can be concluded that the extract with the least cytotoxicity (97.06 ± 31.04), moderate antibacterial activity (average MIC of 3.75 mg/ml) and the extract which best reduced the symptoms of the PVY^N is *M. plumbea* (fresh bulb). The next best extract with moderate cytotoxicity (44.63 ± 1.9), good antimicrobial activity (2.5 mg/ml) and reduced the symptoms of the PVY^N is *C. orbiculata*.

Independently, *B. volubilis* (dry bulb) had the best overall antimicrobial activity (2.5 mg/ml) and *M. plumbea* (dry bulb) had the best inhibitory effect on the presence of the PVY^N virus in the plant cells. Therefore the plant with the least cytotoxicity and best bioactivity which can be considered for a new approach to novel antibacterial and antiviral medication is *M. plumbea*.

7.2 Recommendations for future research

All studies and investigations are endless and there are always some considerations and new ideas to improve future research. To improve the prospective work of the present study, there are few recommendations and suggestions.

Most of the selected plant extracts have shown promising antibacterial activity. Further research is needed to elucidate the active constituents of these plants which may be useful in the development of new and effective antibacterial agents.

According to the literature some of the selected plant extracts and particularly isolated cardiac glycosides showed antiviral potential previously. In this study it was however not possible to determine the antiviral potential of the extracts *in vitro*. Alteration of the range of dilutions, to start at 150 µg/ml with eight dilutions ending at 0.58 µg/ml, would be suggested to obtain the possible desirable result.

Because of the existence of some complexity in the isolated cardiac glycoside especially at the glycone part, using other methods such as HMBC, X-ray crystallography and further NMR analysis with higher resolution may help to accurately elucidate the structure, especially to clarify the sugar part of this complex compound. Due to the time restrictions of this study, there was no opportunity to test the isolated cardenolide against the HSV-2. An appealing experiment to conduct would be the examination of the antiviral activity of the isolated cardenolide and its possible mechanism of action and also determining the glycone activity on the possible inhibitory action of this compound against viruses.

Very promising results were obtained with the antiphytoviral tests and these should be investigated further. *Merwilla plumbea* showed potential to be developed as an antiphytoviral agent. Some aspects that will have to be investigated in future are; toxicity to

the environment, interaction with fertilisers or pesticides, how long it takes for the extract to be broken down to mention only a few. Since there are very little remedies available to treat viral infections in plants, this could be an indication of a very promising field of study in the future.

7.3 References

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Appendix

^{1}H NMR and ^{13}C NMR of isolated compounds from *Strophanthus speciosus* fruit extract

1. Compound A: fraction 3; sub fraction 14-20

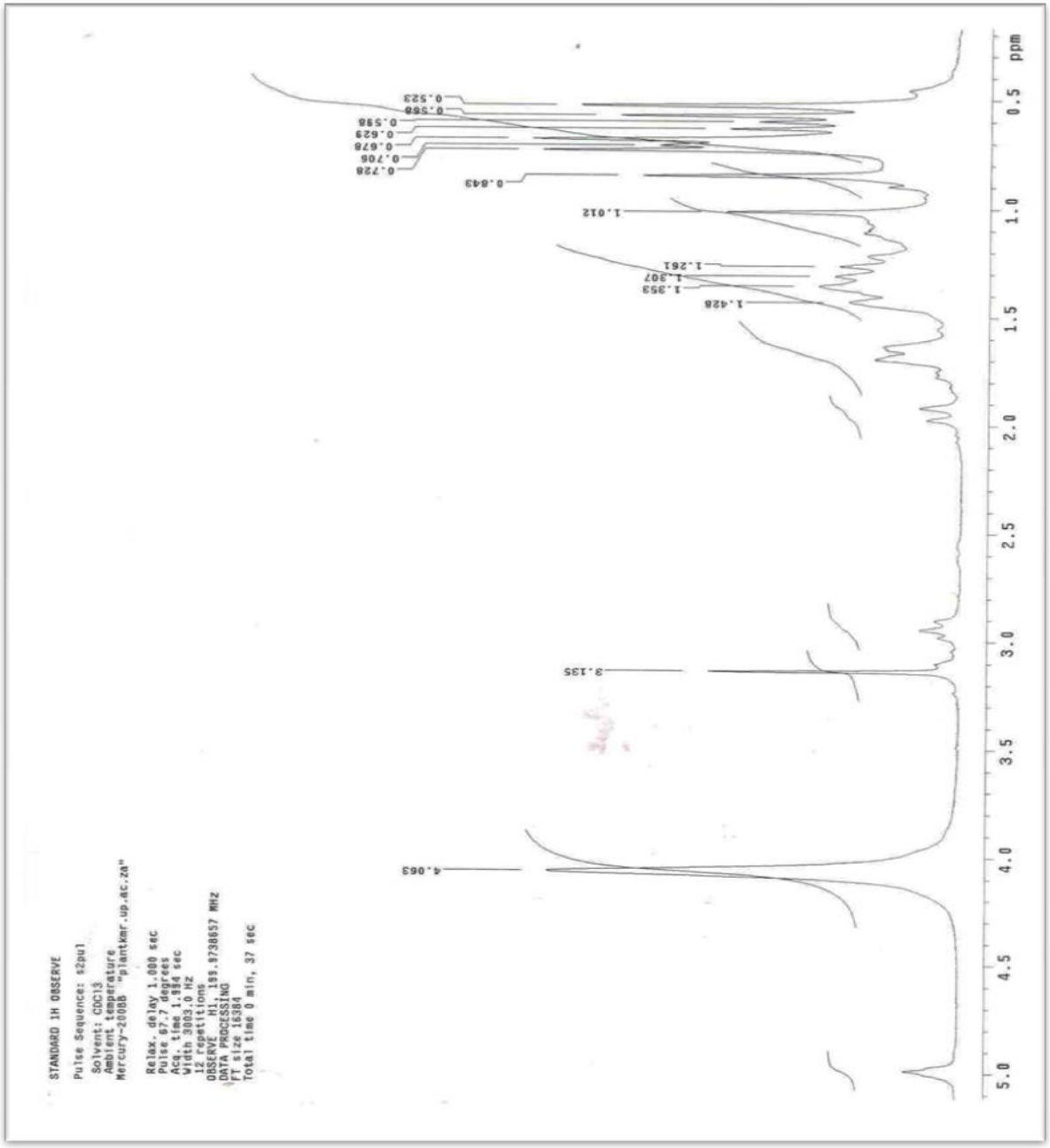

Figure 1: ^{1}H NMR for ursolic acid methyl ester

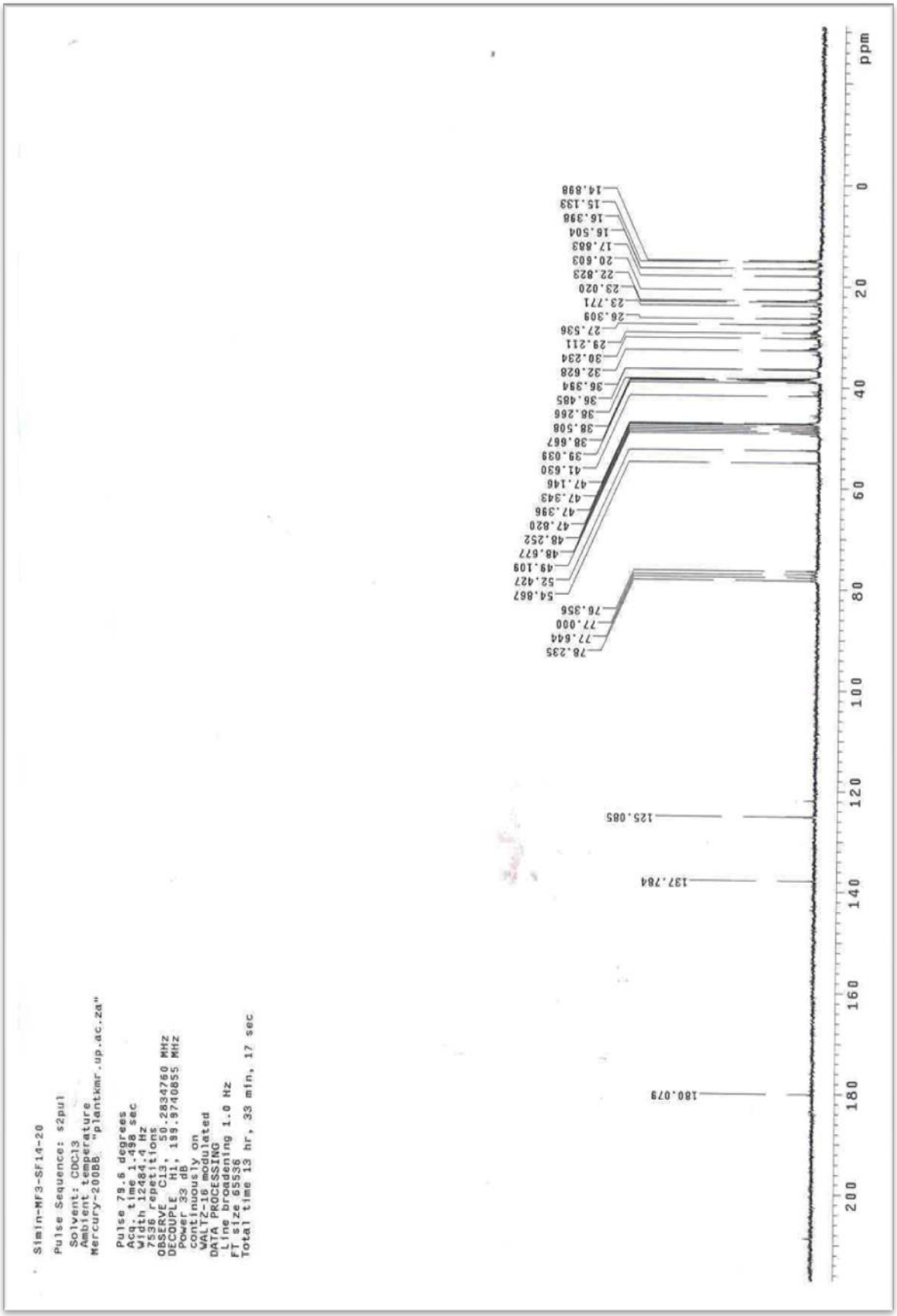

Figure 2: ^{13}C NMR spectra for ursolic acid methyl ester

2. Compound B: fraction 23 and 24

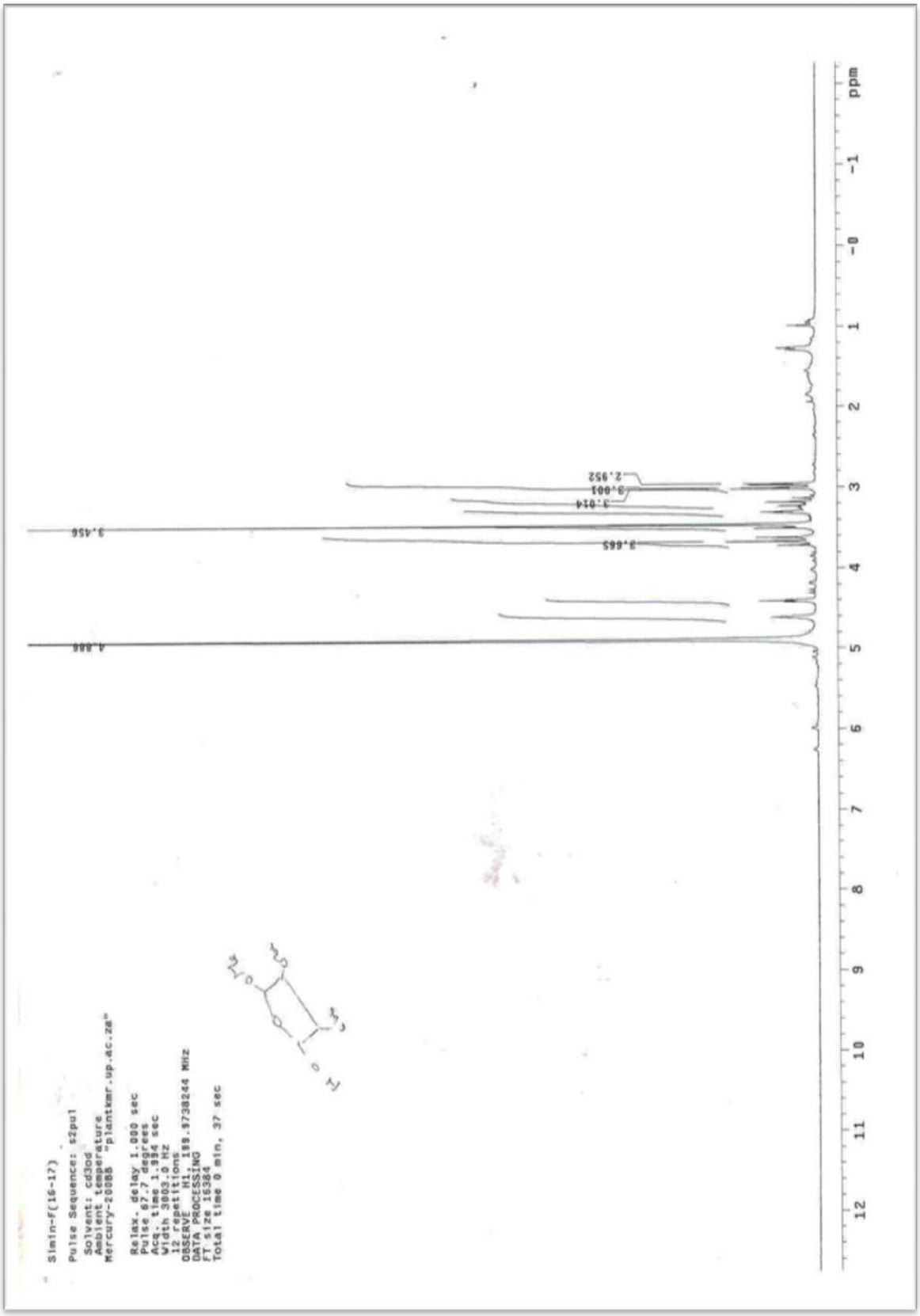

Figure 3: ^{1}H NMR for *myo*-inositol-methyl ether

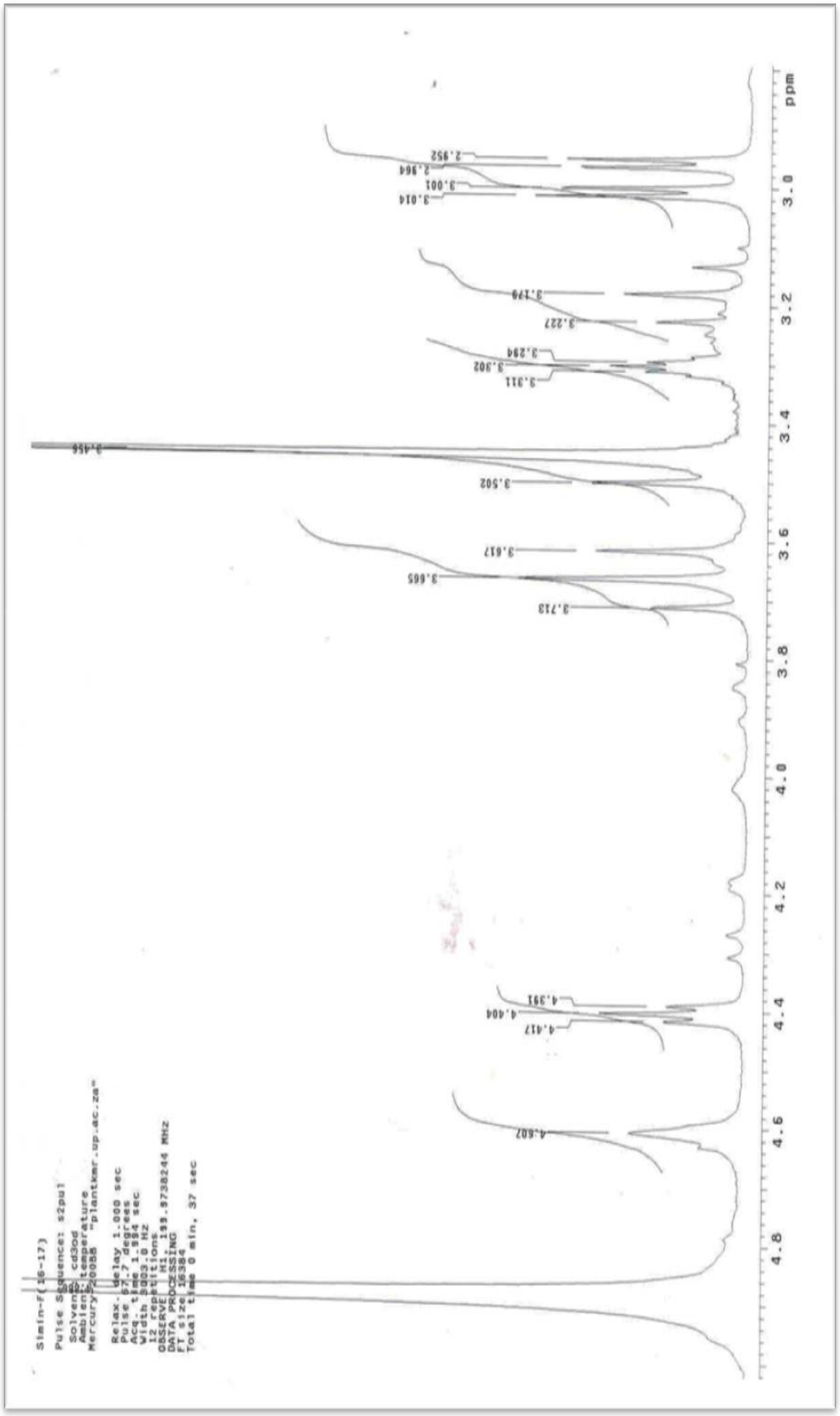

Figure 4: ^{1}H NMR for *myo*-inositol-methyl ether

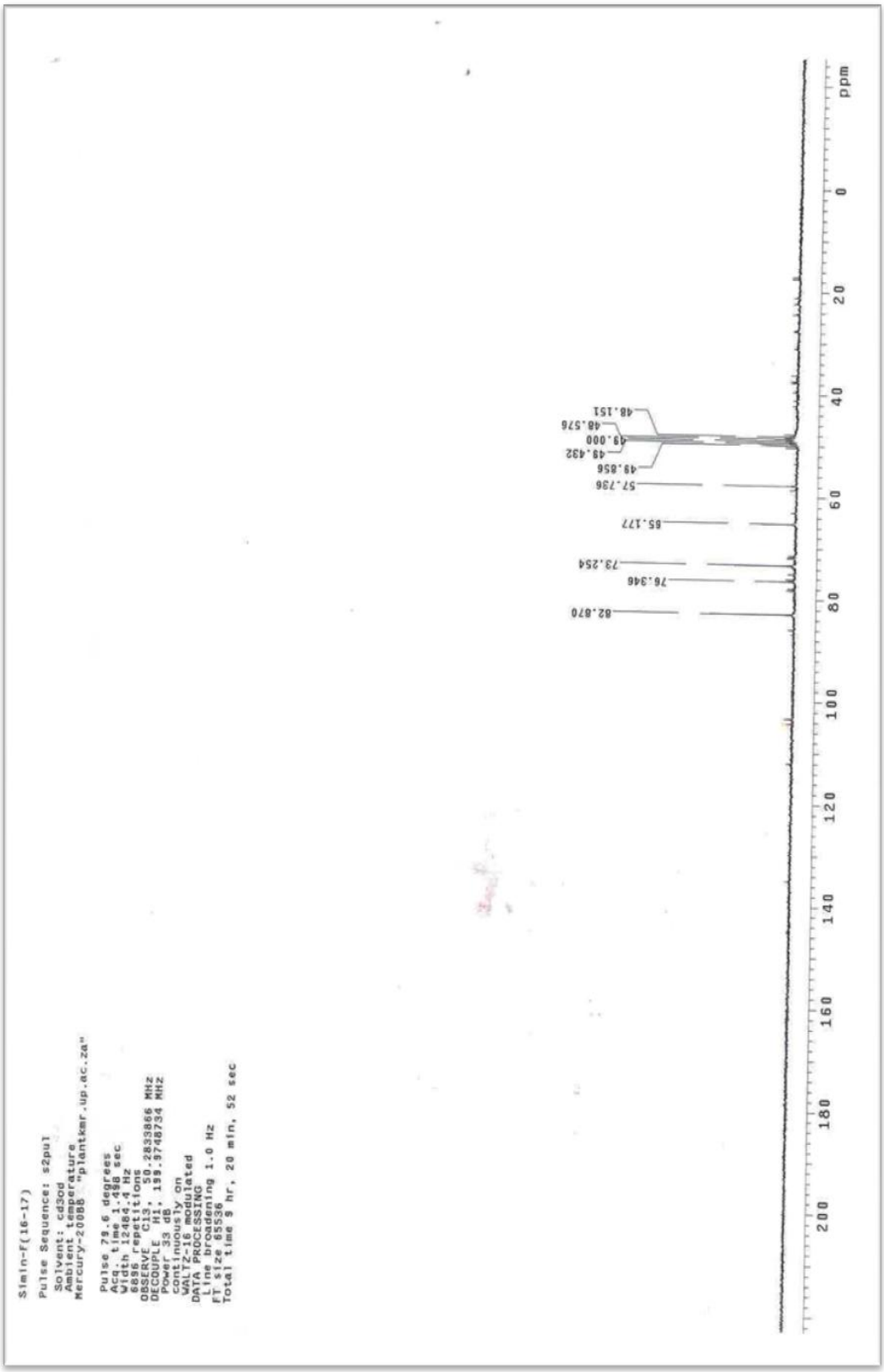

Figure 5: ^{13}C NMR spectra for *myo*-inositol-methyl ether

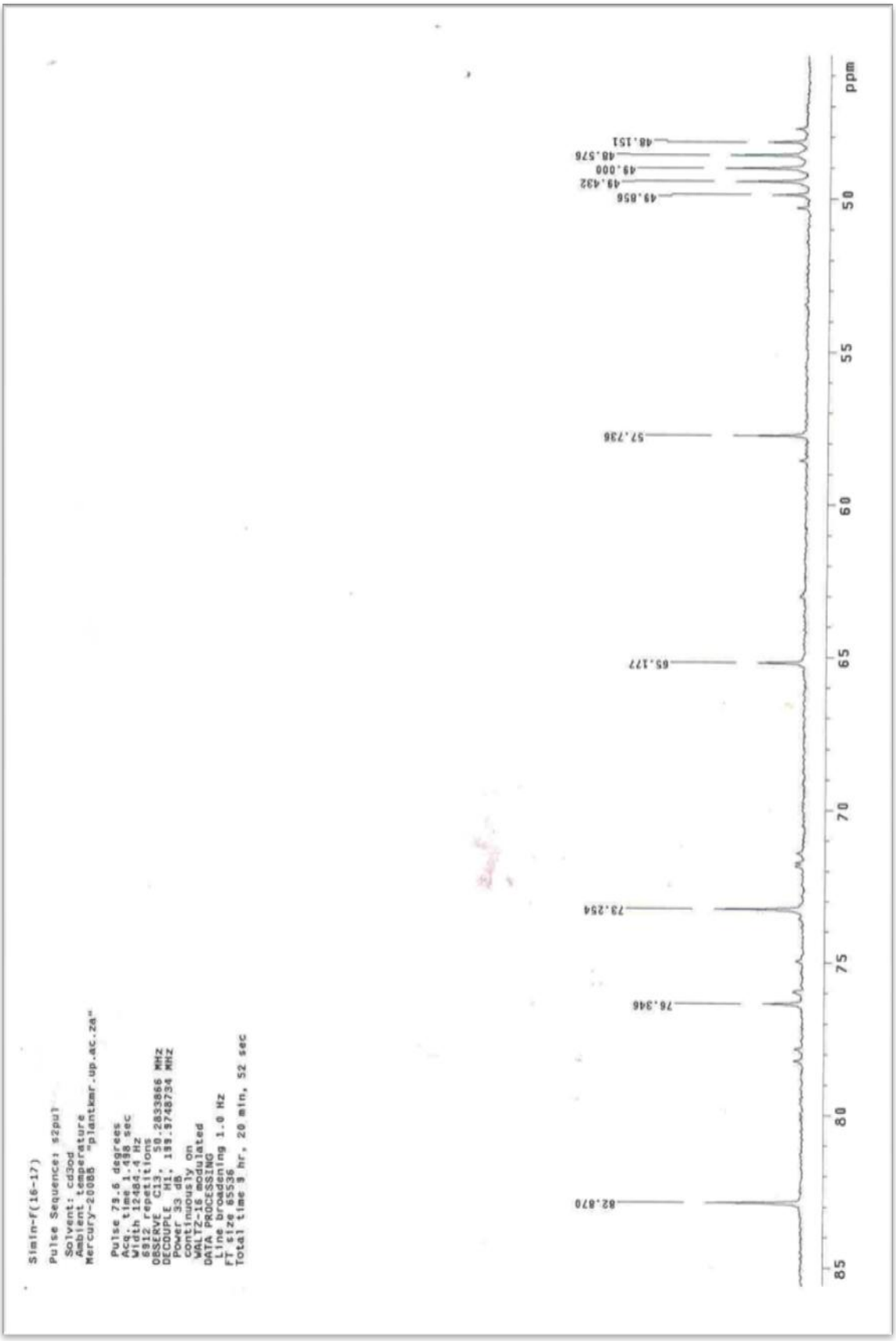

Figure 6: ^{13}C NMR spectra for *myo*-inositol-methyl ether

3. Compound C: fraction 17-18 and 20

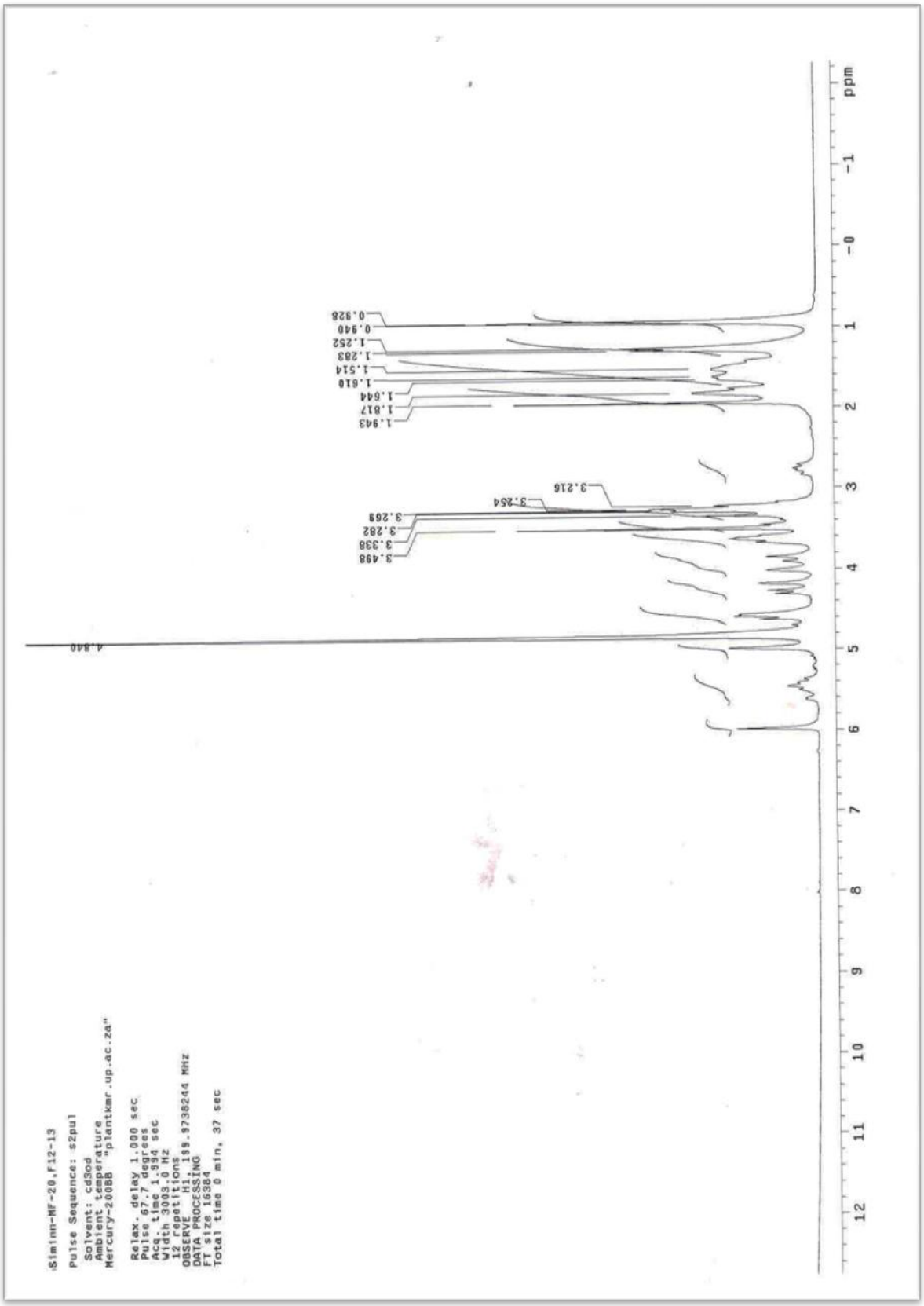

Figure 7: ^{1}H NMR for cardenolide

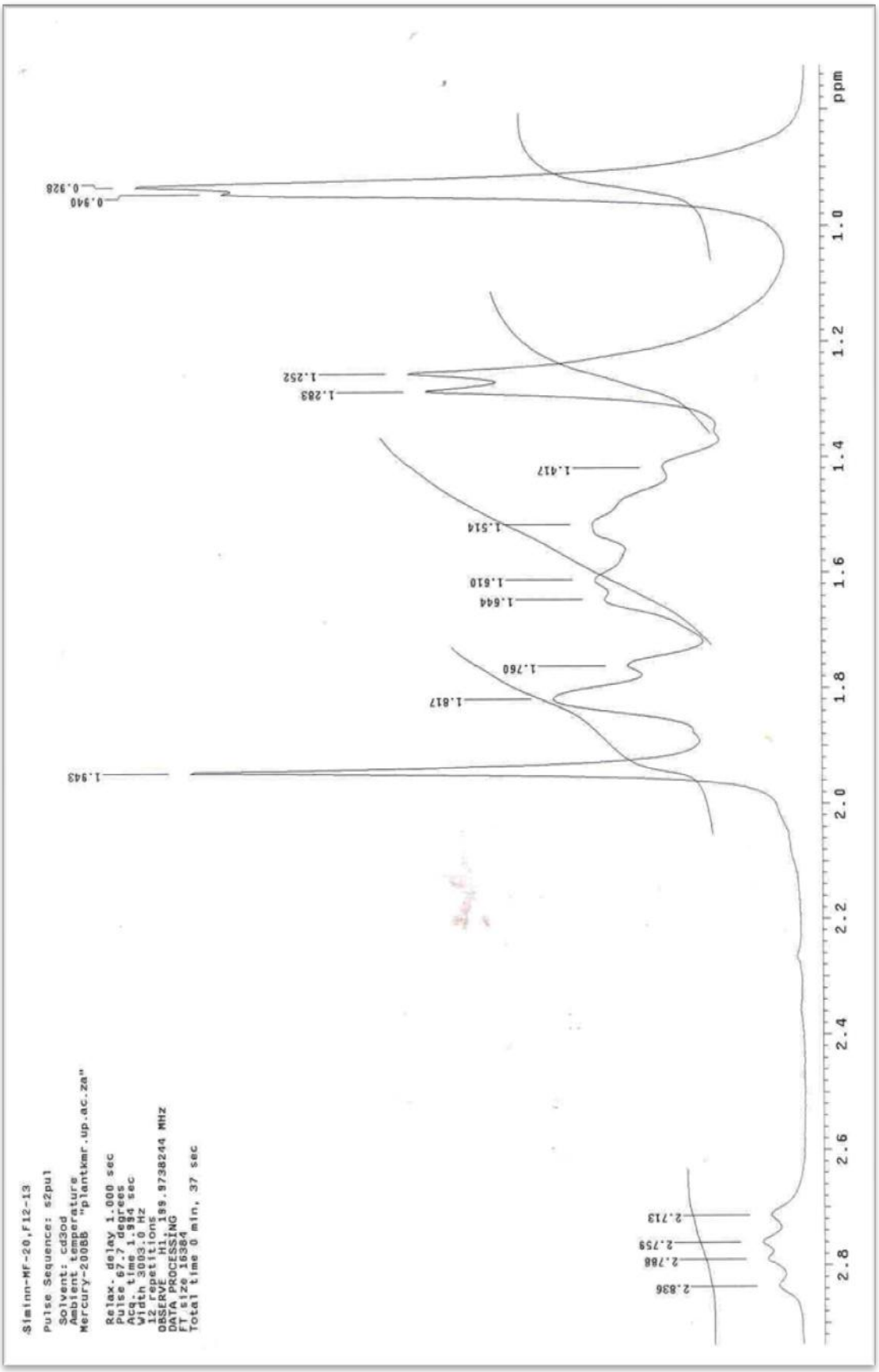

Figure 8: ^{1}H NMR for cardenolide

Appendix

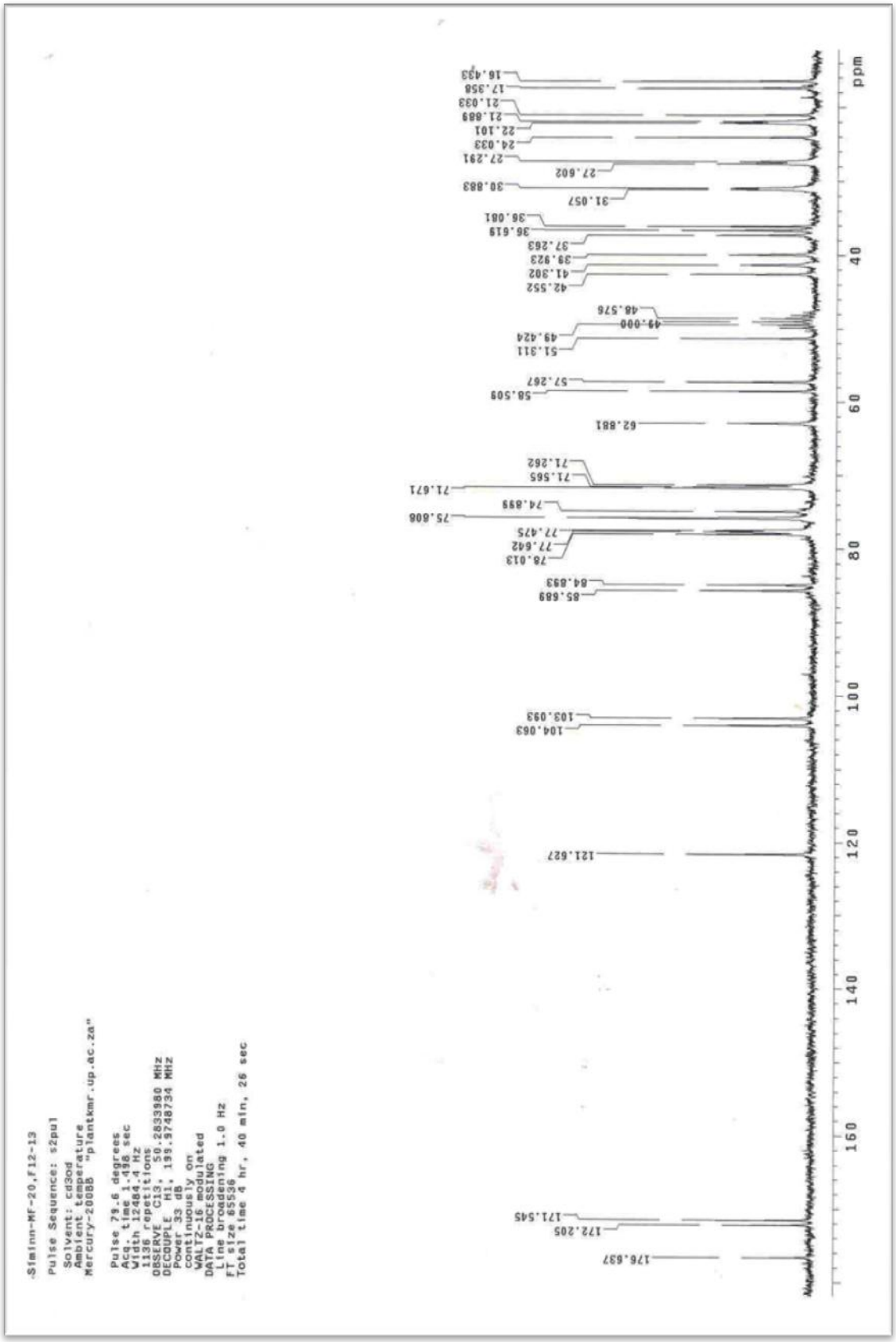

Figure 9: ^{1}H NMR for cardenolide

Appendix

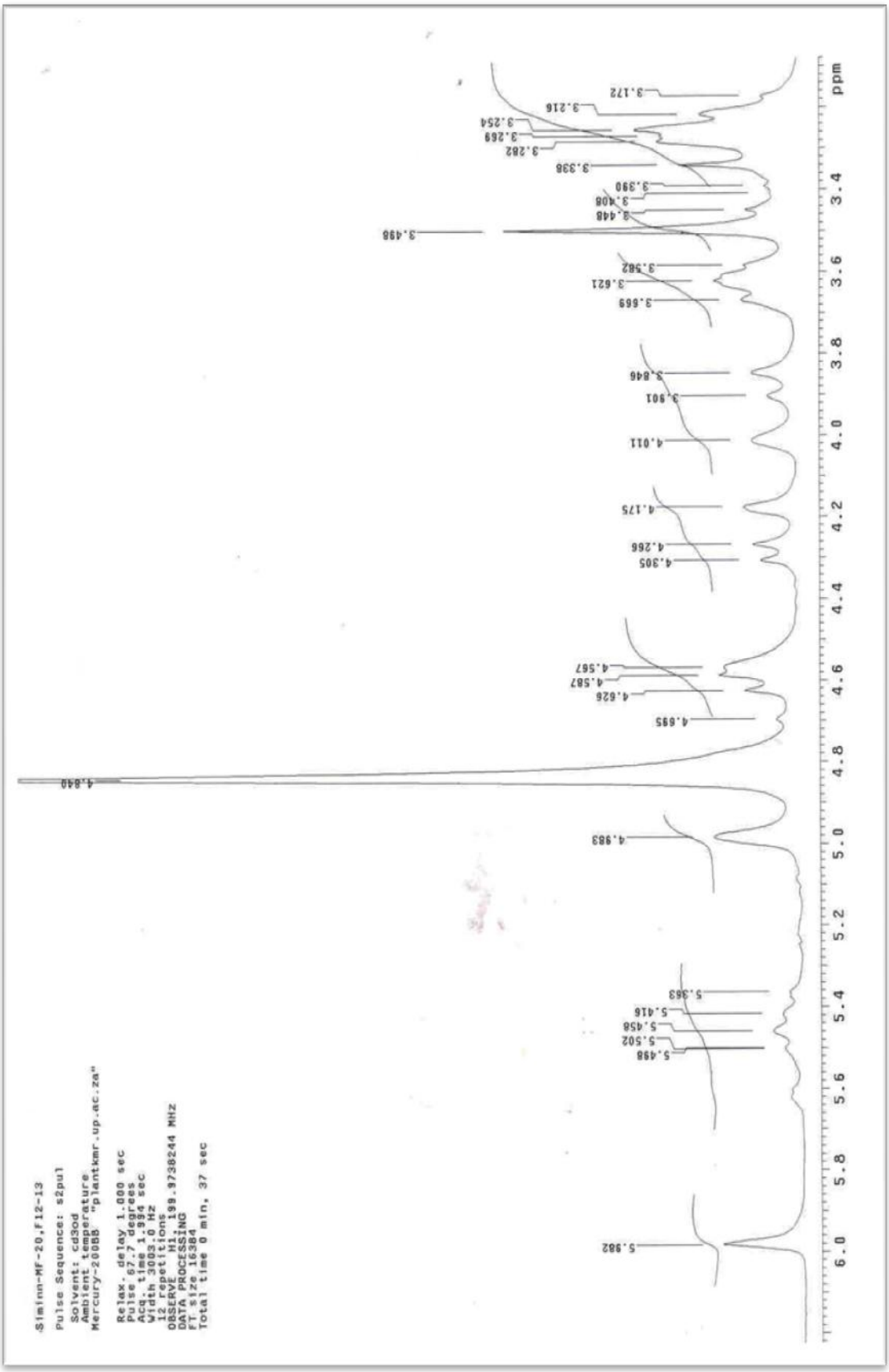

Figure 10: ^{1}H NMR for cardenolide

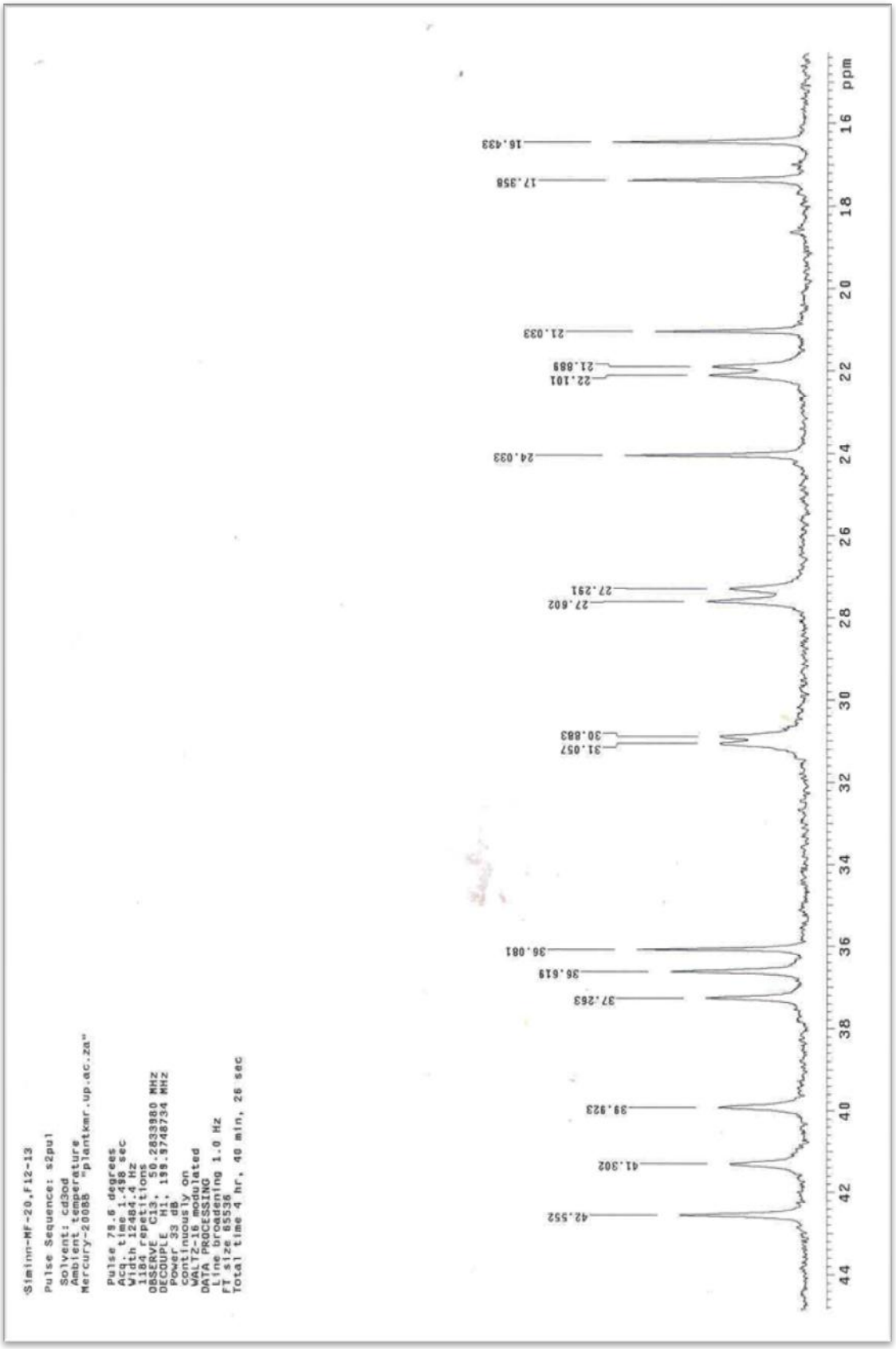

Figure 11: ^{13}C NMR for cardenolide

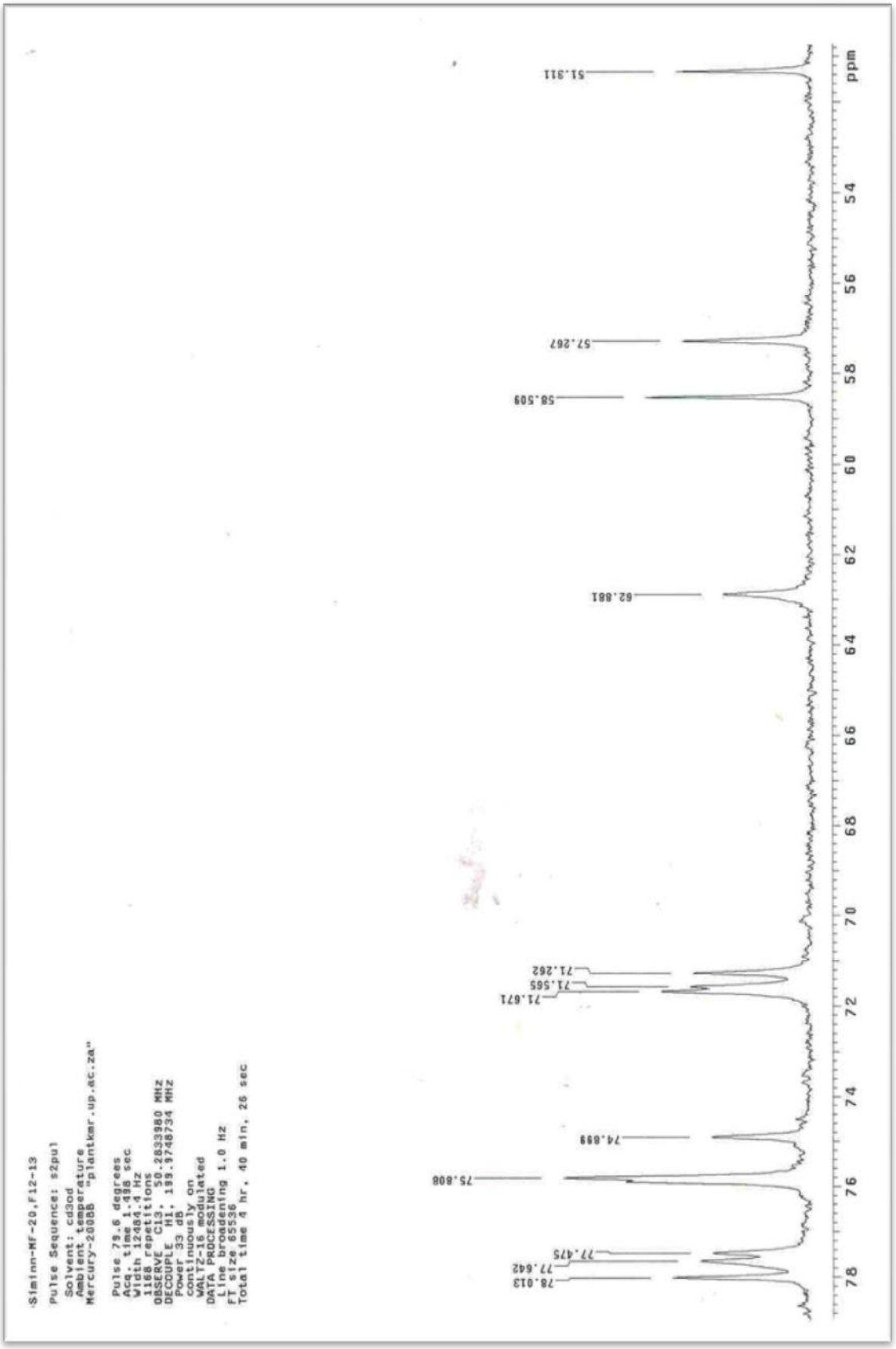

Figure 12: ^{13}C NMR for cardenolide

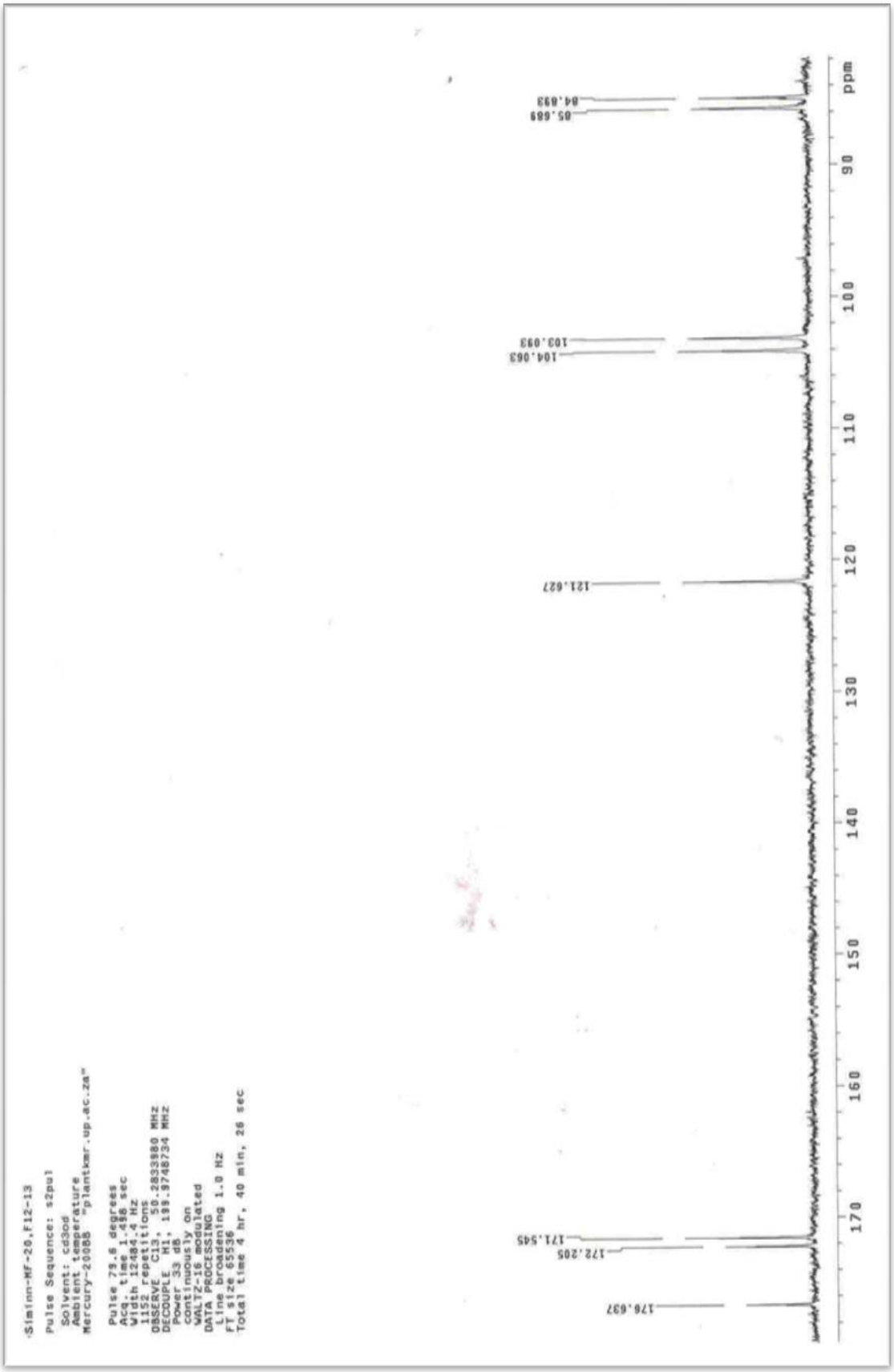

Figure 13: ^{13}C NMR for cardenolide

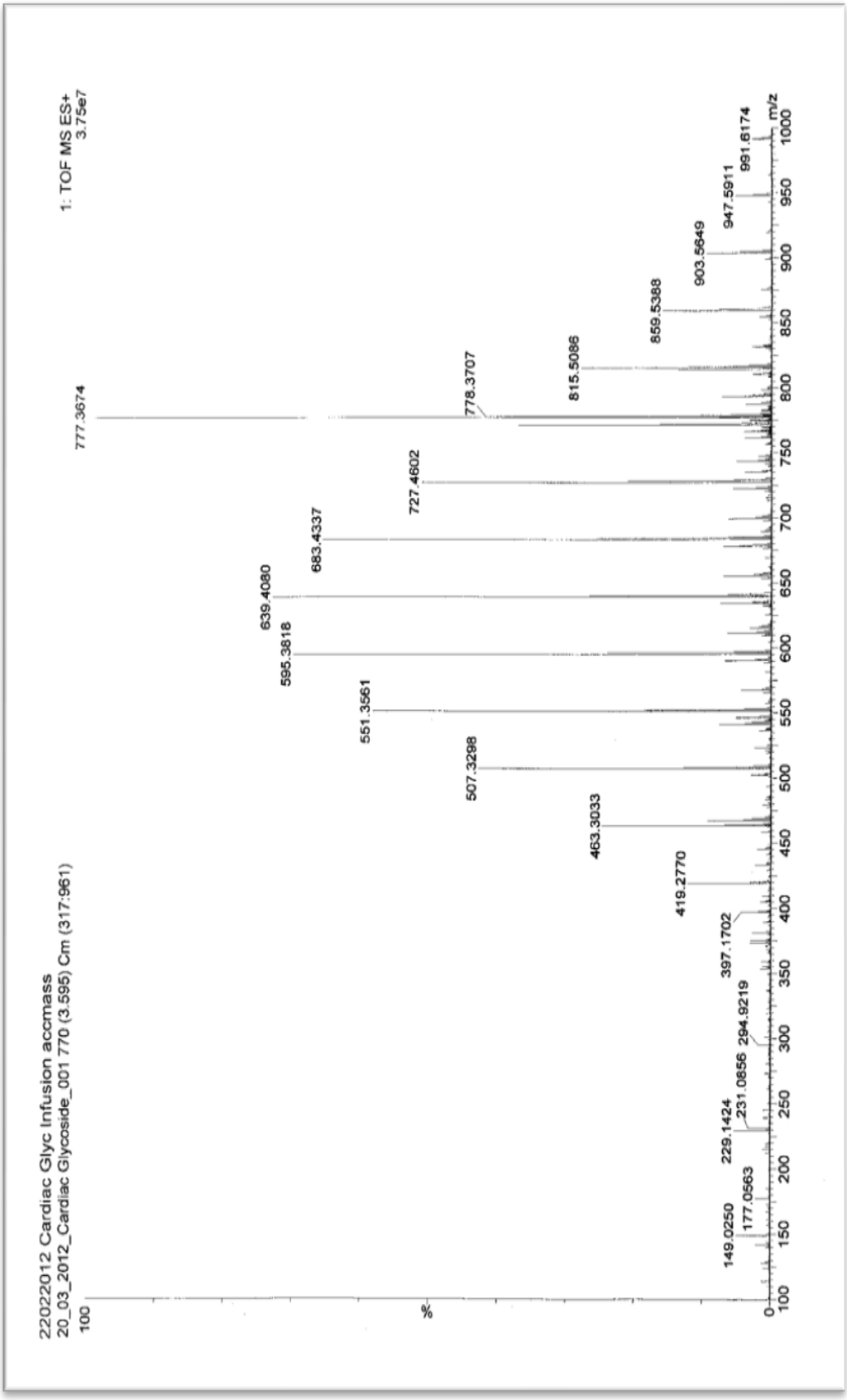

Figure 14: HRMS spectra for cardenolide