IN VITRO AND *IN VIVO* ANTICANCER ACTIVITY OF *GOMPHOCARPUS FRUTICOSUS* EXTRACTS AND DEVELOPMENT OF LIPID-BASED NANOPARTICLES

A DISSERTATION SUBMITTED IN FULFILMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN SCIENCE (BIOLOGICAL SCIENCES)

OF

THE UNIVERSITY OF NAMIBIA

BY

FLORENCE DUSHIMEMARIYA

200815431

September 2019

MAIN SUPERVISOR: Prof Davis Mumbengegwi (Multidisciplinary Research Center: University of Namibia)

CO-SUPERVISOR: Dr Ronnie Bock (Department of Biological Sciences: University of Namibia)

ABSTRACT

Cancer is on the rise globally, with incidences and mortalities increasing in developing countries including in Namibia. The ongoing search for efficacious and safe medicines to treat cancer is often cumbered by poor solubility and toxicity of potential treatments. *Gomphocarpus fruticosus* is a medicinal plant used to treat tumors in Namibia through topical application and it has been shown to have anticancer action against several cell lines. However, due to its cardiotoxic properties, it has not been investigated further. This study was conducted to develop a lipid-based drug delivery system based on *Gomphocarpus fruticosus* extracts, to alleviate the plants toxicity profile while harnessing its anticancer properties. The phytochemistry of *G. fruticosus* harvested in Namibia was investigated using thin layer chromatography and Gas Chromatography-Mass Spectroscopy. The anticancer activity was evaluated against three cells lines (PC3, HeLa and HepG2) and cytotoxicity against primary cell lines (Hek293 and KMST6). Apoptosis induction was investigated using the *APOPercentage*^{TM} assay, while general oxidative stress and caspase induction was investigated using the Reactive oxygen species (ROS) assay in HeLa and Hek293 cells. The toxicity of the *G. fruticosus* extracts was also assessed using *in vivo* toxicity assays in Balb/C mice. Finally, to improve solubility of phytochemicals and reduce non-specific toxicity, a lipid based delivery system was formulated through thin-layer hydration of phosphatidylcholine, cholesterol and plant extract (250:1:10) in phosphate buffered saline The nanoparticles were characterized for physically for zeta potential and polydispersity using Dynamic Light Scattering and Scanning Electron Microscopy. The encapsulation efficiency of the drug delivery system and its stability in media was also investigated before its biological activity was evaluated against the HeLa cervical cancer cell line. Phytochemical analysis showed *G. fruticosus* had alkaloids, coumarins, steroids, anthraquinones, flavonoids and triterpenoids. Furthermore, anti-inflammatory, anticancer and antioxidant compounds such as beta-sitosterol, alpha-amyrin, lupeol and their esters and acetates were detected by GCMS. The plant extract showed selective antiproliferative activity against HeLa cells (IC_{50} 8.35^{-7} ± 0.13 g/L) although the mechanism of cell death was most likely necrosis not apoptosis as expected. This conclusion was supported by detected ROS levels and lack of caspase cleavage. *In vivo* toxicity in Balb C mice was observed (LD_{50} 1.968^{-1} ± 59.16 g/kg) and

classified as category 3 under GHS. Lipid based nanoparticles showed high encapsulation efficiency of 96.51 % with a particle size range between 130-150 nm and narrow particle size spread. The zeta potential was low at -1.6 ± 0.6 mV, contributing to agglomeration and low stability in biological media. Fluorescent microscopy and flow cytometry analysis showed uptake of *G. fruticosus* phytosomes based on the rhodamine fluorescence, treatment of HeLa cells with phytosomes (3.976^{-4} g/L), increased apoptosis. Cells also had reduced viability in a short time space after exposure, alluding to accelerated cell death in comparison to free extract. This study raises the possibility that the toxicity of *G. fruticosus* can be managed using a lipid based delivery system especially for cervical cancer following optimization in *in vivo* cancer disease models. It validates the ethnomedicinal use of *G. fruticosus* and adds value to a plant whose development was previously abandonded due to toxicity. Furthermore, it elucidates the mechanism of action of *G. fruticosus* extracts which was not previously known, while describing the phytochemistry of Namibian *G. fruticosus*. This study can lead to the development of commercially viable, safe and locally sourced treatment alternatives, especially for cervical cancer.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS/ACRONYMS

AIDS	Acquired immune deficiency syndrome
Akt	Protein kinase B
Apaf1	Apoptotic protease activating factor 1
API	Active pharmaceutical ingredient
ATCC	American type collection of cultures
ATP	Adenosine triphosphate
Bak	B-cell lymphoma 2- antagonistic killer
Bax	B-cell lymphoma 2 associated X protein
BCL-2	B-cell lymphoma 2
BCRP	Breast cancer resistance protein
Cdks	Cylin dependent kinases
CM-H_2DCFDA	Chloromethyl derivative of 2',7'-dichlorodihydrofluorescein
DD	Death Domain
DLS	Dynamic light scattering
DMEM	Dulbecco modified eagle's media
DMSO	Dimethyl sulfoxide
DNA	Deoxy Nucleic Acid
DR	Death Receptor
EE	Encapsulation efficiency
EMT	Epithelial to mesenchymal transition

FDA	Food and Drug Administration
FL1	Flow cytometer channel, 533/30 nm
FLICA	Fluorophore inhibitor for caspases
GCMS	Gas chromatography and mass spectroscopy
GHS	Globally Harmonized System of classification and labeling of chemicals
GIT	Gastrointestinal tract
GLP-1	Glucagon-Like Peptide 1
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
IC_{50}	Concentration causing 50% inhibitory activity
IUPAC	International Union of Pure and Applied Chemistry
JAK2	Janus Kinase 2
LCMS	Liquid Chromatography Mass Spectroscopy
LD_{50}	Median Lethal Dose
m/z	Mass to charge ratio
MAPK	Mitogen-activated protein kinase
MCF5	Non-cancerous human mammary glands cells
MCF7	Breast cancer cell line

Mcl1	Induced myeloid leukemia cell differentiation protein 1
MDP1	Multidrug resistance associated protein 1
NAD^+	Nicotinamide adenine dinucleotide
NBRI	National botanical research institute
NCI	National cancer institute
NFkB	Nuclear factor kappa B
NIST	Nation institute of standards and technology
NNCR	Namibia National Cancer Registry
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OECD	Organization for Economic Co-operation and Development
PARP-1	Poly (ADP-ribose) polymerase 1
PBS	Phosphate buffered saline
PDI	Polydispersity index
PIDD	P53-induced death domain
ppm	Parts per million
PS	Phosphatidyl serine
RAIDD	RIP-associated ICH-1/CED-3 protein with death domain
RNAPII	Ribonucleic acid polymerase II
ROS	Reactive oxygen species
SE	Standard Error
SEM	Scanning Electron Microscope

SEPASAL	Survey for Economic Plants of Arid and Semi Arid Lands
STAT3	Signal transducer and activator of transcription 3
TGI	Total growth inhibition
TLC	Thin layer chromatography
TNF	Tumor necrosis factor
TRAF2	tumor necrosis factor (TNF) receptor–associated factor 2
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WST-1	Water soluble tetrazolium salt 1
ZP	Zeta potential

ACKNOWLEDGEMENTS

I am so grateful for the opportunity afforded to me by God, by allowing me to pursue this dream, which etches ever closer to greater things. For aligning all things necessary to make all this possible as follows:

This study would not have been possible, had it not been for the financial support rendered by the German Academic Exchange Program (DAAD), Multidisciplinary Research Center and Department of Biological Sciences (University of Namibia). This created a conducive environment for personal growth and healthy academic pursuits.

Next, I would like to express my deep appreciation and respect to my mentors: Prof Davis. R Mumbengegwi, Dr Ronnie Bock and the late Dr Matthias Adorka for being my biggest supporters. Mentoring me with passion, patience, wise insights, investigating both time, energy and much more, I am sincerely indebted.

I would like to sincerely thank Prof Mervin Meyer, who has been such a kind mentor, who hosted me during laboratory work at the University of Western Cape. His gentle guidance and support during that time is invaluable. Within the short time at UWC, I was profoundly inspired and encouraged. I am also grateful to Dr Jyoti Sharma, Dr Nicole Sibuyi, Mr Yunus Kippie, Ms Taahirah Boltmann, Dr Mustafa Drah and Mr Earl McDonald for their invaluable support, patience and trust during the time I spent in various laboratories (Pharmacy, NanoTechnology, Physics and Astronomy) at UWC. I will never forget your selflessness, tireless contribution to my work and friendship.

My heartfelt appreciation for the assistance of Mr Aloysius Lubega, from Makerere University's Department of pharmacology and therapeutics, for his vital assistance during

animal studies. My thanks also go to Dr Stefan Louw, for his tireless assistance with GC-MS analysis and interpretation. Thank you both so very much.

Last but not least, I would also like to thank my friends and family members for always being supportive. I thank the Windhoek, Kampala and Cape Town Churches of Christ for their support during my development, for encouraging my academic pursuits and taking the time to listen to me. Your understanding in stressful times, cheerfulness and enduring patience made the journey lighter.

DEDICATION

I would like to dedicate this study to DAAD, for the invaluable support given to students in their academic studies: here is to finding influential solutions to challenges that are faced by a multitude of people globally. To my mentors, family and cancer patients.

DECLARATION

I, Dushimemariya Florence, hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

....

Name of student Signature Date

CHAPTER 1: INTRODUCTION

Cancer is a term used to refer to a group of diseases which are characterized by uncontrolled cellular division. The global cancer burden was estimated to be 18.1 million new incidences and 9.6 million deaths respectively in 2018 (Bray *et al.*, 2018). The most prominent cancers remain prostate and breast cancer for males and females respectively. Mathers, Boerma and Ma Fat, (2009) cited cancer as the third leading cause of death while (Bray *et al.*, 2018) cites it as expected to be the leading cause of death and reduction of life expectancy globally. This trend shows the progressive danger that cancer possess on human health. The types of cancer cases reported is changing from cancers related to infection or poverty to those associated with changes in lifestyle linked to economic development (Bray *et al.*, 2012), such as aging populations, inactivity, westernized diets (Thun *et al.*, 2010).

Not only is the cancer burden on the rise globally, but also in Namibia. Namibian cancer statistics have been on the rise, resulting in increases of both morbidity and mortality. The latest cancer incidences as reported by the Namibia National Cancer Registry (NNCR) between 2010-2014 reported a total of 11 248 new cases (Carrara *et al.*, 2017). This represents an alarming hike of 43.4%, as compared to the previous report (Carrara *et al.*, 2011), which puts stress on the health care system. In addition, the completeness of the registry is a concern with some cases being missed, whilst some cancer cases are not formally diagnosed. Cancers such as prostate cancer and Kaposi sarcoma have continued to be the most prevalent among males while cervical and breast cancers are the most prominent in females. The burden of cancer in Namibia has been greatest in the female

population (Carrara *et al.*, 2009, 2011, 2017), unlike the trend seen globally (Bray *et al.*, 2018).

In Namibia, cancers associated with HIV/ AIDS continue to increase, despite improvements in antiretroviral coverage in the country (Bruin and Stefan, 2013) and these include Kaposi sarcoma, Non-Hodgkin lymphoma, eye cancers and cervical cancer (Carrara *et al.*, 2017). The incidence of hepatitis B virus has also been increasing in Namibia, and it is known as a cervical cancer risk factor (Mhata *et al.*, 2017). Namibia has struggled with hepatitis E outbreaks in the past (Isaacson *et al.*, 2000) and has recently experienced a similar outbreak. Hepatitis E infection is suspected to be an associated risk, contributing to liver cancer in individuals already carrying hepatitis B and C (Atsama *et al.*, 2017). Other risk factors include smoking, multiple sexual partners, early onset of sexual activity, use of contraceptives and human papillomavirus infection (Carrara *et al.*, 2017). The Namibia National Cancer Registry (Carrara *et al.*, 2017) further confirms the lack of up to date information linking risk factors such as alcohol, tobacco use, Human papillomavirus, Hepatitis B and Human Immunodeficiency Virus to cancer incidence. Information linking risk factors to disease occurrence will reduce infection as screening initiatives are implemented and strengthened, which would lead to an overall decline in cancer incidence (De Vuyst *et al.*, 2012). Currently, treatment options involve chemotherapy mostly but can also take the form of radiotherapy, surgery or a combination of these. To access effective medical care, cancer patients go through a long referral process for confirmed diagnosis, with often poor prognosis of disease (Dushimemaria and Mumbengegwi, 2015; Dushimemaria, Du Preez and Mumbengegwi, 2017).

To improve access and provide effective alternative anticancer therapies, many researchers have turned to traditionally used medicinal plants (Kalita, Das and Sharma, 2013; WHO, 2013). Medicinal plants continue to be valuable resources for pharmacological-lead drugs such as vincristine and vinblastine: potent anti-leukemia drugs in clinical use (Nirmala, Samundeeswari and Sankar, 2011). However, some plant derived alternative compounds display poor solubility and non-specific toxicity (Nirmala *et al*, 2011). Furthermore, the bio-distribution of pharmacological agents are not always optimal for treatment of cancer. Drug delivery systems have been shown to be effective for improving all these undesirable properties of synthetic medicines. Applying drug delivery technologies may mitigate against these challenges facing use of medicinal plant extracts used in anticancer therapy.

This study will focus on a plant called *Gomphocarpus fruticosus*. The plant is widely distributed around the world and is distinct due to the milky substance exuding from its injured parts as well as it bulb-like fruiting structures. In Namibia, the plant is reportedly used traditionally by the Bergdamara/berdamara/bergdama/daman/damaqua, people who inhabited the mountains near Grootfontein and other mountainous places in Namibia (von Koenen, 2001). It is known to be poisonous, with reports citing death of livestock (SEPASAL: http://apps.kew.org/sepasalweb/sepaweb). It has also been studied by different researchers for anticancer properties and a number of compounds isolated from various organs (Fouche *et al.*, 2008; Mashele and Kolesnikova, 2010). However, owing to its poisonous effects, it was abandoned and not studied or developed further. The encapsulation of the toxic herb (*G. fruticosus*) using a lipid-based delivery system may

present an opportunity to make use of its anticancer properties while targeting its delivery and therefore reduce its toxic effects to none-cancerous cells.

1.1. Statement of the problem

G. fruticosus is a plant used for treatment of tumors in Namibia's traditional setting and therefore has a potential for anticancer treatment. It has received scientific attention in different parts of the world for its anticancer activity *in vitro* against different cell lines with promising outcomes. However, due toits dose limiting toxicity, low therapeutic window and poor bio-distribution properties (Nirmala, Samundeeswari and Sankar, 2011) it has been abandoned and not studied further due The formulation of a lipid-based delivery system for Namibia's *Gomphocarpus fruticosus* extracts may reduce toxicity while improving it biological properties and hence provide an alternative treatment options for to the reduction of cancer morbidity and mortality.

1.2. Research objectives

a) To determine the phytochemical profile of *Gomphocarpus fruticosus.*

b) To investigate the anti-proliferative, cytotoxicity activity of *Gomphocarpus fruticosus* extract and its mechanism of action *in vitro.*

c) To determine the *in vivo* toxicology properties of crude extract of *Gomphocarpus fruticosus.*

d) To formulate, characterize and evaluate, a lipid based-drug delivery system for a *Gomphocarpus fruticosus* extract for *in vitro* anticancer treatment regimen.

1.3. Research hypothesis

The formulation of *Gomphocarpus fruticosus* extract as a nanoparticulate drug delivery system will improve their therapeutic effect against an *in vitro* cancer model as compared to crude plant extract.

1.4. Significance of study

The study which is the first of its kind in Namibia, will lead to a phytosome as a delivery platform for extracts derived from *Gomphocarpus fruticosus*, to be used as alternative therapies for diseases such as cancer. Phytosomes will increase acceptability of herbal medicines and add value to indigenous natural products and downstream industries associated with such products. In addition, it will lead to the documentation of the chemical constituents of *G. fruticosus*, its toxicity *in vivo* and anticancer mechanism of action. The study will also contribute to the gap in knowledge of the anticancer properties of Namibian *G. fruticosus*, there is a lack of the much needed research into the efficacy and safety of medicinal plants. Research such as this will not only serve to document and validate the use of these medicinal plants but will also form the basis of advice to policy makers and also potentially lead to the development of commercially viable, safe and locally produced medicinal products. This will improve access to effective anticancer therapies and thereby improve prognosis and reduce morbidity as resulting from cancer, especially in a resource poor environment.

1.5. Limitation of study

While the study envisions that the combination of nanotechnology and herbal extracts will improve access, result in improved acceptability of *G. fruticosus* as a cancer treatment option or better bio-distributed herbal products but these will not be determined in this study due to availability of resources. In addition, the origin of the phytochemicals is not often defined as being the plant's own or belonging to endophytic fungi, and this will not be ascertained in this study.

1.6. References

Atsama, M. A., Atangana, P. J. A., Noah, D. N., Moundipa, P. F., Pineau, P. and Njouom, R. (2017) 'Hepatitis E virus infection as a promoting factor for hepatocellular carcinoma in Cameroon: Preliminary Observations', *International journal of infections diseases*, *64*, pp. 4–8. doi: 10.1016/j.ijid.2017.08.010.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R., Torre, L. A. and Jemal, A. (2018). Global Cancer Statistics 2018 : GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries, *CA: a cancer journal for clinicians*, *68*, pp. 394–424. doi: 10.3322/caac.21492.

Bray, F., Jemal, A., Grey, N., Ferlay, J. and Forman, D. (2012). Global cancer transitions according to the Human Development Index (2008-2030): a population-based study, *Lancet Oncology*, *13*(13), pp. 790–801. doi: 10.1016/S1470-2045(12)70211-5.

Bruin, G. P. De & Stefan, D. C. (2013). Children with Kaposi Sarcoma in Two Southern African Hospitals : Clinical Presentation , Management , and Outcome', *Journal of tropical medicine*, 2013, p. 6.

Carrara, H., Parken, M., Zietsman, A., Pontac, J., Lawrence, S., Horn, L. & van Schalkwyk, L. (2017). Namibia National Cancer Registry (NNCR) Cancer incidences in Namibia 2010 - 2014. Windhoek.

Carrara, H., Stein, L., Sitas, F., Zietsman, A., Gariseb, B., Rautenbach, N., Pontac, J., Johannesson, I. & Ferlay, J. (2009). Cancer in Namibia 2000-2005. Windhoek.

Carrara, H., Stein, L., Sitas, F., Zietsman, A., Gariseb, B., Rautenbach, N., Pontac, J., Rooi, T. & L, T. (2011) Cancer in Namibia 2006-2009. Windhoek.

Dushimemaria, F. & Mumbengegwi, D. R. (2015). Palliative treatment of cancer in resource poor settings: Traditional medicine perspective, *Journal of Pharmacognosy and Phytotherapy*, *7*(5). doi: 10.5897/JPP2014.0331.

Dushimemaria, F., Du Preez, C. I. & Mumbengegwi, D. R. (2017). Randomized anticancer and cytotoxicity activity of *Guibourtia coleosperma* and *Diospyros chamaethamnus*, *African journal of traditional complementary alternative medicine*, *14*(4), pp. 1–7.

Fouche, G., Cragg, G. M., Pillay, P., Kolesnikova, N., Maharaj, V. J. & Senabe, J. (2008). *In vitro* anticancer screening of South African plants, *Journal of ethnopharmacology*, *119*(3), pp. 455–61. doi: 10.1016/j.jep.2008.07.005.

Isaacson, M., Frean, J., He, J., Seriwatana, J. & Innis, B. L. (2000). An outbreak of hepatitis E in northern Namibia, 1983', *American Journal of tropical medicine and hygiene*, *62*(5), pp. 619–625.

Kalita, B., Das, M. K. & Sharma, A. K. (2013). Novel Phytosome Formulations in Making Herbal Extracts More Effective, *Research Journal of Pharmacy and Technology*, *6*(11), pp. 1295–1301.

von Koenen, E. (2001) Medicinal, poisonous and edible plants of Namibia. Windhoek: Klaus Hess Publishers/Verlag.

Mashele, S. and Kolesnikova, N. (2010) '*In vitro* anticancer screening of *Asparagus laricinus* extracts', *Pharmacologyonline, 2,* pp. 246–252.

Mathers, C. D., Boerma, T. & Ma Fat, D. (2009). Global and regional causes of death, *British Medical Bulletin, 92*(1), pp. 7–32. doi: 10.1093/bmb/ldp028.

Mhata, P., Rennie, T. W., Small, L. F., Nyarang'o, P. M., Chagla, Z. & Hunter, C. J. (2017) Distribution of hepatitis B virus infection in Namibia, *South African medical journal, 107*(10), pp. 882–886. doi: 10.7196/SAMJ.2017.v107i10.12171.

Nirmala, M. J., Samundeeswari, a. & Sankar, P. D. (2011). Natural plant resources in anti-cancer therapy-A review, *Research in Plant Biology, 1*(3), pp. 1–14.

Thun, M. J., DeLancey, J. O., Center, M. M., Jemal, A. & Ward, E. M. (2010). The global burden of cancer: Priorities for prevention, *Carcinogenesis, 31*(1), pp. 100–110. doi: 10.1093/carcin/bgp263.

De Vuyst, H., Alemany, L., Lacey, C., Chibwesha, C. J., Sahasrabuddhe, V., Banura, C., Denny, L. & Parham, G. P. (2012). Comprehensive Control of HPV Infections and Related Diseases in Sub-Saharan Africa, *Vaccine, 30*(5), pp. 32–46. doi: 10.1016/j.vaccine.2012.07.092.

WHO (2013) WHO traditional medicine strategy 2014-2023. Available at: http://apps.who.int/medicinedocs/documents/s21201en/s21201en.pdf (Accessed: 1 August 2017).

CHAPTER 2: LITERATURE REVIEW

2.1. Cancer

Cancer refers to a group of diseases which are mainly characterized by uncontrolled cell division (Ganesan and Muthuchelian, 2011) and their acquired ability to escape programed cell death (Rastogi & Mishra, 2012). In addition to these two characteristics, Hanahan and Weinberg, (2011) further described cancer as a disease which induces the development of new vascular pathways (angiogenesis), perpetuates invasion and metastasis in order to sustain the growing mass of cells. Furthermore, the cancer phenotype is characterized by the ability to escape growth forbidding signals and immune system, sustaining its independent sources of growth-signals, redirecting energy investments, resulting in replicative immortality (Hanahan and Weinberg, 2011). Cancer can develop in any body tissue and exist as either a benign or malignant cancer. Benign cancers develop and remain in the body tissue or organ of their origin, whereas, malignant cancers display migration and invasion from site of origin to additional distal sites in the body (Saunus *et al.*, 2011). It is reported that, breast, prostate, uterus, lung, kidney stomach and colon primary cancers are responsible for metastasis to the maxillofacial region, (Sagheb *et al.*, 2017), via the hematologic, non-lymphatic or vertebral venous plexus pathway. In addition, it is theorized that cancer cells in a primary site are capable of determining or targeting themselves to distant organs for the purpose of establishing secondary metastatic sites using the stromal microenvironment (Saunus *et al.*, 2011).

2.2. Cancer etiology

Etiological agents of cancer fall into various categories such as chemicals, infection, radiation, physical agents, food intake and exercise, hormones. Chemical agents such as arsenic, tobacco, asbestos and benzene are known cancer causers. Tobacco smoking, arsenic, asbestos is a known etiological agent of lung cancer. These chemicals have been shown to be risk factors for a number of cancers, such as tobacco smoking linked to not only lung cancer but eosophagus, larynx, tongue (Carrara *et al.*, 2017) and others such as stomach, bladder, kidney cancer. Physical inactivity contributes to cancer in a two-fold manner through weight gain and adverse effects on the endocrine and immune systems for breast, prostate, pancreatic, colorectal cancer and more (Clague and Berstein, 2012). Physical carcinogens such as ultraviolet rays and biological agents such as the hepatitis viruses (Yeap *et al.*, 2012; Tagliamonte *et al.*, 2015) have all been implicated in the initiation, promotion and progression of the disease (Mulware, 2013). Menopause, hormonal changes, age at first menstruation, number of children, lactation are also reported risk factors for various cancers such as breast and cervical cancer (Calaf *et al.*, 2015). Still, cancer development may be hereditary with cancer predisposition passed down to offspring. A small percentage of observed cancers are caused in this manner, while most commonly inherited cancers are ovarian, breast or colorectal.

The etiological agents of cancer are varied and come in many forms. However, they all have a singular effect on the normal order of the cell cycle, which results in the development of cancer.

2.3. Cell cycle control and cancer development.

The human body is made up of different cells which are grouped into tissues, organs and organ systems. Each cell originates from pluripotent stem cells which undergo differentiation to allow cells to perform specialized functions. This process occurs so that the organism may grow and increase in size but also to allow old cells or damaged cells to be replaced, ensuring the survival of the organism as a whole. Since all these processes are orchestrated by the DNA, it is imperative its replication and subsequent events of the cell cycle proceed in a manner which maintains DNA integrity. DNA integrity is maintained as a cell undergoes a cycle comprising of a DNA synthesis stage and mitosis stages, which are interspaced by two cell growth stages (G1 and G2) and three checkpoints (Dickson and Schwartz, 2009).

During the mitotic phase, three different initiator caspases are restricted by phosphorylation, such that additional cellular processes are inhibited (Parrish, Freel and Kornbluth, 2013), **figure 2.1.** Cyclin B and CdK1 complex phosphorylates procaspase 2, 8 and 9, effectively preventing their activation and their subsequent role in programmed cell death (Parrish, Freel and Kornbluth, 2013). These caspases play unique roles in apoptosis induction. Two distinct pathways lead to apoptosis: The intrinsic and extrinsic pathways (Brentnall *et al.*, 2013). The two pathways both employ a cascade of caspases in order to cause programmed cell death. The intrinsic pathway involves the mitochondria, while the extrinsic pathway is initiated by a death signal received through a transmembrane receptor (Brentnall *et al.*, 2013). Procaspase 8 is recruited on the intracellular side of the transmembrane, when a death signal is received by the death

receptor (DR) on the outside of the membrane and linked to the death domain (DD) and FAS- associated death domain (FADD), to be able to transverse the membrane (McIlwain, Berger and Mak, 2015). This recruitment causes procaspase to be activated by dimerization, leading to activation of procaspase 3, an executioner of apoptosis in type I cells or activation the intrinsic pathway in type 2 cells (McIlwain, Berger and Mak, 2015). Activated caspase 8 cleaves proaopoptotic enzyme Bid, which further aids the dimerization of Bax and Bak. Bax and Bak enable the permealization of the mitochondria membrane by forming pores in the membrane. The formation of the pores results in the release of cytochrome c. In the cytosol, cytochrome c forms an apoptosomes complex with procaspase 9 (initiator caspase) and apoptotic protease activating factor 1(Apaf1) (McIlwain, Berger and Mak, 2015). The resulting apoptosome can now cleave procaspase 3/7 in the cytosol, which also direct the expression of several downstream genes such as Lamin A, PARP-1, Actin leading to apoptosis etc, in the nucleus (Chaabane *et al.*, 2013).

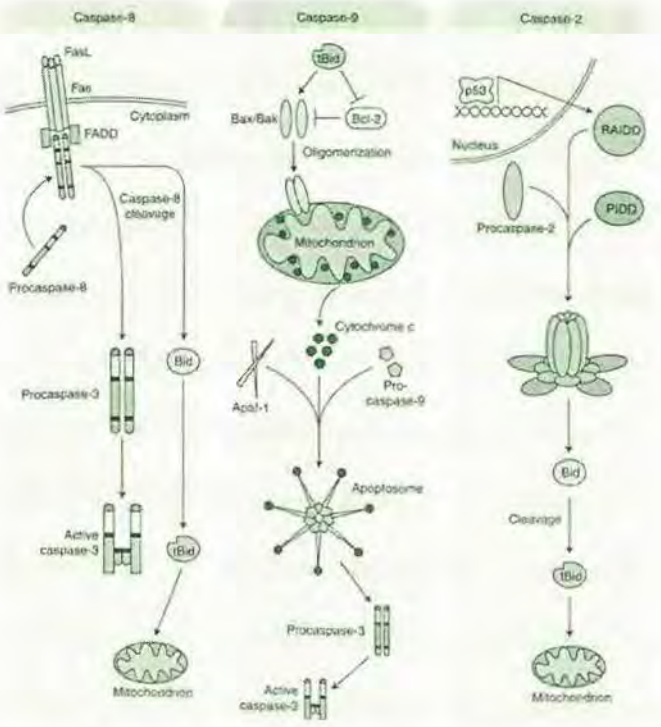


Figure 2.1. Schematic representation of intrinsic and extrinsic pathways, leading to apoptosis activation. Adopted from (Parrish, Freel and Kornbluth, 2013).

For the initiator caspase 2, its role in apoptosis is not well understood (McIlwain, Berger and Mak, 2015), even though its activation appears to occur in a similar as caspases 8 and 9 (dimerization). All three procaspases are found in the cytosol, and similar to procaspases 8 and 9, procaspase 2 associates with p53-induced protein with death domain (PIDD) and RIP- associated ICH-1/CED-3 protein with death domain (RAIDD), under the influence of p53, to form a complex which activates BID, further propagating mitochondrial release of cytochrome c and other mitochondria associated molecules (Parrish, Freel and Kornbluth, 2013). Procaspase 2 is thought to initiate apoptosis from the nucleus in response to heat shock, DNA damage, metabolic distress or cytoskeletal injury (McIlwain, Berger and Mak, 2015).

Alternatively, in response to physical damage such as hypoxia or unrepaired DNA damage, an intrinsic apoptotic pathway dependent on the mitochondria can be initiated (Marchi *et al.*, 2012). This pathway depends on the release of cytochrome c from the mitochondria. Once cytochrome c is released, it binds a protein found within the cytosol called Apaf-1, which orchestrates the aggregation of procaspase molecules in a cascade of events leading to cell death (Nowsheen and Yang, 2012). To date, two pro-apoptotic (Bax and Bid) signals and one anti-apoptotic proteins belonging to the Bcl-2 family have been identified. It is believed that the pro-apoptotic proteins create channels allowing the leaching of cytochrome c from the mitochondria (Nowsheen and Yang, 2012). Failure for damaged cells to undergo apoptosis means that defective cells survive and can undergo the cell cycle, resulting in the multiplication of said cells. Oncogenic activities exert their greatest effect on the check point which is found in the late G1 phase. The G1 checkpoint is a fundament stage in the regulation of the cell cycle since it is at this point when cells are sensitive to external signaling factors (Williams and Stoeber, 2012). When a cell passes this checkpoint, it is no longer sensitive to external signaling and it must commit autonomously to replication. This checkpoint requires a series of factors to control the cell cycle. At this stage, mitogens, either endogenous or exogenous act to encourage cell division.

Faults occurring in the mechanism designed to protect the integrity of the organism, in terms of cell division is what is termed as cancer. Cancerous cells significantly differ from normal cells in cell morphology, gene expression and resulting cell surface properties (Hanahan and Weinberg, 2011). In addition, control on cell growth and division, cell to

cell interaction, protein secretion and cytoskeletal infrastructure further differentiate cancerous from normal cells. It is these hallmarks of cancer that makes the disease debilitating to the organism as a whole. The actions of oncogenes and tumor suppressor genes further play a distinguishing feature between normal and transformed cells (Hanahan and Weinberg, 2011).

2.4. Cancer treatment and chemotherapy

The understanding of cancer as a disease has also come with the advent of numerous treatment options. Currently, the most commonly used treatments are surgery, radiotherapy and chemotherapy. Even though chemotherapy is the most widely used option, a combination with treatment options such as surgery, radiotherapy, is often used to effectively treat the disease, prolong life as well as improve patient's quality of life. In addition, newer options are continually being developed and being subjected to stringent clinical trial before being approved by the food and drug administration (FDA).

Chemotherapy is the most commonly used cancer treatment, because of its wide application against a wide range of cancers. Various chemotherapeutic drugs exist with various mechanisms of action. These mechanisms include actions against the spindle apparatus or those inhibiting critical DNA replication structures such as topoisomerase (Calaf *et al.*, 2015).

Despite being the most commonly used mode of cancer therapy, chemotherapy is accompanied by several disadvantages: the occurrence of side effects experienced by patients undergoing treatment with chemotherapeutic drugs (Mukhtar, Adhami and Mukhtar, 2014). Side effects associated with chemotherapy include nausea, diarrhea /constipation, vomiting, fatigue and hair loss (Mukhtar, Adhami and Mukhtar, 2014), **table 2.1**. Additional side effects include dizziness, depression, headaches, pain, which further compounds the use of existing cancer drugs. To date, clinically relevant cancer drugs such as vinca alkaloids, paclitaxel, colchicine and combretastatins have been documented to cause adverse side effects contributing to dose limitations, further limiting their usability and acceptability for cancer therapy. The mechanism of these side effects can have one or more toxicities: myelosuppression, hematoxicity, gastrointestinal injury, hair follicle injury and cardiotoxicity.

Table 2.1. Side effects associated with the use of chemotherapeutic drugs for cancer treatment.

Type of toxicity	Side effects	Chemo-drug & Source
Myelosuppression	Anaemia	Paclitaxel, Topotecan***
	Thrombocytopenia	5-flurouracil, Carboplatine***
	Neutropenia	Vinblastine, Camptothecin*
Gastroinstestinal toxicity	Oral sores and stomatitis	Cytarabine, Irinotecan***
	Costipation	Vinca alkaloids***
	Diarrhea	Floxuridine, Methotrexate***
Hair follicle toxicity	Alopecia	Etoposide, Doxorubicine***
Neurotoxicity	Hypersensitivity	Cyclophospamide***
Hepatic toxicity	Fibrosis, ductal injury	5-Flurouracil, Streptozocin***

Urinary tract toxicity	Renal failure, urinary retention	Streptozotocin, Mitomycin***
Pulmonary toxicity	Pulmonary fibrosis, pneumonitis	Bleomycin, Methotrexate***
Gonadal toxicity	Sterility, menstrual anomalies	Alkalating drugs***
Occular and ontotoxicity	Sight and hearing loss	Amino glycosides***
Cardiac toxicity	Ischemia, Arrhythmia	Doxorubicin, Paclitaxel**

Key: * Moore (2016), **Curigliono et al. (2016),***Remesh (2012)

Another disadvantage of chemotherapeutics is the development of resistance, which has been documented in literature (Labhasetwar, 2011; Rebucci and Michiels, 2013; Zahreddine and Borden, 2013; Calaf *et al.*, 2015) against a range of cancer phenotypes. Hallmarks of cancer resistance to chemotherapeutics are outlined in **figure 2.2**. Resistant cancers demonstrate the ability to actively export chemotherapeutics out of the target cell. This is facilitated by the increased translation of proteins encoding the ATP-Binding Cassette transports family members. Specific examples are the expression of the breast cancer resistance protein (BCRP), the multidrug resistance protein 1 (MDR1) and the multidrug resistance associated protein 1 (MDP1),all are hyper-expressed in chemotherapy resistant cancers (Housman *et al.*, 2014). Chemotherapy resistant cancers also show resistance to cell death signals, this resistance is achieved by the alteration of apoptotic proteins such as BCL-2, procaspase 9 and Akt. When this occurs, cancer cells become unresponsive to cell death signals, and they continue to grow and divide. Cancer cells can also acquire resistance by enhancing the repair of DNA damage caused by cytotoxic drugs. Since drug-induced damage is one mechanism by which cytotoxic drugs induce apoptosis, cells able to repair their own DNA will survive (Housman *et al.*, 2014).

Epigenetic alterations of tumor suppressor and oncogene, coupled with drug-target changes also contribute to drug resistance in cancer. Epigenetic changes to drug targets influences treatment such that entry into target cells is hindered. Furthermore, metabolism in target cells can alter the drug, to an extent, making it inactive, therefore resulting in drug resistance. Finally, cancer epithelial cells transitioning to mesenchymal (EMT) is another method of cancer drug resistance. During this transition, cancer cells (progenitor) detach from their stromal spaces with the aid of proteases. In addition, the formation of new vascular vessels forms by angiogenesis. It is thus, cancer progenitor cells that contribute to resistance, when they set up new tumors in distant places (Housman *et al.*, 2014; Calaf *et al.*, 2015).

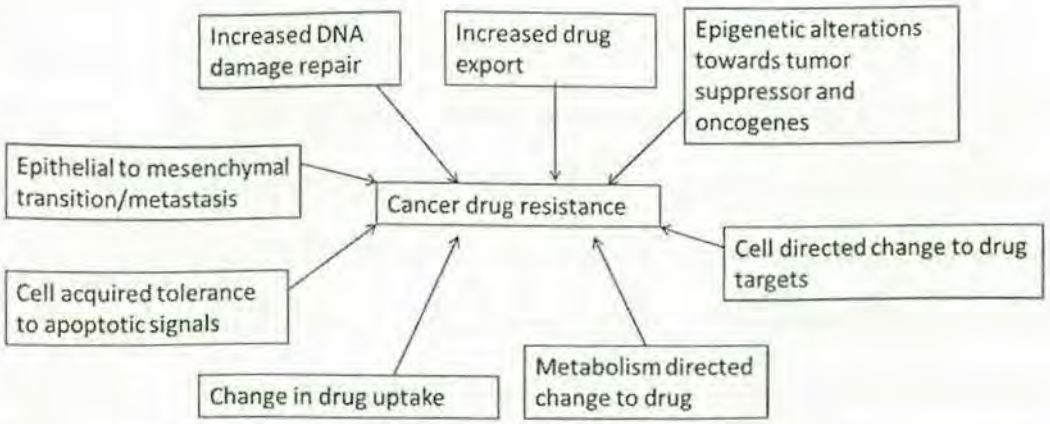


Figure 2.2. Mechanisms employed by cells resistant to chemotherapeutics.

2.5. Complementary and alternative medicine approach to cancer treatment

Complications often encountered during cancer therapy using other conventional methods has opened avenues such as immunotherapy. Immunotherapy has grown increasingly popular with the advent of antibody-based cancer drugs such as trastuzumab. Since its

initial introduction, several antibody-based drugs have also been introduced into mainstream health care programs. The use of antibodies for cancer therapy is relatively new and scientists suggest that the treatment option still requires improvements. An example can be demonstrated in the development of vaccines for hepatocellular carcinoma (HCC), with suggested improvements in both the selected antigen and in intrahepatic immunosuppressive environment (Tagliamonte *et al.*, 2015). In addition, cross-cutting development such as the FDA-approval of radiolabeled anticancer antibodies such as Y-Ibritumomab tiuxetan and I-Tositumomab, for the treatment of non-hodgkin lymphoma, improves the fight against cancer (Ryan and Bodei, 2017).

Another non-conventional cancer treatments gaining popularity globally is the use of herbal medicine. The use of herbal medicines has arguably been popular in developing countries but it is increasing utilized in developed countries in America and Europe (WHO, 2013). Demand for herbal products has risen globally, with many patients spending large sums of money to acquire herbal remedies. An increase in trade of herbal products is also evident with reports of US$ 83.1 billion made by the Chinese Materia Medica in 2012 alone (WHO, 2013). As a result, established traditional systems such as Ayurveda and Chinese medicine, boasting an array of herbals products, deal in export trade to Europe and America. Reasons for the use of herbal medicines varies among cancer sufferers but can range from immune system boosting effects to remediation of side effects or belief that traditional medicine is more efficacious than conventional drugs.

The immune modulating properties of herbal medicines has been an attraction to its use among cancer patients. Since evasion of the immune system is part of the hallmark of cancer development, treatments that can enhance the immune system can be advantageous to cancer patients. Reports suggests that the use of some herbal medicines positively modulates the individual's immune system by elevating cytokine activity and increasing lymphocyte counts, including dendritic cells (Yin *et al.*, 2013). The increase in lymphocytes and cytokines increases the surveillance of cancerous cells by the immune system. Herbal products derived from sources such as *Ganoderma lucidum, Echinacea purpurea* have been known to positively modulate the immune system and positive results have been reported in colorectal and Hepatocellular carcinoma patients (Yin *et al.*, 2013)

Cancer patients sometimes opt to combine herbal remedies with their anticancer prescription drugs in the belief that this offers them an advantage towards managing and curing of the disease (Mwaka, Okello and Orach, 2015). According to Hu et al. (2016), the use of herbal medicines can be an advantage to a patient through synergistic action of more than one active ingredient through multiple mechanisms of action. This can occur by a) treatments working on different targets using a similar pathway, b) same target but via different mechanisms, c) different targets and through different mechanisms, d) supplemental modulation of biological networks. Another report further illustrates the advantages of combining herbal and conventional drugs, which includes immune modulation, among others (Yin *et al.*, 2013). The use of herbal medicine in conjunction with conventional drugs allows a patient to practice "self-medication", a trend which is increasingly popular and allows patients to be in control of their own healthcare.

Cancer patients may prefer to use herbal medicines, whilst taking conventional drugs for cancer treatment in an effort to reduce the side effects, which arise from the use of these drugs thus improving their quality of life (Yin *et al.*, 2013). Some cancer patients prefer the use of herbal medicines because of an increased awareness of the need for spirituality-centered healthcare option. This is based on the principal that herbal medicines not only focus on alleviating symptoms of diseases but rather has a holistic approach to healing disease. According to (Karimi, Majlesi and Rafieian-Kopaei, 2015), herbal remedies aim to help various systems in an individual to regain their ability to fight the disease from within. In a diseased state, these various systems may have become deficient, and can be resuscitated using herbal remedies to allow the entire body to fight as a whole, hence why herbal remedies are thought to have a holistic approach to healing (Karimi, Majlesi and Rafieian-Kopaei, 2015).

According to the WHO, herbal/ traditional medicines is the main source of primary health care for 70-80% of the population in developing countries (Ekor, 2014; Maroyi and Cheikhyoussef, 2015). This is partly because it is the only medical resource in close proximity to their homes. It may also be the only resource which is affordable to them. Mwaka et al. 2015 states that additional factors such as costs of cancer conventional drugs and distance to health care facilities contribute to the popularity of herbal remedies.

The rise in the use of herbal medicines also comes at a period when there is an increased awareness in "self-care", "autonomy", personal responsibility to one's health (Joos,

Glassen and Musselmann, 2012; Mwaka, Okello and Orach, 2015). This development allows a patient to be more in control of their own care (WHO, 2013). This also comes at a time when herbal medicinal patients prefer a more holistic approach to health care. In addition, it is widely believed that herbal medicines are natural, and should be used instead of conventional treatments, since they are more accepted by society.

The scientific validation of herbal medicines and their bioactive components has contributed to the increased attention received by herbal medicines. However, there continue to be negative reports about herbal medicines especially undocumented traditional medicines used with conventional cancer therapy resulting in treatment failure, morbidity and death (Clarke and Mclachlan, 2011; Izzo, 2012). Despite the versatility and beneficial effects of traditional medicines in the treatment of cancer, there is a need to properly validate the use of these medicines, determine their potential side effects and elucidate their modes of action. This will further contribute to identifying the cause of contra-indications arising from the use of these traditional medicines.

2.6. Traditional Medicinal plants

Traditional medicine is a term used to refer to a system of medicine which existed long before the advent of allopathic medicine, and is founded on cultural beliefs and practices handed down from one generation to another, by word of mouth or practice and observation (Dan, Mchombu and Mosimane, 2010). These traditional practices of medicine are used in the prevention, diagnosis and treatment of both physical and mental

illnesses. Examples of such traditional systems include but are not exclusive to Chinese traditional medicine, Ayurveda and Unani (Subramoniam, 2014). These continue to be reliable resources of active medicines which are developed and incorporated into mainstream use. Plant and plant products continue to make up the largest bulk of traditional medicine, with plant parts such as leaves, roots, rhizomes, barks, flowers, fruits seeds all being employed alone or in combination to prepare various medicinal preparations. Medicinal preparations can also take different forms such as tinctures, teas, decoctions, infusions, poultices or concoctions. The resulting preparations have equally varied forms of administrations with topical applications (lotions, creams and oils), oral, anal (enemas), nasal (snuffs, smoke, steaming) or bathing. Records dating back to 2600 BC depict the earliest known records of the use of medicinal plants (Chanda and Nagani, 2013). Traditional medicinal plants continue to play an active role in primary health care for many people, about 80 % of the global or African and Asian population utilize traditional plants to combat various illnesses, including cancer (Ekor, 2014; Oyebode *et al.*, 2016). In addition to this, about 100 million people in Europe are also believed to use herbal and complementary medicines currently.

2.7. Biological active compounds derived from medicinal plants.

Medicinal products derived from these traditionally used plants owe their therapeutic properties to the presence of phytochemicals, which are secondary metabolites produced by the plants or symbiotic microorganisms in the plant (Chandra, 2012). Clinical drugs such as vincristine and vinblastine were first isolated from plant sources such as *Catharanthus roseus* (Nirmala, Samundeeswari and Sankar, 2011).

However, these compounds can have non-desirable properties such as animal toxicity, poor solubility (Nirmala, Samundeeswari and Sankar, 2011) and nonspecific toxicity resulting in low therapeutic indices and thereby preventing inclusion in mainstream usage. There is therefore a need to discover alternative remedies or improvement of existing medicinal compounds to circumvent obstacles facing cancer treatment. **Table 2.2** below lists compounds investigated for anticancer actions, which have been isolated from various plants. Some compounds are FDA approved, while others are at various stages of investigation between the bench top and various stages of clinical trials.

Table 2.2. Compounds isolated from plants demonstrating anticancer activity.

Type	Compound name	Plant name	Literature
Alkaloid	Vinblastine,Vincristine	*Catharathus roseus*	1
	Paclitaxel	*Taxus brevifolia*	2
	Camptothecin	*Camptotheca acuminate*	3
	Berbamine	*Berberis amurensis*	4
	Berberine	*Hydrastis canadensis* *Berberis vulgaris* *Coptis chinesis* *Berberis aquifolium*	5
	Cephalotaxine	*Cephalotaxus harringtonia*	2
	Ellipticine	*Ochrosia borbonica* *Excavatia coccinea* *Ochrosia elliptica*	3
	Curcumin	*Curcuma longa*	6
Coumarin	Daphnoretin	*Wikstroemia indica*	7
Anthraquinone	Emodin	*Rheum palmatum*	8
Flavonoid	Flavopiridol	*Amoora rohituka*	3
Terpenoids	Perrily alcohol	lemongrass, lavender, ginger	9
	Tanshinone IIA	*Salvia miltiorrhiza*	10

	Cucurbitacin	*Cucurbitaceae family*	10
	Triptolide	*Tripterygium wilfordii*	10
	Belulinic acid	*Zizyphus species, Betula alba*	2
Steroid	28-homocastasterone	*Brassica napus*	11

Key: 1 (Sarma 2016), 2 (Prakash et al. 2013), 3 (Nirmala et al. 2011), 4 (Rahmatullah et al. 2014), 5 (Balakrishna & Kumar 2015), 6 (Perrone et al. 2015), 7 (Jiang et al. 2014), 8 (Lin et al. 2012), 9 (Farazuddin et al, 2012), 10 (Huang et al, 2012) and 11 (Mehndiratta et al, 2011).

Alkaloids are often described as heterocyclic bases containing ammonia, with the Nitrogenous bases derived from amino acids (Sarma, 2016). Alkaloids take the role of defending plants against herbivores and other opportunistic enemies of plants such as parasites and pathogens due to their bitter taste. Pharmaceutically, alkaloids are employed as narcotics, poisons, chemotherapeutic drugs and stimulants.

For cancer therapy, several alkaloids are worth mentioning. Vinca alkaloids were initially discovered from the Madagascan periwinkle plant, *Catharanthus roseus* (Sarma, 2016). Vinca alkaloids where discovered amidst research of traditionally renowned hypoglycemic properties from the ornamental periwinkle plant (Sarma, 2016; Sri, 2016). The research lead to the observation that extracts from the plant increase the lifespan of mice implanted with lymphocytic leukemia cells. This lead to isolation of bioactive compounds such as the notable Vincristine and Vinblastine, which are currently FDA approved leukemia drugs (Nirmala, Samundeeswari and Sankar, 2011). Vincristine is currently in clinical use for the treatment of various cancers, including testicular, small lung carcinoma, acute lymphomas, cervical, breast and leukemia (Pandey *et al.*, 2011).

All vinca alkaloids and their synthetic analogs such a Vinolberine, Vindesine and Vinflunine (Sarma, 2016), see **figure 2.3**, treat cancer by disrupting the assembly of the microtubule components during cell division (Mukhtar, Adhami, & Mukhtar, 2014; Nirmala et al., 2011). However, vinca alkaloids also kill normal cells since they have microtubules (Sri, 2016). The use of vincristine and vinblastine has been observed to cause obstruction of the colon and ileum leading to constipation, as they act on the autonomic nervous system of the GIT (Torrisi *et al.*, 2011). In addition, other side effects such as nausea, vomiting, cardiovascular difficulties, problems to the reproductive system have also reported (Berrios, 2016). Vinflunine is a fluorinated derivative of Vinorelbine drug currently approved clinically for primary and secondary treatment of cancers of the urothelium tracts and advanced breast cancer respectively (Sarma, 2016). The fluorinated version has shown promise to be the most potent of all vinca alkaloids (Nirmala, Samundeeswari and Sankar, 2011). Vinca alkaloids are effective against a number of varied cancers, including leukemia, Kaposi sarcoma, breast, testicular, lung cancers and lymphomas (Nirmala, Samundeeswari and Sankar, 2011; Sarma, 2016).

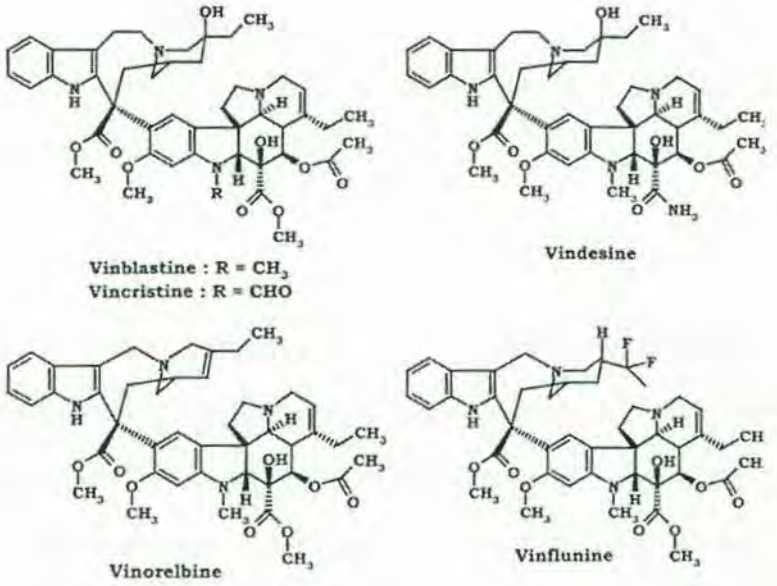


Figure 2.3. Clinically relevant vinca alkaloids and synthetic analogues. Adopted from (Sarma, 2016).

The Pacific Yew plant, *Taxus brevifolia* is the source of clinically relevant anticancer drug Paclitaxel (Taxol) (Prakash et al., 2013). The drug was discovered under the initiative dedicated towards the discovery and development of newer chemotherapeutic compounds from plants. The isolated compound was introduced into the pharmaceutical market in the 1990s. However, the drug was found to have poor solubility in water, which led to the formulation of a derivative, docetaxel, to improve paclitaxel's poor solubility. In addition, paclitaxel is also associated with several side effects, including vomiting, diarrhea and ileum inflammation (by product of neutropenia), all which affect the GIT (Torrisi *et al.*, 2011). Currently, both drugs are still important primary and secondary line drugs for the treatment of Kaposi sarcoma, lung, breast and ovarian cancers (Habli, Toumieh, Fatfat, Rahal, & Gali-Muhtasib, 2017; Prakash et al., 2013). The mode of action of Paclitaxel and its semi-synthetic derivative Docetaxel is similar to the antimitotic activity of the vinca

alkaloids (Barbuti & Chen, 2015; Habli et al., 2017; Nirmala et al., 2011), which is exerted by stabilizing microtubules, thereby preventing disassembly (Aly, Debbab, Kjer, & Proksch, 2010; Habli et al., 2017).

The Chinese ornamental plant, *Camptotheca acuminate* is a source of a clinically approved anticancer drug, camptothecin (Nirmala, Samundeeswari and Sankar, 2011). The active ingredient was isolated from the bark and stems of the tree and introduced into clinical trials by the National Cancer Institute but was withdrawn due to extensive bladder toxicities brought about by poor solubility (Prakash, Kumar and Kumar, 2013). As a result, several derivatives have been synthesized such as irinotecan, topotecan, lurtotecan in an effort to reduce toxicity of the precursor molecule. In addition, in a bid to improve the usability camptothecin, some of its derivatives such as SN-38 (7-ethyl-10-hydroxycamptothecin) have been formulated into lipid-based formulations to counteract the low aqueous solubility and high toxicity. Lipid-encapsulated camptothecin and its derivatives demonstrates reduced GIT toxicity, manifesting as as diarrhea as a result of intestinal lining destruction (Schmid et al., 2014). The mode of action of these complex alkaloids is the inhibition of DNA topoisomerase. The various camptothecin derivatives have been found efficacious against epithelial ovarian cancer, colorectal and small cell lung cancer among others.

Another anticancer drug isolated from a Chinese plant is Berbamine. The active compound was isolated from *Berberis amurensis* and has shown promising activity

against myeloid leukemia cells. It has been shown through research that Berbamine causes apoptosis via a caspase-3-dependent pathway. Caspase 3 has constantly been up-regulated in various cells exposed to berbamine *in vitro* (Rahmatullah, Jahan and Bashar, 2014).

Berberine is an anticancer drug isolated from several plants including *Hydrastis canadensis, Berberis vulgaris, Coptis chinesis, Berberis aquifolium* (Balakrishna and Kumar, 2015). This clinically active drug has been found to be effective against lung, liver, prostate, breast and osteosarcoma cancers. It has also been investigated to show various mechanisms of action leading to apoptosis development (Ho *et al.*, 2009; Lu *et al.*, 2012)

Harringtonine and Homoharringtonine are two anticancer drugs derived from *Cephalotaxus harringtonia*. These two drugs have been found effective against both acute and chronic cases of myeloid leukemia, even therapy resistant cancers (Prakash, Kumar and Kumar, 2013). Cephalotaxine is used as a precursor in the synthesis of both Harringtonine and Homoharringtonine in an esterification reaction, although it has no anticancer activity naturally. Both alkaloid esters inhibit leukemia cells by preventing chain elongation during the synthesis of proteins and therefore, prevent protein synthesis.

Ellipticine is another plant derived anticancer alkaloid whose mode of action is inserting itself into the DNA strand and thereby inhibits the activity of topoisomerase II (Stiborová

et al., 2015). Ellipticine and its analogs also modulate cancerous cell death through different pathways, which includes but not limited to Bcl-2 pathway, mitochondria modulated cell death through the release of cytochrome c and apoptosis inducing factors, Mitogen-activated protein kinases and induction of p53 (Stiborová *et al.*, 2015). The active ingredient, 5,11-dimthyl-6H-pyrido [4, 3b] carbazole was originally isolated from three plants, *Ochrosia borbonica, Excavatia coccinea* and *Ochrosia elliptica* (Nirmala, Samundeeswari and Sankar, 2011; W. Ma *et al.*, 2012) and is believed to be common in plant belonging to Apocynaceae family members. Ellipticine and its derivatives have gained attention from the pharmacological industry due to their wide applications for treatment of a wide range of different cancers and its notably low toxicity (Stiborová *et al.*, 2015). Ellipticine is efficacious against bone, breast, brain, kidney cancers and leukemia (W. Ma *et al.*, 2012).

Phenolic compounds represent a large group of plant derived compounds which are primarily responsible for the bright pigmentation observed in plants. Phenols serve several functions, they deter herbivores, play a role in plant reproduction and defend the plant against disease-causing microbes (Rosa *et al.*, 2016). Pharmaceutically, phenols can be used as antibacterial, anticancer, antiviral and anti-inflammatory agents (Rosa *et al.*, 2016) with varied modes of action. They are also known to possess antioxidant potential, phenolics can be divided into three major groups: (i) Flavonoid polyphenols, (ii) Phenolic acids, (iii) Non-flavonoid polyphenols. **Figure 2.4**. shows several phenols derived from plants.

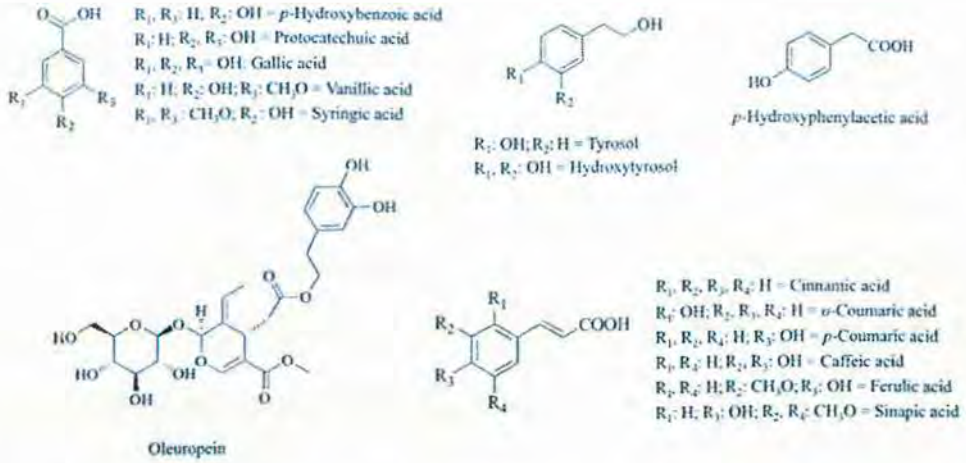


Figure 2.4. Examples of phenolic compounds depicting the characteristic phenol ring. Adopted from (Becerra-Herrera, Sayago and Beltrán, 2017).

Curcumin, a common polyphenol, has various food applications and is used in curries and other spices, turmeric contains about 2% curcumin. Curcumin is also an anticancer compound isolated from *Curcuma longa*, a member of the ginger family (Perrone *et al.*, 2015). Traditionally, the active principal of the plant has been used as an antiseptic, antioxidant, anti-inflammatory and analgesic. Experimentally, curcumin has been found to be effective against colorectal, pancreatic and multiple myelomas (Nirmala, Samundeeswari and Sankar, 2011). Despite its potential use as a treatment option for cancer therapy, curcumin has no definite mode of action even though research has shown various modes including its impact on cell cycle regulation, apoptosis, oncogenic expression, mutagenesis and metastasis regulation (Wilken *et al.*, 2011).

Daphnoretin is an anticancer drug originally isolated from the Chinese plant *Wikstroemia indica* in 1979. Traditionally, *Wikstroemia indica* was used to treat various communicable and non-communicable ailments including tuberculosis, syphilis, arthritis and pertussis

(Jiang *et al.*, 2014; Yang *et al.*, 2014). Daphnoretin has been shown to hold potential for treatment of different cancers such as lung cancer (Jiang *et al.*, 2014), Ehrlich ascites carcinoma and liver cancer cells (Nirmala, Samundeeswari and Sankar, 2011). Jiang et al., (2014) and Nirmala et al., (2011) report that daphnoretin exerts its anticancer effect by suppressing B-cell lymphoma genes involved in the cell cycle, thereby causing cell death and G2/M-phase cell arrest and cell death through a mitochondrial dependent pathway in cervical and lung cancer cells (Jiang *et al.*, 2014; Yang *et al.*, 2014).

Emodin is isolated from the rhizomes of the rhubarb plant, *Rheum palmatum* and has been found to cause death in pancreatic and colon cells (Lin *et al.*, 2012). In addition, emodin has been known to restore programmed cell death in leukemia, breast, prostate, lung, liver and ovarian cancers (Chen *et al.*, 2010; Nirmala, Samundeeswari and Sankar, 2011). Research has shown varied mechanisms by which emodin causes cell death and these includes VEGF receptor signaling (Lin *et al.*, 2012), reactive oxygen species (ROS) mediated DNA damage, mitochondrial mediated caspase cascade among others (Chen *et al.*, 2010).

Flavonoids are a subgroup of phenolics, which can further be divided into xanthones, proanthocyanidins, calchones, flavans, flavonones, flavones and catechins. Flavonoids are widely distributed in different plants and their basic structure is that called flavans. Flavonoids consist of more than on benzene ring in their chemical structure. Scientifically,

flavonoids are known commonly as free-radical scavengers, thus possessing antioxidant properties.

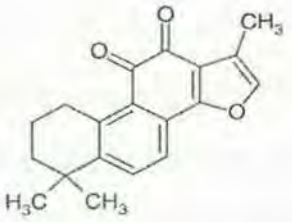
Flavopiridol is a semisynthetic derivative of the alkaloid rohitukine isolated from *Amoora rohituka* (Nirmala, Samundeeswari and Sankar, 2011). Current clinical trials are underway involving flavipiridol in various stages for the treatment of varied cancer types such as non-hodgkin lymphoma, non-small cell lung cancer, colorectal and renal cancers as well as leukemia.

Terpenes are compounds that are commonly found in nature, and can be derived from both animals and plants. In plants, terpenes are commonly found in fruits, flowers, resins or vegetables, where they commonly serve the function of being attractants, deterrents and communication agents, **figure 2.5** shows examples of terpenoids discussed below. Terpenes are composed of repeating units called isoprenes (C_5H_8) which can be connected to form either cyclic or linear structures (Yadav, Yadav and Goyal, 2014). Furthermore, terpenes can be classified into hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes or polyterpenes depending on the total number of isoprene units. Terpenes are referred to as hydrocarbons but the isoprene units can be used to make structures which contain additional functional groups such as carbonyls, hydroxyls, ketones or aldehydes groups, which turns them into terpenoids (Yadav, Yadav and Goyal, 2014). Due to their functions in nature, terpenes and terpenoids are used for their

organoleptic properties in the fragrance, flavor, pharmaceutics and chemical industries (Huang *et al.*, 2012).

Perrily alcohol is a monoterpene isolated from common herbs such as lemongrass, lavender, sage, ginger, perilla, mints etc (Nirmala, Samundeeswari and Sankar, 2011; Farazuddin *et al.*, 2012). Perillyl alcohol has been shown to be effective in a number of cell lines including breast, prostate, colon and lung cancer (Farazuddin *et al.*, 2012). Perillyl alcohol is effective against cancer by causing apoptosis, cell arrest in the G1 phase and differentiation of cancerous cells (Nirmala, Samundeeswari and Sankar, 2011). Clinical trials have shown a reduction of tumor masses after treatment with Perillyl alcohol and especially without adverse side effects (Garcia *et al.*, 2010).

Tanshinone IIA



Triptolide

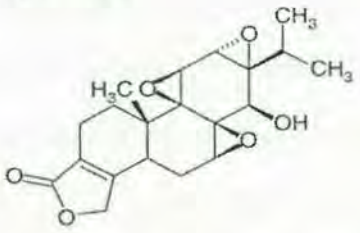


Figure 2.5. Examples of terpenoids with anticancer activity. Adopted from (Huang et al., 2012).

Tanshinone IIA is anticancer compound isolated from *Salvia miltiorrhiza*, a plant used in Chinese medicine for the treatment of heart diseases(Huang *et al.*, 2012; Prakash, Kumar

and Kumar, 2013). Research has shown that the active principal of *Salvia miltiorrhiza* is effective by inhibiting the growth, inducing apoptosis, causing differentiation in several cell lines (Lu, Zhang, Zhang, & Chen, 2009; Ma, Fan, Yu, Xin, & Zhang, 2012), and the prevention of cancerous cell migration (Huang *et al.*, 2012). Huang et al., (2012) further reported the synergistic anticancer effects with other drugs such as cisplatin and doxorubicin. Tanshinone IIA exerts its anticancer effects by binding to DNA in the minor groove, which results in DNA damage and inhibition of the actions of RNAPII, further causing a series of events involving TNF-α, PI3K/AKT, ROS, Bax/Bcl-2 etc (Huang *et al.*, 2012).

Cucurbitacin is a triterpenoid isolated from plants from the cucurbitaceae family. The role of cucurbitacins in plants as insect attractants has led to enormous attention given to these biomolecules. Cucurbitacins demonstrate hepato-protective, anti-proliferative and anti-inflammatory properties (Huang *et al.*, 2012). The mechanism by which cucurbitacins exerts their anticancer activity is through the down-regulation of key signal transducer proteins such as JAK2 and STAT3 (Nirmala, Samundeeswari and Sankar, 2011). In addition, cucurbitacins have been known to cause cell death through JAK2/STAT3 independent pathways such as the MAPK pathway (Lee, Iwanski and Thoennissen, 2010) and inhibition of angiogenesis (Shukla *et al.*, 2016). Additional modes of action have been mitochondrial dependent cell death, PRAPK cleavage, active caspase-3 expression, down-regulation of genes such as Bcl-2, Cyclin D3, Mcl-1 and Bcl-xL (Alghasham, 2013), cell cycle arrest at G2/M, S phase, cell differentiation, cytoskeletal assembly disruption and metastasis inhibition (Huang *et al.*, 2012).

Triptolide is an anticancer compound isolated from a Chinese plant called *Tripterygium wilfordii*, which has been found efficacious against a number of cell lines, such as pancreatic, oral, cervical, colon, thyroid and ovarian cancer (Meng *et al.*, 2014). Triptolide also has immunosuppression and anti-inflammatory activity (Huang *et al.*, 2012; Li *et al.*, 2012). The active principal is a diterpenoid triepoxide and inhibits tumor cell migration, proliferation and induces apoptosis (Li *et al.*, 2012). It also enhances the anticancer activity of chemotherapeutic drugs such as cisplatin both *in vitro* and *in vivo* models of gastric cancer (Li *et al.*, 2012) and the improves the efficacy of radiotherapy (Meng *et al.*, 2014). The mechanism of action of triptolide is not completely understood but it is postulated that it interferes with the transcription factors such as p53, Nuclear Factor-kB, Nuclear Factor-AT, and Heat Shock Factor-1 (Huang *et al.*, 2012). Despite its attributes, triptolide has poor solubility which has resulted in the development of a semi-synthetic derivative, PG490-88 (Nirmala, Samundeeswari and Sankar, 2011), which is currently in clinical trials.

Betulinic acid is a triterpenoid anticancer compound consisting of five fused rings. The active principal has been isolated from various plant sources including *Zizyphus species* and *Betula alba* (Nirmala, Samundeeswari and Sankar, 2011; Prakash, Kumar and Kumar, 2013). It has shown potency against a number of cancer cells (colon, prostate, ovary, lung, melanoma, glioblastoma, medulloblastoma etc), including *in vivo* cancer models, while causing no harm to non-cancerous cells (Periasamy *et al.*, 2014). Betulinic acid exerts it' anticancer actions through mitochondrial-mediated apoptosis induction (Nirmala, Samundeeswari and Sankar, 2011). The poor solubility of betulinic acid limits its

bioavailability, has led to the development of improved semi-synthetic derivatives (Furtado *et al.*, 2017).

Steroids are organic compounds typically comprised of a four-membered ring system, which is synthesized from squalene as the precursor molecule via the triterpene pathway (Sawadogo *et al.*, 2012). Steroids have been isolated from many natural sources including plants, marine organisms, fungi and animals (Saeidnia *et al.*, 2014), which exhibit various biological activities and have been exploited for the treatment of many illnesses. Simoben, Ibezim, Ntie-Kang, Nwodo, & Lifongo, (2015) summarized a number of steroids with anticancer potential isolated from African botanical material, among these were the more common compounds such as stigmasterol and sitosterol (Saeidnia *et al.*, 2014). Other steroids such as those called brassinosteroids are discussed by (Greenwell and Rahman, 2015), 24-epibrassinolide and 28-homocastasterone have been investigated for anticancer effects against leukemia, lung, breast, prostate cervical cancer, myeloma and osterosarcoma cells. Brassinosteroids have been shown to induce apoptosis as well as inhibit cancer cell proliferation through cyclin dependent cell cycle arrest (Mehndiratta *et al.*, 2011; Greenwell and Rahman, 2015). Sawadogo et al., (2012) reported that steroids are useful as anticancer treatment options as they alter the balance of sodium, Potassium, Calcium and hydrogen ions inside cancerous cells, which initiates apoptosis. *Caloropis procera* is a drought resistant plant which is native to India, northern Africa, Brazil, and Southern USA and has been instrumental in the maintenance of health in these local communities. Cardenolides isolated from *C. procera*, have been shown to confer some pharmacological properties to the extracts of this plant, **figure 2.6**. Although much of the

toxic properties of the *C. procera* plant has been attributed to cardenolides, they have also been shown to be responsible for anti-inflammatory, antitumor, selective toxicity, anti-ulcer properties and protection against oxidative stress (Teixeira *et al.*, 2011). With regards to their anticancer properties, cardenolides were found to operate through the inhibition of the sodium ion pump (Sawadogo *et al.*, 2012). Presently, a semi synthetic derivative, UNBS1450, modeled after cardenolide 2"-oxovoruscharin, is available, which demonstrated better pharmacological applications (Teixeira *et al.*, 2011; Sawadogo *et al.*, 2012).

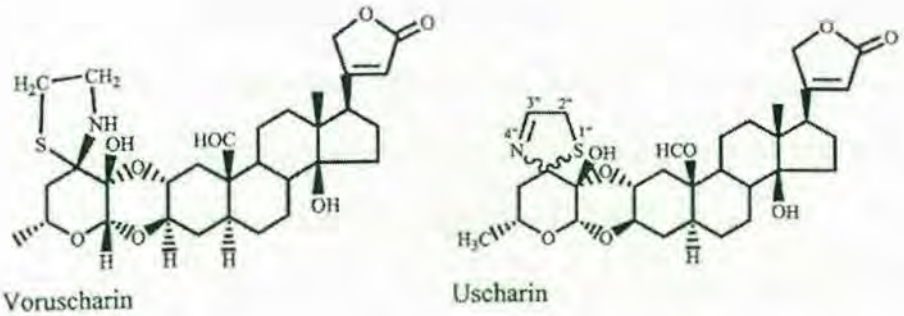


Figure 2.6. Examples of steroids, Voruscharin and Uscharin, belonging to cardenolide group. Adopted from (Sawadogo et al., 2012).

2.8. The use of traditional plants and practices to manage cancer-like symptoms in Namibia.

Namibia is characterized by four distinct biomes: desert, nama karoo, succulent karoo and savanna. It is also known as the most arid country south of the Sahara Desert. About 16% of its entire land is either covered by the Kalahari or the Namib Desert. Despite this,

Namibia has a rich heritage of over 4000 (Maggs, Craven and Kolberg, 1998) plants inhabiting different biotic areas, with about 690 plants endemic to Namibia (Cole *et al.*, 2014) and about 615 plants listed for medicinal use by the SEPASAL database. The plant species which inhabit the different biotic niches develop mechanism to enable them to survive and thrive in these biota and secondary metabolites are a key part of the survival mechanisms. These plants, are a part of the rich traditional medicinal knowledge which is held by the local communities in the areas where they are found. Traditional medicinal practices are usually passed down from elderly community members to the young generation in the form of songs, rituals, stories, observations (Parrotta, Yeo-Chang and Camacho, 2016). However, the preservation or continuity of traditional medicinal practices are at risk of disappearing from communities all together because of the adoption of western life-styles. In Namibia, ethnomedicinal surveys have been undertaken to document Namibia's rich traditional medicine heritage and they report on plant species and their local medicinal uses (Cheikhyoussef et al. 2011; Chinsembu et al. 2011; Chinsembu & Hedimbi 2010; Dan et al. 2010). Knowledge holders in local communities were key informants on symptoms or perceived causes of disease and the use of medicinal plants to treat them Descriptions such as skin infections, breast infections (Dan, Mchombu and Mosimane, 2010), skin infections, Herpes zoster, Herpes simplex, skin rashes (Chinsembu and Hedimbi, 2010) were based on the informants description and the interviewers interpretation, there was no clinical/ formal diagnosis. *L. zastrowiana, L. stuhlmanni, A. senegalinsis, A. stenophylla* are all used for treating skin infections, Herpes zoster, H. simplex and rashes (Chinsembu and Hedimbi, 2010). *Vigna dinteri, acanthosycios naudiniana* are used for treating herpes and inflammation (Chinsembu and Hedimbi, 2010).

Efforts towards documenting traditional knowledge of medicinal plants is commendable, however the validation of the biological activity of these plants and the study of their safety profiles is still lagging (Dushimemaria and Mumbengegwi, 2015).

2.9. Traditional uses of *G. fruticosus* in Namibia

This study focused on a plant called *Gomphocarpus fruticosus* (L.)Aiton f., belonging to the Apocynaceae/ Asclepiadaceae family, **figure 2.7**. *G. fruticosus* has had numerous names but is most commonly known as either *G. fruticosus* or *Asclepias fruticosa*. The plant is listed among the 619 plants on the Survey of Economic Plants in Arid and Semi-Arid Lands (SEPASAL: http://apps.kew.org/sepasalweb/sepaweb) as being used for medicinal purposes. The plant is known locally by many different names: *Kapokbos, Melkbos, Tondel, Wilde kapok* (Afrikaans), *Oruseppa (Otjiherero), |giitama||oob, ||horapob (Khoekhoegowab), Wilde Baumwolle* (German) or Milkweed, Wild cotton (English). The plant is native to south tropical Africa with distribution in countries such as Namibia, Zambia, Zimbabwe, Mozambique, Botswana, Swaziland, Lesotho and South Africa. *G. fruticosus* has various local uses such as arrow poison for hunting, as a vegetable for human consumption. It is used medicinally for treatment of stomach troubles (Fouche *et al.*, 2008; Madureira *et al.*, 2012), asthma (Marzouk, Osman and Gohar, 2016), sores, boils, headaches, toothaches, TB (Madureira *et al.*, 2012) coughs, and other upper pulmonary respiratory complaints (Marzouk, Osman and Gohar, 2016). *G. fruticosus* used as both a potent evacuate and emetic (Fouche et al. 2008), a sedative in treating mental disorders, treatment of venereal infections such as gonorrhea and as an anti-inflammatory decoction. In addition, *G. fruticosus* has been used for the treatment of tumors and several

skin disease including scabies (R. A Mothana et al. 2009). Marzouk et al. (2016) and R. A. A. Mothana et al. (2009) both cited the application of the plant in the remedy of heart related conditions. In Ethiopia, extracts prepared from the root of the milk weed are known to be useful in termination of pregnancy (Araya, Abera and Giday, 2015).

Figure 2.7. *Gomphocarpus fruticosus* erect plant growing in a riverbed. Picture credit: Florence Dushimemariya.

2.9.1. Bioactivity of *G. fruticosus* in literature

Gomphocarpus fruticosus has been screened for antibacterial activity, against *P. aeruginosa* (Madureira et al, 2012). In addition, *G. fruticosus* demonstrated potent activity against parasitic *T. cruzi*, with IC_{50} 2.6 ± 0.5 µg/ml (Mothana et al, 2014). Mothana et al, 2009 reported that the methanolic extract of *G. fruticosus* exhibited anticancer properties against three cancer cell lines: Breast, lung and urinary bladder cancer. The IC_{50} values obtained against these cell lines were all below 10 µg/ml, indicating moderate anticancer activity (log GI_{50} between 0 and 1.10) (Fouche et al, 2006). In another study conducted by Fouche *et al.*, (2008), methanolic and dichloromethane extracts of *G. fruticosus* demonstrated potent activity against MCF7 (breast), Renal (TK10) and Melanoma cancer cells (6.25 µg/ml<TGI <15µg/ml for at least two cell lines). Surprisingly, total growth inhibition (TGI) on metabolically slow cell line TK10, was less than 0 µg/ml. The cell line TK10 usually demonstrates slow metabolism of treatments, which leads to less sensitivity to treatments in comparison to other cell lines such as the MCF7 (Garner and Eastman, 2011).

The methanolic extract of *G. fruticosus* demonstrated cytotoxic properties in MCF-5 cells, indicating its none-selective nature towards cancerous and non-cancerous cell lines (Mothana *et al.*, 2014). In addition, several reports in literature indicate the toxic nature of bioactive compounds derived from species of the apocynaceae family. Exposure to toxins manifests as corneal oedema as a result of Na^+/K^+ ATPase pump inhibition along the corneal endothelium (Amiran, Lang and Yeung, 2011; Pina *et al.*, 2014; Matsuura *et al.*, 2017; Mikkelsen *et al.*, 2017).

2.9.2. Phytochemicals found in *G. fruticosus*

Plant species in the *Gomphocarpus* group contain glycosides of the pregnane and cardenolide variety (Warashina and Noro, 1994; Komissarenko, Chernobai and Komissarenko, 1995). These glycosides are toxic, having cardio-active properties and as a result, are used as arrow poison for hunting. A study by Fouche et al., (2008), further highlighted the anticancer activity of members of the Apocynacea family such as *G. fruticosus* and *G. physocarous*. In addition to cardenolides, the milkweed contains terpenoids (R. A. A. Mothana *et al.*, 2009). In a study conducted by Komissarenko et al. (1995), three glycosides, gomphoside, gomphotin and gomphotoxin were identified in the leaves of *G. fruticosus*. Additionally, Warashina & Noro (1994) identified steroidal glycosides from whole plant methanolic extracts of *G. fruticosus*, which comprised of lineolon and 5 of its derivatives. In a recent report, Marzouk et al. (2016) reported a new steroidal glycoside,lineolon-3-O-[β-D-oleandropyranosyl-(1–4)-β-D-cymaropyranosyl-(1–4)-β-D-cymaropyranoside isolated from *G. fruticosus*. Uzarigenin and deglucourazin have also been identified from leaf extract of the milkweed (Komissarenko, Chernobai and Komissarenko, 1995), whilst coroglaucigenin and corotoxigenin were identified from stem and root extracts (Abe et al. 1994). Coroglaucigenin significantly reduced the viability of human lung cancer cells (ROS and Nrf2 activation-oxidative stress) and also increased the sensitivity of A549 to radioactive rays (Sun *et al.*, 2017). In another study, extracts from *Asclepias curassavica*, demonstrated significant anti-proliferative activity against HepG2 cells, Raji cells and nasopharynx carcinoma cells, IC_{50} 0.01-1.46 μM/ml (Al-snafi, 2015). Other phytoconstituents reported in literature include triterpenoids 3β-taraxerol acetate, 13α-methyl, 27-norolean-14-en, 3β-ol (3β-taraxerol), calotropin, betulinic acid, uscharin, uscharidin, calactin, voruscharin, calotropagenin, afroside and

other glycosides containing ikemagenin or kidjolanin as the aglycon portion (Groeneveld *et al.*, 1990; Marzouk, Osman and Gohar, 2016). These cardenolide and pregnane glycoside identified in *G. fruticosus* and other members of the Apocynaceae family are renowned for cardiotonic actions, anticancer actions against leukemia, antioxidant activity and may likely possess additional biological actions. In another study, an investigation into the phytochemistry of *G. fruticosus* growing in Egypt lead to the identification of flavonoid glycosides containing quercetin, kaempferol and isorhamnetin (Heneidak *et al.*, 2006). However, very little is known about Namibian *G. fruticosus*, which grows in a unique environment.

2.10. Challenges of cancer treatment using chemotherapeutics agents

The most common route for anticancer bioactives/ drug administration is the oral route, through the alimentary canal, followed by dissolution, absorption and transport to various sites. This is the most preferred route of drug administration because it is less invasive and therefore leads to high patient compliance. However, despite being the most preferred method of drug administration, it is not without problems. Drug administration through the oral route has the challenge of poor bioavailability and patient to patient variability. Drugs or bioactive compounds need to be stable enough to remain intact as they pass through the stomach cavity where they are exposed to gastric juices (Kushwaha *et al.*, 2012). In addition, the bioactive compound needs to be sufficiently absorbed from the alimentary canal into the blood system. This is followed by the potential elimination of the bioactive compound by the hepatic portal system, further decreasing its bioavailability. As much as these are real challenges faced during drug delivery, the two most common

challenges for cancer chemotherapy is poor solubility and toxicity. These challenges have led to the development of numerous drug delivery systems in an effort to improve bioavailability, targeted delivery, stable formulations, extended release tablets and even formulations which can be injected directly into the blood system to improve bioavailability. Nanoparticles have gained popularity and have the advantage of multiple dosage forms, which can be taken orally, as aerosols and parenterally through the muscle or directly into blood circulation (Mudshinge *et al.*, 2011; De Smet *et al.*, 2013; El-Sherbiny, El-baz and Yacoub, 2015).

Even though chemotherapy is the most commonly used cancer treatment option, it is not without challenges. One common disadvantage is that the chemotherapeutic agents are often toxic, and due to non-specific distribution, side effects occur. This places dose-limitations on existing drug candidates reducing the dosage reaching the tumorous sites and causing side effects (Ebrahimifar *et al.*, 2017). Furthermore, the non-discriminant distribution of chemotherapy also contributes to treatment failure as a result but more importantly to side effects and toxicity. These experienced adverse effects have been influential factors in attempts to improve treatment-related toxicity associated with drugs such as taxol, vinflurine, vincristine such as neuropathy, anemia, neutropenia, sight loss, and neurotoxicity (Habli *et al.*, 2017). These attempts has been either to improve existing drugs or discover new therapeutic drugs. In addition to side effects, cancer cases can often demonstrate resistance to treatment interventions, reducing treatment options (Labhasetwar, 2011; Rebucci and Michiels, 2013; Zahreddine and Borden, 2013).

Available drugs on the markets such as paclitaxel, camptothecin have poor solubility in aqueous solvents (Nirmala et al., 2011). Poor solubility of bioactive compounds is influenced by their large molecular weight, which prevents their passive diffusion across the membrane bi-layer (Sundaraganapathy and Leena, 2016). The bioactive compounds demonstrate poor lipid solubility, caused by their polarity (Agrawal, Gupta and Chaturvedi, 2012; Sundaraganapathy and Leena, 2016), exacerbating their poor bioavailability properties possibly leading to treatment failure in *in vivo* studies. Poor solubility of prominent drugs such as paclitaxel, campothecin prevents parenteral administration of drug, which would improve bioavailability by 100%, and therefore require the use of drug carriers (Kushwaha *et al.*, 2012). It is against this background that liposome and other lipid based formulations have emerged as a potential solution. Liposomal based drug formulations are expected to improve the efficacy of treatments, reduce the clearance or degradation of treatments from the body, thereby increasing half-lives and reducing toxicity via site-targeted delivery (Dickson and Schwartz, 2009).

In addition, the drug discovery and development pipeline takes a long time, currently being approximated to take about 23 years (Cummings *et al.*, 2017). The success rate of a drug making it to the market is about 1 in 5000 compounds screened in the research and development pipeline. For the most part, the poor success rate is mainly attributed to high toxicities as can be expected from the rise in poor aqueous-soluble drugs making it through the drug research and discovery pipeline (Ma *et al.*, 2012; Gupta, Kesarla and Omri, 2013).

2.11. Lipid-based phytosomes for improved bioactive delivery

Drug or bioactive compounds are often ineffective owing to barriers encountered whilst bioactive compounds/ drugs are in the body. One of such challenges is the poor solubility of drug/ bioactive compounds in the body, resulting in poor bioavailability. Bioavailability is a term used to describe the degree and rate by which the active pharmaceutical ingredient reaches the blood circulatory system, thereby being able to access the point of action (Mehta *et al.*, 2016). As a result, technologies aimed at mitigating against these challenges have been developed such as liposomes, nano-emulsions, nanocrystals, hydrogels, solid lipid formulations, and phytosomes (Gupta, kesarla, Omri et al., 2013). Major components of these delivery structures are phospholipids (Li *et al.*, 2015). Phospholipids have an advantage over other materials used for active pharmaceutical ingredient (API) delivery such as carbon and silica (De Jong and Borm, 2008), are biodegradable and shows bio-compatibility when used *in vivo*, an important aspect of drug delivery (Mehta *et al.*, 2016). Phospholipid based drug delivery systems developed over the years continue to play a paramount role in the fight against cancer. Ebrahimifar et al., (2017) reported that nano-particulate encapsulated carboplatin showed increased efficacy against ovarian cancer in mice in comparison to carboplatin alone. Another example is 5-fluorouracil (5-FU), an anticancer drug with poor solubility in water, severe toxicity when administered intravenously and a half-life of close to 22 min, limits bioavailability. However, lipid based nanoparticles of 5-FU proved to improve its efficacy in *in vitro* studies (Shenoy, Gude and Murthy, 2013). Several lipid based drugs have been approved by the FDA, with a myriad of applications, including amphotericin B, cyclosporin A and topotecan (Bulbake *et al.*, 2017).

A phytosome as compared to liposomes (**figure 2.8**), is a lipid based delivery system which was specifically developed for the delivery of herbal remedies (Awasthi, Kulkarni and Pawar, 2011). Similar to single compounds, herbal remedies also face challenges pertaining to poor solubility, which eventually leads to poor bioavailability (Shivanand and Kinjal, 2010). Phytosomes, which are comprised of a lipid moiety and herbal components enclosed within the lipid bi-layer is able to mitigate this challenge. Herbal bioactives often comprise of water soluble (Shivanand and Kinjal, 2010) and water insoluble portions, both which are essential for treatment (Shivanand and Kinjal, 2010). Water soluble components are often poorly absorbed due to the lipid-rich nature of cells lining the GIT or when administered topically (Pawar and Bhangale, 2015) or the large size of these compounds (Shivanand and Kinjal, 2010). These two reasons can lead to treatment failure due to poor bioavailability. Additionally, encapsulation of herbal bioactive components to form phytosomes serves to protect contents from degradation of the gastrointestinal tract (GIT) juices, further leading to improved bioavailability (Pawar and Bhangale, 2015). Phytosomes also provide for opportunities for targeted delivery. Targeted delivery involves surface modifications which adds functionality to phospholipids such that they accumulate at disease sites (Mehta *et al.*, 2016). Other research shows the targeting of phytosomes to receptors found on cancerous cells or specific to certain tissues such as folate receptors in breast cancer cells and others, to achieve targeted delivery (Fasehee *et al.*, 2016). Research has shown several methods used to confer targeted delivery, including the delivery of cinnamaldehyde using magnetite nanoparticles (Wani *et al.*, 2014). Wani et al., demonstrated the reduced viability of breast cancer cells treated with encapsulated cinnamaldehyde at lower extract concentrations, in comparison to free cinnamaldehyde. Additionally, the use of poly-ethyleneglycol (PEG)

has become increasingly popular to ensure sustained stability of phytosomes/nanoparticles, resulting in increased bioavailability (Li *et al.*, 2015; Fasehee *et al.*, 2016). Furthermore, due to the lipid nature of phytosomes, increased absorption results, significantly increasing bioavailability and hence reducing dose needed to achieve therapeutic efficacy and this has been demonstrated in several studies (Wani *et al.*, 2014; Wu *et al.*, 2017) and reviews (Pawar and Bhangale, 2015; Mehta *et al.*, 2016).

As a result, phytosomes are able to effectively improve the solubility and reduce nonspecific toxicity by encapsulating plant extracts both within the lipid bi-layer as well as within the nanostructure core region. Within the lipid bi-layer, phytocompounds can form chemical bonds with the phosphate "head" regions (Awasthi, Kulkarni and Pawar, 2011), thereby increasing the caring capacity of phytosomes, in comparison to liposomes.

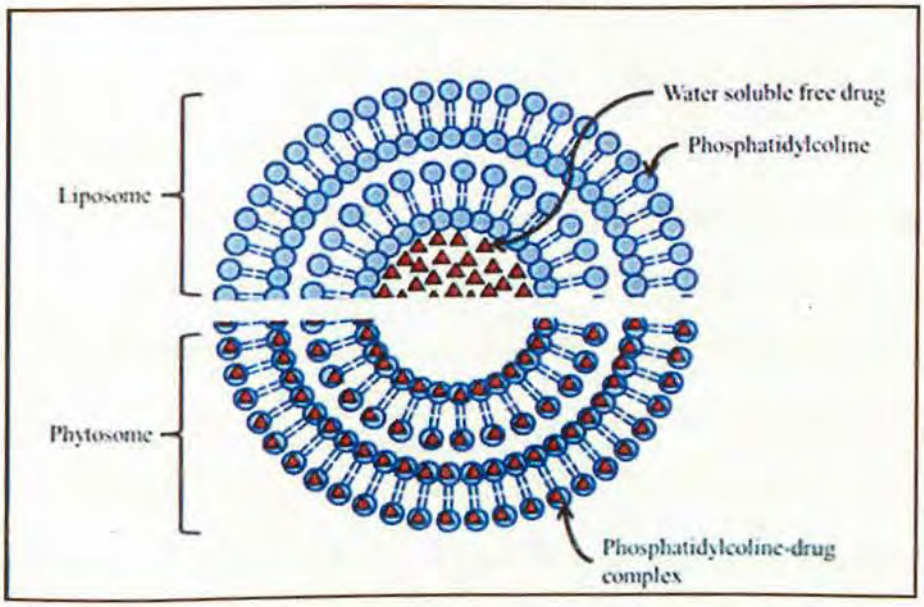


Figure 2.8. Presentation of phytosome as compared to a liposome. Adapted from (Awasthi, Kulkarni and Pawar, 2011).

The nano-size of phytosomes gives this drug formulation an advantage. Since particles below 30 nm are eliminated through the renal excretion system, while large particles are picked up for degradation by the body's defense system comprised of mononuclear phagocytic cells and destroyed. Particles with sizes ranging from 150-300 nm accumulate in major body organs such as the spleen, liver, heart, bone marrow, stomach and kidneys (Gaumet *et al.*, 2008; Chime and Onyishi, 2013). The size of phytosomes also allows them to escape through fenestration on vascular vessels, therefore avoiding transport to the hepatic portal system, leading to increased half-life and bioavailability through accumulation at disease sites such as solid tumors' and in the skin. Therefore, the use of a phytosome as a delivery system not only improves bioavailability of herbal medicines, but it also targets the accumulation of a combination of phytoconstituents into susceptible cancerous cells.

This study will focus on *G. fruticosus*. Even though the plant has been researched numerous times and literature shows that it is poisonous to livestock, and that cardio-active principals were responsible for it toxicity, the mechanism of action has not yet been determined. Its anticancer activity has also been reported and despite its potent activity, the plant has been abandoned owing to observed toxicities. Besides this, the Namibian *G. fruticosus* grows in a unique ecological niche, which affects its phytochemical content. This study aims to investigate Namibian *G. fruticosus* for its phytochemical content as a contrast to already existing literature. In addition, in an effort to mitigate cited toxicities, a herbal-based delivery system will be developed in the form of a phytosome and evaluated against cancer models that are prominent in Namibia and beyond.

2.12. References

Abe, F., Mori, Y., Okabe, H. & Yamauch, T., 1994. Steroidal constituents from the roots and stems of *Asclepias fruticosa*. *Chemical and pharmaceutical bulletin, 42*(9), pp. 1777–1783. doi: 10.1248/cpb.37.3229.

Agrawal, V. K., Gupta, A. and Chaturvedi, S., 2012. Improvement in bioavailability of class-III drug:phytolipid delivery system. *International journal of pharmacy and pharmaceutical sciences*, *4*(1), pp. 37–42.

Al-snafi, A. E., 2015. Chemical Constituents and Pharmacological Effects of *Asclepias Curassavica* – a Review. *Asian Journal of Pharmaceutical Research*, *5*(2), pp. 83–87.

Alghasham, A. A., 2013. Cucurbitacins - a promising target for cancer therapy. *International Journal of Health Sciences*, *7*(1), pp. 77–89.

Amiran, M. D., Lang, Y. and Yeung, S. N., 2011. Corneal endothelial toxicity secondary to Asclepias fruticosa, *Eye, 25*, pp. 961–963. doi: 10.1038/eye.2011.59.

Araya, S., Abera, B. and Giday, M., 2015. Study of plants traditionally used in public and animal health management in Seharti Samre District, Southern Tigray, Ethiopia. *Journal of ethnobiology and ethnomedicine, 11*, p. 22. doi: 10.1186/s13002-015-0015-5.

Awasthi, R., Kulkarni, G. T. and Pawar, V. K., 2011. Phytosomes: An approach to increase the bioavailability of plant extracts. *International Journal of Pharmacy and Pharmaceutical Sciences*, *3*(2), pp. 1–3.

Balakrishna, A. and Kumar, M. H., 2015. Evaluation of synergetic anticancer activity of berberine and curcumin on different models of A549, Hep-G2, MCF-7, Jurkat, and K562 cell lines. *BioMed Research International, 2015*, p. 7. doi: 10.1155/2015/354614.

Barbuti, A. M. and Chen, Z. S. (2015). Paclitaxel through the ages of anticancer therapy: Exploring its role in chemoresistance and radiation therapy, *Cancers, 7*(4), pp. 2360–2371. doi: 10.3390/cancers7040897.

Becerra-Herrera, M., Sayago, A. and Beltrán, R., 2017. Exploring antioxidant reactivity and molecular structure of phenols by means of two coupled assays using fluorescence probe (2,3-diazabicyclo[2.2.2]oct-2-ene, DBO) and free radical (2,2-diphenyl-1-picrylhydrazyl, DPPH·). *Journal of Chemical Sciences, 129*(9), pp. 1381–1390. doi: 10.1007/s12039-017-1331-1.

Berrios, C., 2016. Severe Motor Polyneuropathy Secondary to the Use of Vincristine in a Patient with Acute Lymphoblastic Leukaemia in the Period of April 2013. *Journal of Pediatric care*, *3*(1), pp. 1–6. doi: 10.21767/2471-805X.100022.

Brentnall, M., Rodriguez-Menocal, L., De Guevara, R. L., Cepero, E. and Boise, L. H. (2013) Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis, *BMC Cell Biology*, *14*(1), p. 32. doi: 10.1186/1471-2121-14-32.

Bulbake, U., Doppalapudi, S., Kommineni, N. & Khan, W., 2017. Liposomal formulations in clinical use: An updated review. *Pharmaceutics, 9*(2), pp. 1–33. doi:

10.3390/pharmaceutics9020012.

Calaf, G. M., Zepeda, A. B., Castillo, R. L., Figueroa, C. A., Arias, C., Figueroa, E. and Farías, J. G., 2015. Molecular aspects of breast cancer resistance to drugs (Review). *International Journal of Oncology*, *47*(2), pp. 437–445. doi: 10.3892/ijo.2015.3055.

Carrara, H., Parken, M., Zietsman, A., Pontac, J., Lawrence, S., Horn, L. & van Schalkwyk, L. (2017). *Namibia National Cancer Registry (NNCR) Cancer incidences in Namibia 2010 - 2014*. Windhoek.

Chaabane, W., User, S. D., El-Gazzah, M., Jaksik, R., Sajjadi, E., Rzeszowska-Wolny, J. and Łos, M. J., 2013. Autophagy, apoptosis, mitoptosis and necrosis: Interdependence between those pathways and effects on cancer. *Archivum Immunologiae et Therapiae Experimentalis*, *61*, pp. 43–58. doi: 10.1007/s00005-012-0205-y.

Chanda, S. and Nagani, K., 2013. *In vitro* and *in vivo* Methods for Anticancer Activity Evaluation and Some Indian Medicinal Plants Possessing Anticancer Properties: An Overview. in *IC Journal Journal of Pharmacognosy and Phytochemistry*, *2*(2), pp. 2668735–5. doi: 10.1007/s13197-011-0276-5.

Chandra, S., 2012. Endophytic fungi: novel sources of anticancer lead molecules. *Applied microbiology and biotechnology*, *95*(1), pp. 47–59. doi: 10.1007/s00253-012-4128-7.

Cheikhyoussef, A., Shapi, M., Matengu, K. and Mu Ashekele, H., 2011. Ethnobotanical study of indigenous knowledge on medicinal plant use by traditional healers in Oshikoto region, Namibia. *Journal of ethnobiology and ethnomedicine*, *7*, p. 10. doi: 10.1186/1746-4269-7-10.

Chen, Y. Y., Chiang, S. Y., Lin, J. G., Yang, J. S., Yi-Shih, M., Liao, C. L., Lai, T. Y., Tang, N. Y. and Chung, J. G., 2010. Emodin, aloe-emodin and rhein induced DNA damage and inhibited DNA repair gene expression in SCC-4 human tongue cancer cells. *Anticancer Research*, *30*(3), pp. 945–952. doi: 30/3/945 [pii].

Chime, S. A. and Onyishi, I. V., 2013. Lipid-based drug delivery systems (LDDS): Recent advances and applications of lipids in drug delivery. *African Journal of Pharmacy and Pharmacology*, *7*(48), pp. 3034–3059. doi: 10.5897/AJPPX2013.0004.

Chinsembu, K. C. and Hedimbi, M., 2010. An ethnobotanical survey of plants used to manage HIV/AIDS opportunistic infections in Katima Mulilo, Caprivi region, Namibia, *Journal of ethnobiology and ethnomedicine*, *6*, p. 25. doi: 10.1186/1746-4269-6-25.

Chinsembu, K. C., Hedimbi, M. and Mukaru, W. C., 2011. Putative medicinal properties of plants from the Kavango region, Namibia. *Journal of Medicinal Plants Research*, *5*(31), pp. 6787–6797. doi: 10.5897/JMPR11.1135.

Clague, J. and Berstein, L., 2012. Physical activity and cancer. *Current Oncology Reports*, *14*(6), pp. 550–558. doi: 10.1016/j.jmb.2008.10.054.The.

Clarke, S. J. and Mclachlan, A. J., 2011. Interaction between complementary and alternative medicine with conventional anticancer medicine. *Cancer Forum*, *35*(1), p. 6. Available at: https://cancerforum.org.au/wp-content/uploads/2015/06/Mar_2011_Forum5.pdf (Accessed: 10 October 2017).

Cole, D., Mallet, M., Maggs-Kolling, G., Iileka, R., Gottlieb, T., Nott, K., Chimwamurombe, P., Bennett, B., Adel-Sheehama, S. den, Plessis, P. du and Shikongo, S., 2014. *Indigenous plant products in Namibia: The Commercialisation of Indigenous Natural Plant Products in Namibia*. 1st edn. Windhoek: Venture Publications.

Cummings, J., Lee, G., Mortsdorf, T., Ritter, A. and Zhong, K., 2017. Alzheimer's disease drug development pipeline: 2017. *Alzheimer's & Dementia: Translational Research & Clinical Interventions*, *3*, pp. 367–384. doi: 10.1016/j.trci.2017.05.002.

Curigliono, G., Cardinale, D., Dent, S., Criscitiello, C., Aseyev, O., Lenihan, D. and Cipolla, C. M., 2016. Cardiotoxicity of Anticancer Treatments: Epidemiology, Detection, and Management. *CA: A cancer journal for clinicians*, *66*, pp. 309–325. doi: 10.3322/caac.21341.

Dan, V., Mchombu, K. and Mosimane, A., 2010. Indigenous medicinal knowledge of the San people: the case of Farm Six, Northern Namibia. *Information Development*, *26*(2), pp. 129–140. doi: 10.1177/0266666910367479.

Dickson, M. A. and Schwartz, G. K., 2009. Development of cell-cycle inhibitors for cancer therapy. *Current Oncology*, *16*(2), pp. 36–43.

Dushimemaria, F. and Mumbengegwi, D. R., 2015. Palliative treatment of cancer in resource poor settings: Traditional medicine perspective. *Journal of Pharmacognosy and Phytotherapy*, *7*(5). doi: 10.5897/JPP2014.0331.

Ebrahimifar, M., Nili-Ahmadabadi, A., Akbarzadeh, A., Shahemabadi, H, E., Hasanzadegan, M., Moradi-Sardareh, H., Madadizadeh, H. and Rezaee-diyan, J., 2017. Preparation, Characterization and Cytotoxic Effects of Pegylated Nanoliposomal Containing Carboplatin on Ovarian Cancer Cell Lines. *Indian Journal of Clinical Biochemistry*, *32*(2), pp. 230–234.

Ekor, M., 2014. The growing use of herbal medicines: Issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology*, *4*, pp. 1–10. doi: 10.3389/fphar.2013.00177.

El-Sherbiny, I. M., El-baz, N. M. and Yacoub, M. H., 2015. Inhaled nano- and microparticles for drug delivery. *Global Cardiology Science and Practice*, *2*, pp. 1–14. doi: 10.5339/gcsp.2015.2.

Farazuddin, M., Sharma, B., Khan, A. A., Joshi, B. and Owais, M., 2012. Anti-cancer-efficacy-of-perillyl-alcohol-bearing-plga-microparticles. *International Journal of Nanomedicine*, 7, pp. 35–47. doi: 10.2147/IJN.S24920.

Fasehee, H., Dinarvand, R., Ghavamzadeh, A., Esfandyari-Manesh, M., Moradian, H., Faghihi, S. and Ghaffari, S. H., 2016. Delivery of disulfiram into breast cancer cells using folate-receptor-targeted PLGA-PEG nanoparticles: *In vitro* and *in vivo* investigations. *Journal of Nanobiotechnology, 14*(1), pp. 1–18. doi: 10.1186/s12951-016-0183-z.

Fouche, G., Cragg, G. M., Pillay, P., Kolesnikova, N., Maharaj, V. J. and Senabe, J., 2008. *In vitro* anticancer screening of South African plants. *Journal of ethnopharmacology*, *119*(3), pp. 455–61. doi: 10.1016/j.jep.2008.07.005.

Fouche, G., Senabe, J., Khorombi, E., Kolesnikova, N., Maharaj, V. J. and Nthambeleni, R., 2005. High throughput screening of South African plants for anti-cancer properties. p. 6665.

Furtado, N. A. J. C., Pirson, L., Edelberg, H., Miranda, L. M., Loira-Pastoriza, C., Preat, V., Larondelle, Y. and André, C., 2017. Pentacyclic Triterpene Bioavailability: An Overview of *In Vitro* and *In Vivo* Studies. *Molecules*, *22*, p. 400. doi: 10.3390/molecules22030400.

Ganesan, R. M. and Muthuchelian, K., 2011. Antitumor potential of an acetogenin isolated from the seed extracts of Annona squamosa Linn. *Journal of Cancer Research and Experimental Oncology*, *3*(8), pp. 95–104. doi: 10.5897/JCREO11.037.

Garcia, D. G., Amorim, L. M. F., De Castro Faria, M. V., Freire, A. S., Santelli, R. E., Da Fonseca, C. O., Quirico-Santos, T. and Burth, P., 2010. The anticancer drug perillyl alcohol is a Na/K-ATPase inhibitor. *Molecular and Cellular Biochemistry*, *345*(1–2), pp. 29–34. doi: 10.1007/s11010-010-0556-9.

Garner, K. M. and Eastman, A., 2011. Variations in Mre11/Rad50/Nbs1 status and DNA damage-induced S-phase arrest in the cell lines of the NCI60 panel. *BMC cancer*, *11*(1), p. 206:1-13. doi: 10.1186/1471-2407-11-206.

Gaumet, M., Vargas, A., Gurny, R. and Delie, F., 2008. Nanoparticles for drug delivery: The need for precision in reporting particle size parameters. *European Journal of Pharmaceutics and Biopharmaceutics*, *69*(1), pp. 1–9. doi: 10.1016/j.ejpb.2007.08.001.

Greenwell, M. and Rahman, P. K. S. M. (2015) 'Medicinal Plants: Their Use in Anticancer Treatment. *International Journal of pharmaceutical sciences and research*, *6*(10), pp. 4103–4112. doi: 10.13040/IJPSR.0975-8232.6(10).4103-12.

Groeneveld, H. W., Steijl, H., Van Den Berg, B. and Elings, J. C., 1990. Rapid, quantitative HPLC analysis of Asclepias fruticosa L. 1 and *Danaus plexippus* L. 2 cardenolides. *Journal of Chemical Ecology*, *16*(12), pp. 3373–3382.

Gupta, S., Kesarla, R. and Omri, A., 2013. Formulation strategies to improve the bioavailability of poorly absorbed drugs with special emphasis on self-emulsifying systems. *ISRN pharmaceutics*, *2013*, p. 16. doi: 10.1155/2013/848043.

Habli, Z., Toumieh, G., Fatfat, M., Rahal, O. N. and Gali-Muhtasib, H., 2017. Emerging cytotoxic alkaloids in the battle against cancer: Overview of molecular mechanisms. *Molecules*, *22*, p. 250. doi: 10.3390/molecules22020250.

Hanahan, D. and Weinberg, R. A., 2011. Hallmarks of cancer: The next generation. *Cell*, *144*(5), pp. 646–674.

Heneidak, S., Grayer, R. J., Kite, G. C. and Simmonds, M. S. J., 2006. Flavonoid glycosides from Egyptian species of the tribe Asclepiadeae (Apocynaceae, subfamily Asclepiadoideae). *Biochemical Systematics and Ecology*, *34*(7), pp. 575–584. doi: 10.1016/j.bse.2006.03.001.

Ho, Y. T., Lu, C. C., Yang, J. S., Chiang, J. H., Li, T. C., Ip, S. W., Hsia, T. C., Liao, C. L., Lin, J. G., Wood, W. G. and Chung, J. G., 2009. Berberine induced apoptosis via

promoting the expression of caspase-8, -9 and -3, apoptosis-inducing factor and endonuclease G in SCC-4 human tongue squamous carcinoma cancer cells. *Anticancer Research*, *29*(10), pp. 4063–4070. doi: 29/10/4063 [pii].

Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N. and Sarkar, S., 2014. Drug resistance in cancer: An overview. *Cancers*, *6*(3), pp. 1769–1792. doi: 10.3390/cancers6031769.

Hu, X.-Q., Sun, Y., Lau, E., Zhao, M. and Su, S.-B., 2016. Advances in synergistic combinations of chinese herbal medicine for the Treatment of Cancer. *Current cancer drug targets*, *16*, pp. 346–356. doi: 10.1172/JCI9818.Use.

Huang, M., Lu, J.-J., Huang, M.-Q., Bao, J.-L., Chen, X.-P. and Wang, Y.-T., 2012. Terpenoids: natural products for cancer therapy. *Expert opinion on investigational drugs*, *21*(12), pp. 1801–1818. doi: 10.1517/13543784.2012.727395.

Izzo, A. A., 2012. Interactions between herbs and conventional drugs: Overview of the clinical data. *Medical Principles and Practice*, *21*, pp. 404–428. doi: 10.1159/000334488.

Jiang, H.-F., Wu, Z., Bai, X., Zhang, Y. and He, P., 2014. Effect of daphnoretin on the proliferation and apoptosis of A549 lung cancer cells *in vitro*. *Oncology Letters*, *8*(3), pp. 1139–1142. doi: 10.3892/ol.2014.2296.

De Jong, W. H. and Borm, P. J., 2008. Drug delivery and nanoparticles: Applications and hazards', *International Journal of Nanomedicine*, *3*(2), pp. 133–149.

Joos, S., Glassen, K. and Musselmann, B., 2012. Herbal medicine in primary healthcare in Germany: The Patient's perspective. *Evidence-based Complementary and Alternative Medicine*, *2012*, p. 10. doi: 10.1155/2012/294638.

Karimi, A., Majlesi, M. and Rafieian-Kopaei, M., 2015. Herbal versus synthetic drugs; beliefs and facts. *Journal of Nephropharmacology*, *4*(1), pp. 27–30. doi: 10.3724/SP.J.1042.2011.00175.

Komissarenko, N. F., Chernobai, V. T. and Komissarenko, A. N., 1995. New cardenolides from the leaves of Gomphocarpus fruticosus. *Chemistry of Natural compounds*, *31*(6), pp. 694–700.

Kushwaha, S. K. S., RastogI, A., Rai, A. k and Singh, S., 2012. Novel Drug Delivery System for Anticancer Drug: A Review. *International Journal of Pharmatech research*, *4*(2), pp. 542–553.

Labhasetwar, V., 2011. Drug resistance in cancer therapy. *Drug Delivery and Translational Research*, *1*, pp. 407–408. doi: 10.1007/s13346-011-0047-x.

Lee, D. H., Iwanski, G. B. and Thoennissen, N. H., 2010. Cucurbitacin: ancient compound shedding new light on cancer treatment. *The Scientific World Journal*, *10*, pp. 413–418. doi: 10.1100/tsw.2010.44.

Li, C. J., Chu, C. Y., Huang, L. H., Wang, M. H., Sheu, L. F., Yeh, J. I. and Hsu, H. Y., 2012. Synergistic anticancer activity of triptolide combined with cisplatin enhances apoptosis in gastric cancer *in vitro* and *in vivo*. *Cancer Letters*, *319*(2), pp. 203–213. doi:

10.1016/j.canlet.2012.01.006.

Li, J., Wang, X., Zhang, T., Wang, C., Huang, Z., Luo, X. and Deng, Y., 2015. A review on phospholipids and their main applications in drug delivery systems. *Asian Journal of Pharmaceutical Sciences*, *10*, pp. 81–98. doi: 10.1016/j.ajps.2014.09.004.

Lin, S. Z., Wei, W. T., Chen, H., Chen, K. J., Tong, H. F., Wang, Z. H., Ni, Z. L., Liu, H. Bin, Guo, H. C. and Liu, D. L., 2012. Antitumor activity of emodin against pancreatic cancer depends on its dual role: Promotion of apoptosis and suppression of angiogenesis. *PLoS ONE*, *7*(8), pp. 1–15. doi: 10.1371/journal.pone.0042146.

Lu, J.-J., Bao, J.-L., Chen, X.-P., Huang, M. and Wang, Y.-T., 2012. Alkaloids isolated from natural herbs as the anticancer agents. *Evidence-based complementary and alternative medicine :* eCAM, 2012, p. 12. doi: 10.1155/2012/485042.

Lu, Q., Zhang, P., Zhang, X. and Chen, J., 2009. Experimental study of the anti-cancer mechanism of tanshinone IIA against human breast cancer. *International Journal of Molecular Medicine*, *24*, pp. 773–780. doi: 10.3892/ijmm.

Ma, H., Fan, Q., Yu, J., Xin, J. and Zhang, C., 2012. Anticancer activities of tanshinone microemulsion against hepatocellular carcinoma *in vitro* and *in vivo*. *Molecular Medicine Reports*, *7*, pp. 59–64. doi: 10.3892/mmr.2012.1129.

Ma, W., Lu, S., Pan, P., Sadatmousavi, P., Yuan, Y. and Chen, P., 2012. Pharmacokinetics of Peptide Mediated Delivery of Anticancer Drug Ellipticine. *PLoS ONE*, *7*(8). doi: 10.1371/journal.pone.0043684.

Madureira, A. M., Ramalhete, C., Mulhovo, S., Duarte, A. and Ferreira, M.-J., 2012. Antibacterial activity of some African medicinal plants used traditionally against infectious diseases. *Pharmaceutical biology*, *50*(4), pp. 481–489.

Maggs, G. L., Craven, P. and Kolberg, H. H., 1998. Plant species richness , endemism , and genetic resources in Namibia. *Biodiversity and conservation*, *7*, pp. 435–446.

Marchi, S., Giorgi, C., Suski, J. M., Agnoletto, C., Bononi, A., Bonora, M., De Marchi, E., Missiroli, S., Patergnani, S., Poletti, F., Rimessi, A., Duszynski, J., Wieckowski, M. R. and Pinton, P., 2012. Mitochondria-Ros Crosstalk in the Control of Cell Death and Aging. *Journal of Signal Transduction*, *2012*, pp. 1–17. doi: 10.1155/2012/329635.

Maroyi, A. and Cheikhyoussef, A., 2015. A comparative study of medicinal plants used in rural areas of Namibia and Zimbabwe. *Indian Journal of Traditional Knowledge*, *14*(3), pp. 401–406.

Marzouk, A. M., Osman, S. M. and Gohar, A. A., 2016. A new pregnane glycoside from *Gomphocarpus fruticosus* growing in Egypt. *Natural Product Research*, *30*(9), pp. 1060–1067. doi: 10.1080/14786419.2015.1107059.

Mashele, S. and Kolesnikova, N., 2010. *In vitro* anticancer screening of *Asparagus laricinus* extracts. *Pharmacologyonline*, *2*, pp. 246–252.

Matsuura, K., Hatta, S., Terasaka, Y. and Inoue, Y., 2017. Extensive bilateral corneal edema 6 weeks after cataract surgery: Keratopathy due to *Asclepias physocarpa*: a case

report. *BMC Ophthalmology*, *17*, p. 5. doi: 10.1186/s12886-017-0400-z.

McIlwain, D. R., Berger, T. and Mak, T. W., 2015. Caspase Functions in Cell Death and Disease. *Cold Spring Harbor Perspectives in Biology*, *5*, p. a026716. doi: 10.1101/cshperspect.a026716.

Mehndiratta, S., Kumar, S., Meena, A. K., Koul, S., Suri, O. P. and Dhar, K. L., 2011. A review on plants a useful source of anti-cancer drugs. *Journal of Pharmacy Research*, *4*(1), pp. 264–271.

Mehta, A., Neha, J., Grobler, A. and Vandana, B., 2016. Role of Novel Drug Delivery Systems in Bioavailability Enhancement : At A Glance. *International Jouranl of Drug Delivery Technology*, *6*(1), pp. 7–26.

Meng, C., Zhu, H., Song, H., Wang, Z., Huang, G., Li, D., Ma, Z., Ma, J., Qin, Q., Sun, X. and Ma, J., 2014. Targets and molecular mechanisms of triptolide in cancer therapy. *Chinese journal of cancer research*, *26*(5), pp. 622–6. doi: 10.3978/j.issn.1000-9604.2014.09.01.

Mikkelsen, L. H., Hamoudi, H., Gül, C. A. and Heegaard, S., 2017. Corneal toxicity following exposure to *Asclepias tuberosa*. *The Open Ophthalmology Journal*, *11*, pp. 1–4. doi: 10.2174/1874364101711010001.

Moore, D. C., 2016. Drug-Induced Neutropenia: A Focus on Rituximab-Induced Late-Onset Neutropenia. *P & T*, *41*(12), pp. 765–768.

Mothana, R. A. A., Gruenert, R., Bednarski, P. J. and Lindequist, U., 2009. Evaluation of the in vitro anticancer, antimicrobial and antioxidant activities of some Yemeni plants used in folk medicine. *Pharmazie*, *64*(4), pp. 260–268. doi: 10.1691/ph.2009.8789.

Mothana, R. A., Al-Musayeib, N. M., Al-Ajmi, M., Cos, P. and Maes, L., 2014. Evaluation of the In Vitro Antiplasmodial, Antileishmanial, and Antitrypanosomal Activity of Medicinal Plants Used in Saudi and Yemeni TraditionalMedicine. *Evidence-based complementary and alternative medicine*, *2014*, p. 7. doi: 10.1055/s-0033-1351928.

Mothana, R. A., Lindequist, U., Gruenert, R. and Bednarski, P. J., 2009. Studies of the in vitro anticancer, antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from the island Soqotra. *BMC Complementary and Alternative Medicine*, *9*(7), pp. 1–11. doi: 10.1186/1472-6882-9-7.

Mudshinge, S. R., Deore, A. B., Patil, S. and Bhalgat, C. M., 2011. Nanoparticles: Emerging carriers for drug delivery. *Saudi Pharmaceutical Journal*, *19*, pp. 129–141. doi: 10.1016/j.jsps.2011.04.001.

Mukhtar, E., Adhami, V. M. and Mukhtar, H., 2014. Targeting Microtubules by Natural Agents for Cancer Therapy. *Molecular cancer Therapeutics*, *13*(2), pp. 275–284. doi: 10.1158/1535-7163.MCT-13-0791.

Mulware, S. J., 2013. Trace elements and carcinogenicity: a subject in review. *Biotechnology*, *3*, pp. 85–96. doi: 10.1007/s13205-012-0072-6.

Mwaka, A. D., Okello, E. S. and Orach, C. G., 2015. Barriers to biomedical care and use

of traditional medicines for treatment of cervical cancer: an exploratory qualitative study in northern Uganda. *European Journal of Cancer Care*, *24*, pp. 503–513. doi: 10.1111/ecc.12211.

Nirmala, M. J., Samundeeswari, a. and Sankar, P. D., 2011. Natural plant resources in anti-cancer therapy-A review. *Research in Plant Biology*, *1*(3), pp. 1–14.

Nowsheen, S., Yang, E. S. and Biology, I., 2012. The Intersection Between Dna Damage Response and Cell Death Pathways. *Experimental Oncology*, *34*(3), pp. 10–18.

Oyebode, O., Kandala, N.-B., Chilton, P. J. and Lilford, R. J., 2016. Use of traditional medicine in middle-income countries: a WHO-SAGE study. *Health Policy and Planning*, *31*, pp. 984–991. doi: 10.1093/heapol/czw022.

Pandey, M., Debnath, M., Gupta, S. and Chikara, S. K., 2011. Phytomedicine : An ancient approach turning into future potential source of therapeutics. *Journal of pharmacognosy and phytotherapy*, *3*(3), pp. 27–37.

Parrish, A. B., Freel, C. D. and Kornbluth, S., 2013. Cellular mechanisms controlling caspase activation and function. *Cold Spring Harbor Perspectives in Biology*, *5*, pp. a008672. doi: 10.1101/cshperspect.a008672.

Parrotta, J., Yeo-Chang, Y. and Camacho, L. D., 2016. Traditional knowledge for sustainable forest management and provision of ecosystem services. *International Journal of Biodiversity Science, Ecosystem Services & Management*, *12*(1–2), pp. 1–4. doi: 10.1080/21513732.2016.1169580.

Pawar, H. A. and Bhangale, B. D., 2015. Phytosome as a Novel Biomedicine : A Microencapsulated Drug Delivery System. *Journal of Bioanalysis & Biomedicine*, *7*(1), pp. 6–12. doi: 10.4172/1948-593X.1000116.

Periasamy, G., Teketelew, G., Gebrelibanos, M., Sintayehu, B., Gebrehiwot, M., Karim, A. and Geremedhin, G., 2014. Betulinic acid and its derivatives as anti-cancer agent: A review. *Archives of Applied Science Research*, *6*(3), pp. 47–58.

Perrone, D., Ardito, F., Giannatempo, G., Dioguardi, M., Troiano, G., Russo, L. L., Lillo, A. D., Laino, L. and Muzio, L. L., 2015. Biological and therapeutic activities, and anticancer properties of curcumin (Review). *Experimental and Therapeutic Medicine*, *10*, pp. 1615–1623.

Pina, S., Pedrosa, C., Santos, C., Feijóo, B., Pego, P., Vendrell, C., Santos, M. J. and Prieto, I., 2014. Ocular Toxicity Secondary to *Asclepias physocarpa* : The Balloon Plant. *Case Reports in Ophthalmological Medicine*, *2014*, pp. 1–5. doi: 10.1155/2014/829469.

Prakash, O., Kumar, A. and Kumar, P., 2013. Anticancer Potential of Plants and Natural Products : A Review. *American journal of pharmacological sciences*, *1*(6), pp. 104–115. doi: 10.12691/ajps-1-6-1.

Rahmatullah, M., Jahan, R. and Bashar, A., 2014. A review on Berbamine–a Potential Anticancer Drug. *World Journal of Pharmacy and Pharmaceutical Sciences*, *3*(8), pp. 95–110.

I: Xanthones, Quinones, Steroids, Coumarins, Phenolics and other Classes of Compounds. *Anti-cancer agents in medicinal chemistry*, *15*(9), pp. 1092–1111. doi: 10.2174/1871520615666150113110241.

De Smet, L., Ceelen, W., Remon, J. P. and Vervaet, C., 2013. Optimization of drug delivery systems for intraperitoneal therapy to extend the residence time of the chemotherapeutic agent. *The Scientific World Journal, 2013*, p. 7. doi: 10.1155/2013/720858.

Sri, A., 2016. Pharmacological Activity of Vinca Alkaloids. *Journal of Pharmacognosy and Phytochemistry*, *27*(3), pp. 27–34.

Stiborová, M., Černá, V., Moserová, M., Mrízová, I., Arlt, V. M. and Frei, E., 2015. The Anticancer Drug Ellipticine Activated with Cytochrome P450 Mediates DNA Damage Determining Its Pharmacological Efficiencies: Studies with Rats, Hepatic Cytochrome P450 Reductase Null (HRN^{TM}) Mice and Pure Enzymes. *International journal of molecular science, 16*, pp. 284–306. doi: 10.3390/ijms16010284.

Subramoniam, A., 2014. Present scenario, challenges and future perspectives in plant based medicine development. *Annals of Phytomedicine*, *3*(1), pp. 31–36.

Sun, M., Pan, D., Chen, Y., Li, Y., Gao, K. and Hu, B., 2017. Coroglaucigenin enhances the radiosensitivity of human lung cancer cells through Nrf2 / ROS pathway. *Oncotarget*, *8*(20), pp. 32807–32820.

Sundaraganapathy and Leena, P. N., 2016. Developmènt and evaluation of anti-cancer activity of phytosome formulated from the root extract of *Clerodendron paniculatum* Linn. *International Journal of Pharmacognosy and Phytochemical Research*, *8*(11), pp. 1778–1781. doi: 10.21276/ijprhs.2016.05.11.

Tagliamonte, M., Petrizzo, A., Napolitano, M., Luciano, A., Arra, C., Maiolino, P., Izzo, F., Tornesello, m, L., Aurisicchio, L., Ciliberto, G., Buonaguro, F, M. and Buonaguro, L., 2015. Novel metronomic chemotherapy and cancer vaccine combinatorial strategy for hepatocellular carcinoma in a mouse model. *Cancer Immunology, Immonotherapy, 64*, pp. 1305–1314.

Teixeira, F. M., Ramos, M. V, Soares, A. A., Oliveira, R. S. B., Almeida-Filho, L. C. P., Oliveira, J. S., Marinho-Filho, J. D. B. and Carvalho, C. P. S., 2011. *In vitro* tissue culture of the medicinal shrub *Calotropis procera* to produce pharmacologically active proteins from plant latex. *Process Biochemistry*, *46*, pp. 1118–1124.

Torrisi, J. M., Schwartz, L. H., Gollub, M. J., Ginsberg, M. S., Bosl, G. J. and Hricak, H., 2011. *CT Findings of Chemotherapy-induced Toxicity: What Radiologists Need to Know about the Clinical and Radiologic Manifestations of Chemotherapy Toxicity 1, Radiology*. Available at: www.rsna.org/rsnarights. (Accessed: 21 August 2018).

Wani, K. D., Kadu, B. S., Mansara, P., Gupta, P., Deore, A. V., Chikate, R. C., Poddar, P., Dhole, S. D. and Kaul-Ghanekar, R., 2014. Synthesis, characterization and *in vitro* study of biocompatible cinnamaldehyde functionalized magnetite nanoparticles (CPGF Nps) for hyperthermia and drug delivery applications in breast cancer. *PLoS ONE*, *9*(9). doi: 10.1371/journal.pone.0107315.

Warashina, T. and Noro, T., 1994. Steroidal glycosides from *Asclepias fruticosa* L. *Chemical and Pharmaceutical Bulletin*, *42*(2), pp. 322–326.

WHO., 2013. *WHO traditional medicine strategy 2014-2023*. Available at: http://apps.who.int/medicinedocs/documents/s21201en/s21201en.pdf (Accessed: 1 August 2017).

Wilken, R., Veena, M. S., Wang, M. B. and Srivatsan, E. S., 2011. Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. *Molecular Cancer*, *10*, p. 12.

Williams, G. H. and Stoeber, K., 2012. The cell cycle and cancer. *Journal of pathology*, *226*(October 2011), pp. 352–364.

Wu, W., Zu, Y., Wang, L., Wang, L., Wang, H., Li, Y., Wu, M., Zhao, X. and Fu, Y., 2017. Preparation, characterization and antitumor activity evaluation of apigenin nanoparticles by the liquid antisolvent precipitation technique. *Drug delivery*, *24*(1), pp. 1713–1720. doi: 10.1080/10717544.2017.1399302.

Yadav, N., Yadav, R. and Goyal, A., 2014. Chemistry of terpenoids. *International Journal of Pharmaceutical Sciences Review and Research*, 27(2), pp. 272–278.

Yang, Z. Y., Kan, J. T., Cheng, Z. Y., Wang, X. L., Zhu, Y. Z. and Guo, W., 2014. Daphnoretin-induced apoptosis in HeLa cells: A possible mitochondria-dependent pathway. *Cytotechnology*, *66*(1), pp. 51–61. doi: 10.1007/s10616-013-9536-8.

Yeap, S. K., Tamilselvan, S., Omar, A., Ho, W. Y. and Beh, B. K., 2012. A review of risk factors , incidence and solutions for hepatocellular carcinoma. *Scientific research and essays*, *7*(2), pp. 94–99. doi: 10.5897/SREX11.007.

Yin, S.-Y., Wei, W.-C., Jian, F.-Y. and Yang, N.-S., 2013. Therapeutic Applications of Herbal Medicines for Cancer Patients. *Evidence-Based Complementary and Alternative Medicine*, *2013*, p. 15. doi: 10.1155/2013/302426.

Zahreddine, H. and Borden, K. L. B., 2013. Mechanisms and insights into drug resistance in cancer *Frontiers in Pharmacology*, *4 MAR(March)*, pp. 1–8. doi: 10.3389/fphar.2013.00028.

CHAPTER 3. PHYTOCHEMICAL PROFILING, OF NAMIBIA'S *GOMPHOCARPUS FRUTICOSUS*, USING TLC AND GC-MS.

Florence Dushimemariya[1], Stefan Louw[2] & Davis R. Mumbengegwi[1*]

1. Multi-disciplinary Research Center, University of Namibia

2. Department of Chemistry and Biochemistry, University of Namibia

*Corresponding author: Davis R. Mumbengegwi; email: dmumbengegwi@unam.na

3.1. Abstract

Cancer cases are increasing globally not just in developed countries. In the past two decades, cancers were more prevalent in developed countries in comparison to developing regions and this was attributed to lifestyles characterized by delayed childbirth, inactive lifestyle leading to obesity, unhealthy eating habits and exposure to tobacco smoke or coal mining. However, a shift in cancer incidences has been observed during the same period with an increasing burden occurring in developing countries because of more westernized lifestyles. With limited treatment options for cancer, medicinal plants represent an avenue that has not been fully explored for bioactive leads, despite there being potent plant-derived anticancer drugs such as vincristine and vinblastine in clinical use. In this study the presence of phytochemicals in *Gomphocarpus fruticosus* was investigated using thin layer chromatography (TLC) and gas chromatography-mass spectroscopy (GC-MS). Extracts were prepared using four solvents, to target chemicals of varying polarities. The methanolic, aqueous, dichloromethane and chloroform extracts were screened for the presence of alkaloids, anthraquinones, coumarins, flavonoids, steroids and triterpenoids.

TLC showed that the chloroform and dichloromethane extracts were positive for the presence of all selected classes of phytochemicals. The aqueous extract showed the presence of polar phytochemicals such as anthraquinones and flavonoids whilst the methanolic extract showed the presence of all selected phytochemicals, except coumarins. GC-MS analysis revealed the presence of carboxylic/fatty acids, triterpenols including α-amyrin, lupeol and their esters as well as a phytosterol, β-sitosterol. These compounds were reported to possess various biological activites such as anti-inflammatory, anticancer and analgesic properties. The presence of fatty acids and other phytocomponents rationalize the use the *G. fruticosus* for various purposes, including cancer treatment and its mode of preparation and administration. Observations in this study corroborate the traditional use of *G. fruticosus* and supports the need for further attention towards this plant as a candidate herbal treatment for cancer treatment and palliation.

Key words: GC-MS, *G. fruticosus*, Phytochemicals,

3.2. Introduction

Medicinal plants are gaining attention as sources of bioactive molecules active against many diseases, especially since the initial discovery of the vinca alkaloids from the Madagascar periwinkle plant, *Catharanthus roseus* (Nirmala, Samundeeswari and Sankar, 2011). Various plant derived anticancer compounds are currently in clinical use, including drugs such as paclitaxel, vincristine, vinblastine, ellipticine. However, the search for better anticancer drugs continues and its being driven by high incidences of treatment related toxicities associated with available cancer treatments, therapy resistant cancers and poor

aqueous solubility of drugs in the current development pipeline (Nirmala, Samundeeswari and Sankar, 2011; Rebucci and Michiels, 2013).

Traditional medicinal plants that have been used successfully in local communities have been validated in the laboratory as having pharmacological activity based on the presence of bioactive compounds contained in extracts from the plants or even proof of activity against disease causing pathogens (Subba, Srivastav and Kandel, 2016). These bioactive compounds are secondary metabolites and have been shown to have number of anti-proliferative mechanisms in cancerous cells. Secondary metabolites such as alkaloids, phenols, steroids, terpenoids, saponins have been reported to possess anticancer properties (Nirmala, Samundeeswari and Sankar, 2011). Scientific reports on traditional plants with anticancer activity have contributed to cancer patients using them together with clinically prescribed treatments (Moreira *et al.*, 2014). Generally, medicinal plants with a long history of usage are perceived to be safe but the WHO recommends the determination of the toxicity profiles of traditional herbal treatments. This is especially important because traditional remedies are being taken concomitantly with conventional drugs (Moreira *et al.*, 2014) and there is a risk that harmful drug-drug interactions can occur (Clarke and Mclachlan, 2011; Izzo, 2012). It is therefore imperative that traditional medicinal plants be assayed to determine potential toxicities and used to support their use in meeting the primary health care needs of people in resource poor communities (WHO, 2013).

Gomphorcarpus fruticosus is a traditionally known plant, especially for its toxic properties. It has a wide distribution around central Namibia, where it is often seen along the roadside and riverbeds. *G. fruticosus* grows to a height between 0.5m to about 4 m. It is characterized by the milky latex which exudes its tissues when injured. Locally, *G. fruticosus* is known commonly as the milkweed or wild cotton in English. It has several names in Namibia's local languages. It is known in Afrikaans as *melkbos, tontelbos* or *wilde kapok,* whereas, in Otjiherero, it is called *oruseppa.* In Khoekhoegowab/Nama, it is called *||giitama||oob* and *Wilde baumwolle* in German. *G. fruticosus* has various uses traditionally in Namibia and other neighboring countries. According to the Survey for Economic Plants of Arid and Semi-Arid Lands (SEPASAL), it is used for fuel, animal folder, poison used in hunting, as an ornamental plant and as a source of medicine. The Bergdamara/bergdama/berdamara/damaqua/berdamara people in Namibia use crushed leaves to make a tea capable of curing cancer, especially when the tea prepared from crushed leaves is taken orally and used as a bath, which can be coupled with topical application of pulverized leaves (von Koenen, 2001). The treatment is then followed by wrapping body in a blanket. Further medicinal claims in Namibia and elsewhere include anti-TB, anti-duiretic, anti-diarrhea or even as a tranquilizer (von Koenen, 2001). However, poisonous effects in small livestock have been reported, which can lead to death, when large quantities of the leaves are ingested.

The aim of this study was to determine the phytochemical profile of *Gomphorcarpus fruticosus* extracts using TLC, and further explore the methanolic extract for bioactive compounds using Gas Chromatography-Mass Spectroscopy.

3.3. Methods and materials

3.3.1. Plant collection and processing

Gomphocarpus fruticosus was collected in the Otjozondjupa region of Namibia using a plant collection form, Appendix A, **figure 3A**. A voucher specimen, FD14, was prepared in a plant press, for identification and deposited with the herbarium at the National Botanical Research Institute (NBRI). The aerial/ above ground parts including leaves, fruiting structures and stalks were harvested and placed in brown paper bags. Plant material was sorted to remove debris before being cut into pieces (1^{-2}-2^{-2} m) to facilitate drying on an open bench top for 3 weeks. Dry plant material was then ground into a powder using an industrial miller. The ground plant material was labelled and stored at -20 °C until needed.

3.3.2. Plant extract preparation

Crude plant extracts were prepared by maceration of powdered plant material (10 g) and 1^{-1} L solvent. Distilled methanol, dichloromethane, Chloroform or water at room temperature were used as solvents for a period of 24 hrs. After the maceration period, extracts were filtered through a Whatman filter paper, using gravity filtration. Filtration was followed by rotary evaporation (Heliodoph) using a water-bath set at 333.15 K, pressure set at 7000 Pa. To further remove traces of extraction solvent, extracts were lyophilized on a freeze dryer for at least 4 hrs or until they could easily be scrapped off the round bottom flask wall. Dry extracts were weighted and stored in air-tight containers at 253.15 K until further use.

3.3.3. Phytochemical analysis

Phytochemical analysis using thin layer chromatography was conducted on all prepared plant extracts. Methods used in this analysis were based on literature publication by Harborne (1998). Dry plant (10 g) extracts was reconstituted in (1^{-3} L) relevant extraction solvent before analysis. **Table 3.1**, shows the selected phytochemicals analyzed, the corresponding mobile solvents and chromogenic reagents. Aluminium sheets precoated with silica 60 (Merck), with dimensions: $2^{-1} \times 2^{-1}$ m, were used in the analysis. The dissolved plant extracts ($\approx 7^{-5}$ L) was spotted on a marked spot on the line of origin using a capillary tube and allowed to dry before placing the silica plate into a mobile solvent saturated tank. At the end of the experiment, the mobile solvent front was marked with a soft pencil. Chromogenic reagents were used to visualize different spots on the plate and were recorded as either present of absent per plant extract.

Table 3.1. Chromogenic reagents and mobile solvents used for selected phytochemicals.

Phytochemical	Mobile phase	Chromogenic reagent	Positive control
Alkaloid	Chloroform: Ethanol (9:1)	Dragendorrf's reagent	Quinine
Anthraquinone	Ethyl acetate: Methanol:Water 100:17:13	10% Potassium hydroxide in methanol	Alizarin
Coumarin	Chloroform	1.3 g Copper sulphate, 17.3 g Sodium Citrate, 10 g anhydrous sodium carbonate, 100 ml water	Coumarin
Flavonoid	Butanol: Acetic acid: Water 4:1:5	10% Antimony chloride in chloroform	Quercetin
Terpenoid	Hexane: Ethyl acetate 17:3	Liebermann reagent	β-sitosterol

Steroid	Chloroform: Acetone 9:1	Aqueous Phosphoric acid and methanolic phosphoric acid, heat plate at 120 °C	β-sitosterol

3.3.4. GC-MS analysis

Plant extracts prepared from the methanolic maceration of the shoot plant material of *G. fruticosus* were analyzed using Gas Chromatography Mass spectroscopy (GC-MS). Briefly, the stored methanol extract was reconstituted in methanol at 1 g/L. The extract was sonicated in a water bath set at 298.15 K for 30 min, to ensure complete dissolution. For the GC-MS analysis, 1^{-5} L of the extract was injected at a split ratio of 1/10, using a Thermo Scientific focus GC coupled to an ITQ 700 MS. Xcalibur software, version 2.1, was used for data acquisition. A column coated with 5% phenyl, 95% dimethylpolysiloxane (film thickness of 2.5^{-7} m) with the following dimensions: 30 m × 2.5^{-4} m I.d. (Thermo Scientific) was used for the chromatography. Chromatographic conditions were as follows: gas carrier was helium at a flow rate of 1^{-3} L/min. The injector was operated at 493.15 K, while the oven temperature was programmed as 313.15-573.15 K at a rate of 275.15 K per min and held for 50 min. Mass spectrometer conditions were as follows: Ionization voltage of 70 eV, ion source temperature of 523.15 K, with an interface temperature of 523.15 K, mass range of m/z 25 to 625. The chemical compounds within the methanolic extract were tentatively identified by comparison of the mass spectra to those in the National Institute of Standards and Technology (NIST) 11 mass spectra data base. The identities of the compounds were further supported by comparison of their Kovatz retention indices (RIs) to those in NIST 11 RI database. Kovatz retention index for each compound was calculated based on the elution time of a series of even

alkanes, C_{10}-C_{40}, analyzed under the same GCMS conditions as the plant extracts according to Lucero *et al.*, (2009). $RI = 100[n + (N - n)((\log RTunknown - v) - (\log RTsmaller\ alkane - v)/(\log RT\ larger\ alkane - v) - (\log RT\ smaller\ alkane - v))]$

Where n is the length of the smaller alkane, N is the length of the larger alkane, RT is the retention time in min and v is column void time, i.e. 1.32 min.

3.4. Results and discussion

Phytochemicals are secondary metabolites produced by plants as a survival mechanism but are not essential for growth. They can be used to deter herbivores, microbes or even other plants, or as a means to adapt to the plant's harsh environment. In other instances, plants produce phytochemicals as a response to plant pathogens (Kennedy and Wightman, 2011). These phytochemicals can be exploited as bioactive molecules with various applications such as treating different illness. In this study, selected phytochemicals relevant for cancer therapy were investigated in four different extracts produced from the ground shoot plant of *G. fruticosus*. **Table 3.2** shows the selected phytochemicals found in solvents of varying polarities.

Table 3.2. Phytochemical analysis of *G. fruticosus* extract for selected bioactive compounds using TLC.

Phytochemical	**Solvent**			
	Chloroform	**Dichloromethane**	**Methanol**	**Water**
Alkaloid	+++	++	+++	-

Anthraquinone	++	+++	++	+++
Coumarins	++	+	-	-
Flavonoids	+	++	+	+
Steroids	++	+++	++	-
Terpenoids	+	++	+	-

Key: +++ = Intense presence, ++ = Moderate presence, + = Low presence, - = Absent

The order of polarity begins with chloroform as the least polar solvent, followed by, dichloromethane, methanol and finally water as the most polar solvent. The chloroform and dichoromethane extracts showed the presence of all selected phytochemicals. The aqueous extract showed the presence of anthraquinones and flavonoids only. The methanolic extract showed the presence of anthraquinones, steroids, terpenoids and flavonoids. Anthraquinones and flavonoids were found in all solvents used, regardless of polarity. Alkaloids, coumarins, steroids and triterpenoids were found in less polar solvents, indicating that non-polar solvents were suitable for extracting these compounds. The findings in this study revealed alkaloids in methanolic extract, which is different from another study conducted in Kenya (Omwenga, Okemo & Mbugua, 2012). Alkaloids from plant sources are known to confer several useful biological properties, including chemo-protective properties, antimicrobial properties, antioxidant and anticancer activity (Kaur and Arora, 2015). Flavonoids, coumarins and anthraquinones all belong to a class of compounds called phenolics. In a report by Heneidak *et al.*, (2006), flavonoids were detected by HPLC in the 80% aqueous methanolic extract prepared from *G. fruticosus* stems. These included quercetin, isorhamnetin, kaempferol and their 3-O-β-rutinosides (Heneidak *et al.*, 2006). Another report cites the presence of glycosides, steroidal glycosides and 5,11- epoxymegastigmanes from polar extracts from the leaves of *G. fruticosus* (Abe & Yamauchi 2000).

Phenolics are among important group of phytochemicals found in plants, with structural diversity, which contributes to its diverse pharmacological actions in man (Ghasemzadeh and Ghasemzadeh, 2011). This structural diversity also increases opportunities to discover useful compounds for development as drugs. The reported pharmacological actions exhibited by members of the phenolics family have become of interest to the scientific community, with their collective members possessing antioxidant activity. Coumarins, submembers of the phenolics family, possess a myriad of biological properties, including, antioxidant, antimicrobial, anticancer, anti-inflammatory properties, among others (Chikezie, Ibegbulem and Mbagwu, 2015). Flavonoids are secondary metabolites known to exhibit antiviral, anticancer, anti-inflammatory, antioxidant, protective effects against diseases of the cardiovascular system and liver-protective effects against toxin damage (Kumar and Pandey, 2013). The results found in this study are consistent with previous reports of *G. fruticosus* possessing flavonoids (Heneidak *et al.*, 2006). In another study conducted in Kenya (Omwenga, Okemo & Mbugua, 2012), also collaborated these observations as flavonoids were found in the methanolic extract presumably prepared from whole ground plant material. In addition, several species belonging to the Apocynaceae family also demonstrated various phytochemicals in different solvents (Joselin *et al.*, 2012).

Terpenoids were detected in all extracts, except that prepared using water. Whereas terpenoids were observed in this study, another study by Omwenga and colleagues found the contrary (Omwenga, Okemo & Mbugua, 2012). No terpenoids were detected in the methanolic extract prepared from *G. fruticosus*. In a study conducted on Yemen *G.*

fruticosus, terpenoids were detected in the methanolic extract (R. A. Mothana *et al.*, 2009). Terpenoids have been shown to possess various biological effects, which are being exploited, including antiviral, antifungal, antiparasitic, anticancer properties and as an anodyne (Sülsen *et al.*, 2017). Terpenoids are commonly found in members of the Apocynaceae family (Marzouk et al. 2016; Mothana et al. 2009). As members of the terpenoids, triterpernoids possess antimicrobial, anti-inflammatory (Chikezie, Ibegbulem and Mbagwu, 2015) activity. In a study on Egyptian grown *G. fruticosus*, triterpenoids such as betulinic acid, 3β-taraxerol, 3β- taraxerol acetate were isolated (Marzouk, Osman and Gohar, 2016). A number of triterpenoids such as alisol, pachymic acid, cucurbitacins and celastrol have been demonstrated to confer actions such as anti-angiogenesis, apoptosis induction, cell cycle arrest, autophagy, all which contribute to their anticancer activity (Huang *et al.*, 2012).

Similar to triterpenoids, steroids were detected in all prepared extracts, except the aqueous extract. This finding is also supported by a report by Warashina and Noro, (1994), which reported the presence of steroidal glycosides from the methanolic extract prepared from shoot plant material of *G. fruitcosus*.

Water displayed limited extractive capacity of selected phytochemicals, in comparison to the other three solvents, **table 3.2**. The aqueous extract was only able to extract anthraquinones and flavonoids, among all six selected phytochemicals. In contrast, solvents such methanol and dichloromethane are better able to extract a wide range of

compounds (polar and semi-polar) from plant material, **table 3.2**. In addition, organic extracts usually exhibit more biological action in comparison to their aqueous equivalents (Fouche *et al.*, 2008). Moreover, organic solvents often have lower boiling points, which preserves the integrity of plant compounds, as some extracts may contain heat labile compounds. This is because water is a polar solvent, which limits its extractive power. Consequently, the methanolic extract was explored further because methanolic extracts have a consistent extractive power/potential, which is important for reproducibility of results.

Gas chromatography coupled with mass spectroscopy (GC-MS) was used to identify the volatile and semi-volatile compounds in the methanolic plant extract derived from powdered shoot plant material of *G. fruticosus*. The GC-MS analysis revealed the presence of 33 prominent compounds, **figure 3.1**. Tentative identities of the compounds were done by comparison of mass spectra to those in the NIST 11 mass spectra data base. Furthermore, the calculated retention indices were compared to those in the NIST RI database and other literature sources, which further added confidence to the tentative identities of the compounds. The extract consisted of carboxylic acids, triterpenoids, and their ester derivatives. Retention index comparisons matched with literature values for a similar column, except for compounds **3, 6-8, 10, 16-17, 20**.

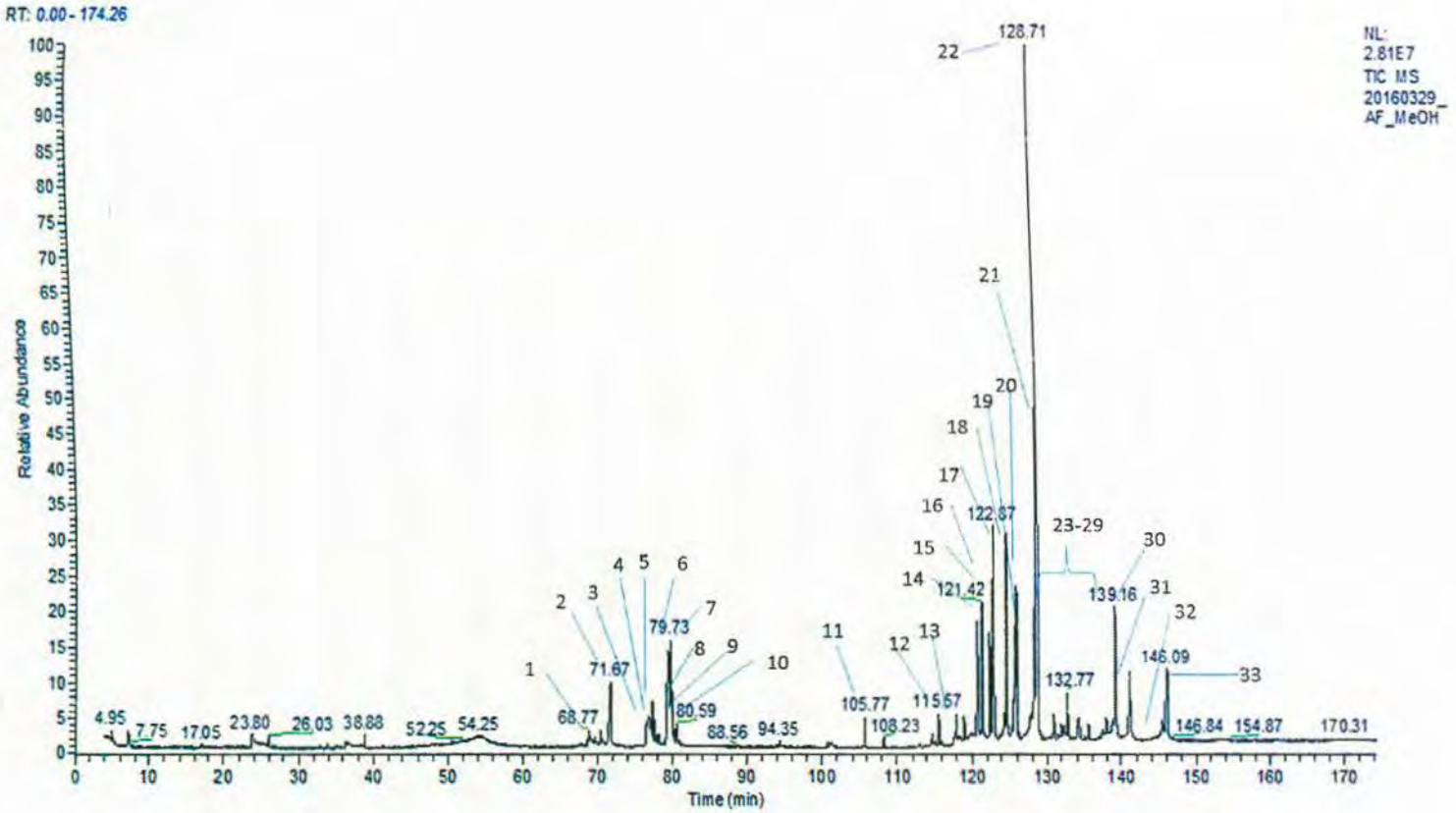


Figure 3.1. Total ion chromatogram of the methanolic extract of *G. fruticosus*. Highlighted peaks denote (**2**) Hexadecanoic acid, (**11**) Squalene, (**14**) Unknown triterpenoid, (**16**) α- amyrin, (**17**) Lupeol and (**15, 18, 21-33**) group of unknown triterpenols and their derivatives

Several triterpenes were identified in the methanolic extract of *G. fruticosus*, eluting in the middle of the chromatogram, **figure 3.1**. Compound **11** was assigned tentative identity as squalene, which eluted at 105.77 min. It is an important precursor in the synthesis of cholesterol in plants, animals and humans. Mass spectra data which aided this assignment were its fragmentation pattern. Its molecular ion a m/z 410 was observed, in addition to peaks at m/z 55, 69, 81, 95, 121, 136, 149, 203, 231, 259, 299, 341 and 367 (Dande, Bonde and Pandita, 2013). However, in literature, it is noted that the base peak is indicated as either 55 or 69 (Dande, Bonde and Pandita, 2013), which is contrary to observation in this study, base peak m/z 81. Squalene is used as a calmative in cosmetic products, being able to relieve eczema, aged skin and hair (Popa *et al.*, 2014), especially for squalene outsourced from plant sources (Popa *et al.*, 2014). This is also in part to squalene's role as a UV-radiation protector (Kostyuk *et al.*, 2012). Additionally, squalene is conferred to possess antioxidant properties (Huang, Lin and Fang, 2009). In addition, squalene continues to be used traditionally for the treatment of various ailments. Literature attributes the beneficial effects of consuming a diet rich in squalene such as olive-oil rich meals to low incidences of cardiovascular diseases (Popa *et al.*, 2014). In the pharmaceutical industry, squalene has gained popularity as ad adjuvant used in several vaccines (Sayers *et al.*, 2012) and an aid in the formulation of parenterally delivered drugs (Fox, 2009). Evidence also shows several articles indicating the protective effect of squalene from chemotherapeutic drugs such as cisplatin, carboplatin (Das *et al.*, 2008) and doxorubicin.

Compound **16**, eluting at 122.74 was identified as α-amyrin. Key mass chromatogram features used for this identification were the distinct base peak at m/z 218.12 (Amorim, 2016; Gasca *et al.*, 2017). However, no molecular ion was visible, as was expected at m/z 426 (Amorim, 2016). This may be due to the instability of the resulting cation. α-Amyrin is a triterpenoid isolated from plant sources and has various biological properties. It has been isolated from various plant sources, and together with β-amyrin, can be found in resin, leaves, bark and woody plant material. The biological activity of α-amyrin includes antioxidant, anti-inflammation and anticancer action against several cell lines. In addition, α-amyrin has been known to confer antibacterial and antifungal activity. In a review by El-Kashef and colleagues, several triterpenoids and sterols were discussed including α-amyrin, its acetate derivative, in members of the Apocynaceae family (El-Kashef *et al.*, 2015). The acetate derivative of α-amyrin, compound **19**, Urs-12-en-3-ol, acetate, (3a)- is also reported to possess antibacterial activity, in addition to other properties such as anesthetizing potential (Ananthi and Kumari, 2013). Its mass chromatogram presented with molecular ion at m/z 468.13, in addition, its bass peak was clearly visible at m/z 218.11, another peak at m/z 203 (Amorim, 2016). In addition to a peak appearing at m/z 408.20, corresponding to loss of the acetate group from the molecule, M: CH_3COOH.

Compound **17** was identified as lupeol, eluting at 122.87 min. Diagnostic ions leading to this identification were molecular ion at m/z 426 and other peaks characteristic of lupeol at m/z 411, 393, 383 (De Carvalho *et al.*, 2010; Suttiarporn *et al.*, 2015). Lupeol is a common triterpene found in various plants, including fruits and vegetables. lupeol and its derivatives possess several biological properties, including anti-inflammatory modulation,

antiparasitic (antiplasmodial and antileishmania), antitumor and antimicrobial activity against both gram positive and negative laboratory strains (Wal *et al.*, 2015), lupeol has been shown to induce the expression of Fas receptors in prostate cancer cells, leading to apoptosis, in addition to its well-known inhibition of the topoisomerase II enzyme (Wal *et al.*, 2011). Furthermore, Wal *et al.*, (2011) discussed the pro-protective effects of lupeol from damage induced by carcinogenic toxins such aflatoxin. Similarly, lupeol was found to be instrumental in preventing tumorigenesis induced by 7,12-dimethylben(a)anthracene in the oral cavity, *in vivo* (Palanimuthu *et al.*, 2012). Furthermore, evidence shows that the acetate derivative of lupeol, compound **20**, Lup-20(29)-en-3-ol, acetate eluted at 125.92 min. The compound showed mass spectra corresponding to Lupeol acetate in literature. A molecular ion was observed at m/z 468, followed by other ions at m/z 408.20, 218.14, 204.14, and base peak at 189.15 (Calixto *et al.*, 2017). Literature search shows that compound **17** has anti-inflammatory potential (Kadhim, Sosa and Hameed, 2016).

Table 3.4 and **figure 3.1** shows a collection of 16 compounds that could not be identified sufficiently. Some of the compounds demonstrated no visible molecular ion. Compound **14** demonstrated no noticeable molecular ion, but demonstrated two distinct peaks at m/z 203 and 218, which were of almost equal intensity. Compound **14** demonstrated a molecular ion at m/z 426, with two unique peaks at m/z 133 and 287. Compounds **18, 23** and **26** demonstrated similar diagnostic peaks with compound **14**. Distinct molecular ions are observable at m/z 468, 481 for compounds **18** and **23** respectively. After the molecular ion, distinct peaks are observable probably corresponding to the loss of acetic acid M-60 for compound **18** and M-88 (butanoic acid), for compound **23**. No distinct peak is

observed corresponding to the anticipated loss of pentanoic acid from compound **26**, M-93. On the other hand, compounds **25, 27, 30** and **31** had a prominent base peak at m/z 218. Molecular ions for these four compounds are observable at m/z 496, 510 for **25** and **27**, while m/z 522 seems to be the molecular ion for compounds **30** and **31**. Compounds **21, 22, 24, 28** and **29** shared a similar feature of a distinct peak at m/z 189. Furthermore, compounds **21** and **22** both demonstrated a molecular ion at m/z 468 and both showed a peak corresponding to the loss of acetic acid from the parent structure, M-60. For compound **24**, no noticeable peak was seen corresponding to the loss of propanoic acid, M-74, while **28** and **29** also did not show the expected loss of the butanoic (M-88) and pentanoic acid (M-93) respectively. Finally, compounds **32** and **33** had similar mass spectra patterns. No distinct molecular ion was visible for both these compounds. However, they both demonstrated a prominent m/z 189, with another distinct peak at m/z 409, which could not be explained. The two peaks did not have a similar abundance. The mass spectra were similar to that of pentacyclic triterpenols.

The majority of compounds tentatively identified in this study were triterpenoids, whereas betulinic acid, 3β-taraxerol and its acetate derivative are the only literature sightings of triterpenoids isolated in *G. fruticosus* (Marzouk, Osman and Gohar, 2016). Further research is required to confirm the identities of these compounds using appropriate standards, analyzed under similar experimental conditions. In this study, about 16 compounds could not be identified due to the limitations imposed by the NIST mass spectra data base. We recommend the use of additional techniques such as High Pressure-Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectroscopy

(LCMS). This is to further determine a complete array of chemicals found in Namibian *G. fruticosus*, since GCMS often requires derivatization for optimal detection (Ruiz-Matute *et al.*, 2011) while masking is also possible (Scalbert *et al.*, 2009).

Table 3.3 also shows the different compounds identified as carboxylic or fatty acids and their esters. Carboxylic acids included compounds **1, 2, 6, 7, 8** and **10**. These included two compounds consisting of 16 carbons each, **1** (branched hexadecanoic acid) and its unbranched compound, **2**, which are isomers and were saturated. Both showed a similar mass spectra with diagnostic ions at m/z 55, 73, 87,115, 129, 157, 185, 213, 227. Most importantly, the molecular ion was visible at m/z 256 as well as base peak at m/z 87 (Moussa and Almaghrabi, 2016). Compounds **6** was identified as octadecadienoic acid, a polyunsaturated carboxylic acid. Diagnostic mass spectra was similar to that observed in literature (Liu *et al.*, 2016). The molecular ion was clearly visible at m/z 280.09. Three additional diagnostic ions were observed at m/z 150.10, 95.10, including base peak at m/z 67.06, similar to 9,12-octadecadienoic acid (Liu *et al.*, 2016). Compounds **7** and **8** contained one double bond, and the carbon chain was 18 carbons long. Both compounds had the same molecular weight, 282 Da. The molecular ion, (M^+), was not easily visible in both compounds. However, the presence of other diagnostic ions such as m/z 55, 83, 97 and 264 contributed to the tentative identification for compound **7**, despite the base peak not being observed at m/z 55 as expected (Zayed, Abd El-Kareem and Zaky, 2017) but rather at m/z 79, in this study. On the other hand, compound **8** demonstrated diagnostic ionization mass spectra fragments similar to published literature report of oleic acid. The base peak was observed at m/z 55, in addition to fragmentation peaks at m/z 69, 83, 97,

264. A peak corresponding to the loss of water, ($[M-18]^+$, m/z =264) was clearly visible in both compounds, while the ion formed by the McLafferty rearrangement, expected to be observed at m/z 60, (HC=C(OH)-OH) had less abundance than expected. Compound **10**, eluting at 80.59 was identified as the unsaturated analogue of compounds **7** and **8**, since it also had a carbon chain consisting of 18 carbons. Fragmentation peaks leading to this assignment include the observation of a peak at m/z 255, corresponding to the loss of an ethyl radical (29 Da). The McLafferty rearrangement ion is also visible at m/z 60, also not highly pronounced. Literature indicates the presence of fragmentation ions observable at m/z 55, 57,60, 69, 71, 73 and molecular ion at m/z 284 as defining features of octadecanoic acid (Aali *et al.*, 2018). Base peak is expected at 73, which contradicts observations in this study, since base peak was observed at m/z 55 (Aali *et al.*, 2018). Among the compounds identified in the methanolic extract of *G. fruticosus*, 4 esters were found. These esters comprised of compound **4** (Methyl octadecadienoate), compound **5** (Methyl octadecenoate) and compound **9** (Ethyl octadecadienoic acid). Compound **4** was identified on the basis of its fragmentation pattern, producing its molecular peak at m/z 294, with additional peaks, and abundance patterns similar to 9, 12-octadecadenoic acid, methyl ester (Zayed et al, 2017). Its base peak was easily distinguishable at m/z 67 and other peaks at m/z 55, 81, 95 (Zayed et al, 2017). The presence of diagnostic ions such peaks at m/z 264, 222, 97, 55 and molecular ion at m/z 296 were used to identify compound **5** as a methyl ester of octadecenoic acid. A report shows these as distinguishing fragmentation ions of 8-octadecenoic and 10-octadecenoic acids (Zayed et al, 2017). However, prominent peaks such as m/z 74 and 69 were absent from the mass spectra in this study. Compound **9** was identified as an ethyl ester due to the observation of the peak at m/z 262, corresponding the loss of ethanol from the parent compound. Furthermore, the

presence of the peak at m/z 279, corresponding to the loss of the ethyl radical ($\cdot CH_2CH_3$), which is characteristic of carboxylic acids. In addition, fragmentation pattern similar to a literature example, 9,12-octadecadienoic acid ethyl ester, with peaks at m/z 41,55,67,81, 95, 109, 123, 136, 150, 164 and 178 were observed in this study, despite the column being different (Igwe and Okwu, 2013). These fatty acids are commonly seen in plant derived extracts during GC-MS, especially in methanolic/ alcoholic extracts and most of these fatty acids are reported to possess biological activity. The presence of fatty acids in *G. fruticosus*, especially those that are polyunsaturated may explain its action (treatment for cancer) and mode of delivery (topical application of pulverized leaves) (von Koenen, 2001), since fatty acids facilitates the absorption of active principles through the skin and mucosa membranes (Loftsson *et al.*, 2016). In addition, carboxylic acids are cited to possess anti-inflammatory and anticancer actions, which are all relevant in herbal candidates for cancer therapy and palliation.

Compound **12** was identified as phenolic compound α-tocopherol, eluting at 115.57 min. This compounded had a molecular ion at m/z 430.22, the next diagnostic ion was seen at m/z 205.10, which corresponds to the loss of the long alkyl side chain on the structure. This was confirmed as a fragmentation pattern expected for ɣ-tocopherol (López-Cobo *et al.*, 2017). Other diagnostic ions were observed in this study at m/z 121.11, 165.08 and the molecular ion at 430.22 were observed similar to another report by Saha *et al.*, (2015). α-Tocopherol is also known as Vitamin E, has several other isomers, including β-tocopherol, ɣ-tocopherol, of which, α-tocopherol has the highest relative abundance in plants, in comparison to the other isomers. α-Tocopherol is cited as possessing antioxidant

activity (Franco *et al.*, 2014), which is beneficial in the prevention of cancer development. Yu *et al.*, 2010, described the anti-proliferative and pro-apoptotic activities vitamin E, it reduced the proliferation and increased apoptosis in human breast cancer cells, implanted in mice. In fact, evidence suggests that not only is α-tocopherol pro-apoptotic but that other isomers of the molecule also possess anticancer effects (Constantinou, Papas and Constantinou, 2008). As such, tocopherol is proposed for consideration as an adjuvant for cancer therapy. In spite of the beneficial properties of α-tocopherol, it blocks the effects of protein kinase inhibitors, which can lead to treatment failure when it is taken concomitantly with anticancer drugs as a supplement (Pédeboscq *et al.*, 2012).

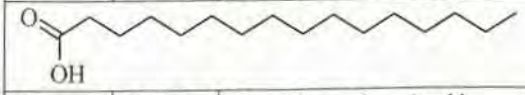
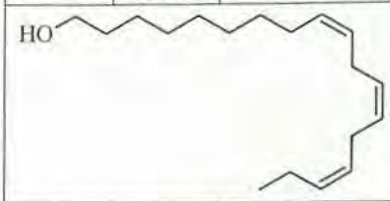
Compound **13** was identified as β-sitosterol or stigmast-5-en-3β-ol and eluted at 120.72 min, **table 3.3**. Its molecular formula ion was easily distinguishable at m/z 414.20. Other diagnostic ions were consisted with expectations for this compound such as m/z 105, 145, 178, 213, 255, 329 and 381, exception being the m/z 57 peak, which was not observed in this study (Saha *et al.*, 2015). Another important ion was observed at m/z 273, corresponding to M:141, the loss of the side chain (Kamboj and Saluja, 2011). β-Sitosterol is a phytosterol, which is structurally similar to cholesterol. It's among the top three phytosterols, contributing the greatest proportion to human diet. In a review by Saeidnia *et al.*, (2014), β-sitosterol is described to have biological activity able to bring about beneficial effects against cancer, inflammation, pain relief, chemoprevention, anti-angiogenic, antioxidant, anti-diabetic effects, anti-parasitic activity, anti-cholesterol, immuno-modulating effects among others. The chemo-preventative effects of β-sitosterol were further demonstrated in both *in vitro* and *in vivo* disease models against colon cancer

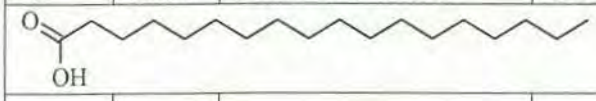
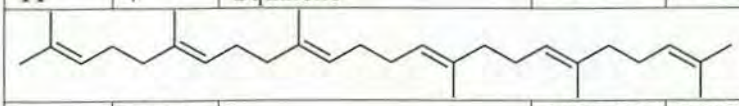
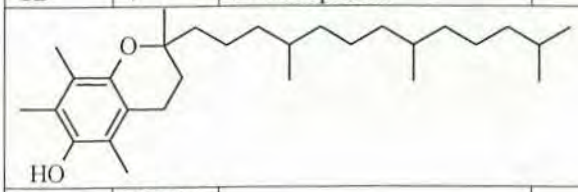
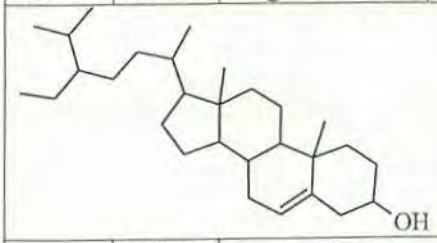
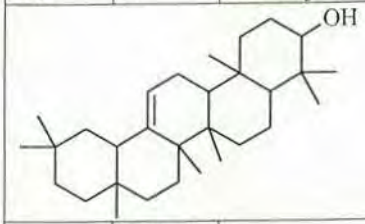
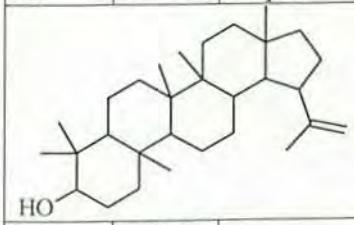
(Baskar *et al.*, 2010). β-sitosterol is known to be anti-cancerous against several cell-lines through its role of inducing apoptosis, preventing angiogenesis, antioxidant/anti-inflammatory modulation (Baskar *et al.*, 2010). Despite the published data on the biological effects of β-sitosterol, the compound has not been endorsed as a known anticancer drug. Consumption of β-sitosterol containing food stuff has been found to contribute to the presence of Estrogen receptor breast cancer tumors (Grattan, 2013). In cancer therapy, ER positive tumors are readily responsive to anticancer treatments while the ER negative tumors are generally resistant. Its therefore, a known suspicion that β-sitosterol may be beneficial, especially to patients suffering from breast and colon cancer (Novotny, Abdel-hamid and Hunakova, 2017).

The detection of β-sitosterol in the methanolic extract was collaborated with findings of steroidal glycosides identified from *G. fruticosus* methanolic extract, including several aglycone components (Warashina and Noro, 1994). In fact, a similar study, Marzouk et al, 2016, reported similar steroidal compounds, using NMR. The study reported the sighting of unknown pregnane glycosides in addition to cardenolides and triterpenoids. These include uzarigenin, afroside, uscharin, uscharidin, voruscharin, calotropin, calotropagenin, calotoxin, calactin, gomphoside, β-sophoroside, 17α-hydroxycalactin, 17α-hydroxyafroxide, gomphotin, gomphotoxin detected methanolic extracts prepared using aerial parts of the *G. fruticosus* (Abe, Mori, Okabe, & Yamauch, 1994; Groeneveld, Steijl, Van Den Berg, & Elings, 1990; Komissarenko, Chernobai, & Komissarenko, 1995; Marzouk et al., 2016) and more isolated from roots or whole plant extracts including coroglaucigenin and corotoxigenin (Abe et al., 1994; Warashina & Noro, 1994).

Compound **3**, was the only alcohol identified, which eluted at 76.73 min. According to mass spectra comparison with NIST database, compound **3** was identified as octadecatrien-1-ol. The position and orientation of its double bonds could not be determined but future studies can use dimethyl disulfide derivatization to locate the double bonds (Ding and Löfstedt, 2015). Its molecular ion at m/z 264.18 was detected. In addition, a prominent base peak at m/z 79.07 was also observed, coupled with a peak at m/z 108, while other distinguishing peaks at m/z 208 and 236 were not observed (Liu *et al.*, 2016). This fatty acid/alcohol has antibacterial and antioxidant potential (Gopalakrishnan and Udayakumar, 2014). This report is consistent with the findings in this study and (Gopalakrishnan and Udayakumar, 2014), since in all three cases, the alcoholic compound was identified in the methanolic extract.

Table 3.3. GCMS analysis of the methanolic extract of *G. fruticosus*.

Peak #	**RT (min)**	**Compound name**	**RI[a]**	**RI[b]**	**MF**	**MW (g/mol)**
1	68.77	branched hexadecanoic acid	1916.78	1904[A]	$C_{16}H_{32}O_2$	256
2	71.67	Hexadecanoic acid	1974.24	1970.1[C], 1968[F]	$C_{16}H_{32}O_2$	256
						
3	76.73	Octadecatrien-1-ol*	2086.09	2058[A]	$C_{18}H_{32}O$	264
						
4	77.3	methyl octadecadienoate*	2098.91		$C_{19}H_{34}O_2$	294
5	77.58	methyl octadecenoate*	2105.17		$C_{19}H_{36}O_2$	296
6	79.48	octadecadienoic acid*	2147.05	2214[G]	$C_{18}H_{32}O_2$	280
7	79.73	octadecenoic acid*	2152.49	2216[G]	$C_{18}H_{34}O_2$	282

8	79.87	octadecenoic acid*	2155.52	2216[G]	$C_{18}H_{34}O_2$	282
9	80.3	Ethyl octadecadienoic acid*	2164.812	2144-2177[F]	$C_{20}H_{36}O_2$	308
10	80.59	Octadecanoic acid	2171.05	2127.4[F]	$C_{18}H_{36}O_2$	284
						
11	105.77	Squalene	2827.31	2838[B]	$C_{30}H_{50}$	410
						
12	115.57	α-Tocopherol	3131.37	3142[C], 3112[D]	$C_{29}H_{50}O_2$	430
						
13	120.72	Stigmast-5-en-3β-ol	3301.58	3297[D]	$C_{29}H_{50}O$	414
						
16	122.74	α-Amyrin	3325.15	3376[A]	$C_{30}H_{50}O$	426
						
17	122.87	Lupeol	3373.55	3443[E]	$C_{30}H_{50}O$	426
						
19	125.75	Urs-12-en-3-yl acetate	3474.81	3486.6[A]	$C_{32}H_{52}O_2$	468

20	125.92	Lup-20(29)-en-3-yl acetate	3480.87	3516[E]	$C_{32}H_{52}O_2$	468

Key: RT= Retention time, MF= molecular formula, MW: Molecular weight. *= position and orientation of double bond unknown. RI[a]: Calculated value. RI[b]: Literature value. Sources of RI[b]:[A]NIST 11 RI database, [B]Kowalski (2005), [C]Nadaf, Halimi and Mortazavi (2012), [D]Stojanović *et al.*, (2011), [E]Díaz *et al.*, (2018), [F]Babushok, Linstrom and Zenkevich, (2011),[G] Fernandes *et al.*, (2017).

Table 3.4. Unidentified compounds by GCMS found in *G. fruticosus* shoot methanolic extract.

Compound #	RT (min)	Identification status
14	121.42	Unknown
15	122.36	Unknown pentacyclic triterpenol
18	124.58	Unkown triterpernyl acetate
21	128.38	Unknown triterpenyl acetate
22	128.71	Triterpenyl acetate
23	130.89	Triterpenyl butanoate
24	131.89	Triterpenyl propanoate
25	132.2	Triterpenyl butanoate
26	132.77	Triterpenyl pentanoate
27	134.21	Triterpenyl pentanoate
28	135.53	Triterpenyl butanoate
29	137.93	Triterpenoyl pentanoate
30	139.16	Triterpenyl ester of compound **14** (Hexenoate ester)
31	141.13	Triterpenyl ester of compound **14** (Hexenoate ester)
32	145.48	Unknown triterpenol
33	146.09	Unknown triterpenol

This study has found that thin layer chromatography (TLC) results are consistent with findings by other researchers (El-Kashef *et al.*, 2015) and GCMS results in **table 3.3**. In this study, the aqueous extract displayed few selected phytochemicals by thin layer chromatography although this is the solvent primarily used in traditional settings. This is collaborated by reports that indicate that the aqueous extract often shows poor bioactivity (Adeonipekun, Adeniyi and Aminu, 2014).

The lack of visible phytochemicals does not necessary indicate the absence of the selected phytochemicals due to masking on thin layer chromatography. Gas Chromatography coupled with Mass Spectroscopy have a higher resolving power in comparison to TLC, and hence was employed in this study. However, GCMS also has limitations in that it captures mainly volatile and semi-volatile compounds. Literature sources also show that *G. fruticosus* has glycosides (Abe et al., 1994; Fumiko Abe & Yamauchi, 2000; Heneidak et al., 2006; Komissarenko et al., 1995; Marzouk et al., 2016), which were not detected using GC-MS, but derivatization practices using trimethylsilyl (Louw *et al.*, 2011) can aid in the detection and identification of these compounds in future work. In addition, future research on Namibia's *G. fruticosus* can focus on identifying bioactive components by fractionation. In addition, methods such as LCMS, HPLC can be used to better give insights into phytochemicals contained therein, in future. This study serves as baseline for future studies and contribute to the knowledge gap authenticating claims of traditionally used plants, inform policy makers and serve to communicate dangers of the plant to the public.

3.5. Conclusions

The findings in this study provide evidence of the properties of extract derived from the powdered material of *G. fruticosus* and support its use in the traditional setting. The presence of lupeol, β-sitosterol and α-amyrin which have both anticancer and anti-inflammatory properties could contribute to cancer treatment and palliation whilst carboxylic acids, especially polyunsaturated compounds, may also explain the traditional use of the plant, permitting its easy permeation through the skin. Traditionally, *G. fruticosus* is used to treat diarrhea, pain, respiratory infection and as an arrow poison. However, toxic effects need to be explored further, while mechanisms of action and bioactivity of plant extracts on Namibian-relevant cancers is imperative in order to meet the healthcare gaps experienced by cancer suffers in Namibia and beyond.

3.6. References

Aali, N. M. El, Bugrein, S. N., El-fellah, M. F., Layas, Y. F. and Almesmari, W. K., 2018. GC / Mass Analysis of *Adiantium capillus* veneris Linn. *Acta Scientific Microbiology, 1*(5), pp. 30–44. doi: 10.31080/ASMI.2018.01.0050.

Abe, F., Mori, Y., Okabe, H. and Yamauch, T., 1994. Steroidal constituents from the roots and stems of *Asclepias fruticosa*. *Chemical and pharmaceutical bulletin, 42*(9), pp. 1777–1783. doi: 10.1248/cpb.37.3229.

Abe, F. and Yamauchi, T., 2000. 5 , 11-Epoxymegastigmanes from the Leaves of *Asclepias fruticosa* 1). *Chemical Pharmaceutical bulletin, 48*(12), pp. 1908–1911.

Adeonipekun, P. A., Adeniyi, T. A. and Aminu, S. O., 2014. Investigating the phytochemicals and antimicrobial activities of shoot and root of *Pycreus smithianus* (Ridl.) C. B. Clarke (Family Cyperaceae). *Journal of Botany, 2014*, p. 5. doi: 10.1155/2014/761613.

Amorim, D. E., 2016. Chemical constituents of *Pseudobrickellia brasiliensis* leaves (Spreng .) R . M . King & H. *Revista Brasileira de Plantas Medicinais, 18*(2): pp. 408–414.

Ananthi, P. and Kumari, B. D. R., 2013. Gas Chromatography - Mass Spectrometry Analysis of Methanolic seed and root Extracts of *Rorippa indica* L. *International Journal of ChemTech Research, 5*(1), pp. 299–306.

Babushok, V. I., Linstrom, P. J. and Zenkevich, I. G., 2011. Retention Indices for Frequently Reported Compounds of Plant Essential Oils. *Journal of Physical and Chemical Reference Data, 40*(4). doi: 10.1063/1.3653552.

Baskar, A. A., Ignacimuthu, S., Paulraj, G. M. and Al Numair, K. S., 2010. Chemopreventive potential of β-Sitosterol in experimental colon cancer model - an *In vitro* and *In vivo* study. *BMC Complementary and Alternative Medicine, 10*, p. 24. doi: 10.1186/1472-6882-10-24.

Calixto, N. O., Cordeiro, M. S., Giorno, T. B. S., Oliveira, G. G., Lopes, N. P., Fernandes, P. D., Pinto, A. C. and Rezende, C. M., 2017. Chemical Constituents of *Psychotria nemorosa* Gardner and Antinociceptive Activity. *Journal. of Brazilian chemical society, 28*(5), pp. 707–723. doi: 10.21577/0103-5053.20160219.

De Carvalho, T. C., Polizeli, A. M., Turatti, I. C. C., Severiano, M. E., De Carvalho, C. E., Ambrósio, S. R., Crotti, A. E. M., De Figueiredo, U. S., Vieira, P. C. and Furtado, N. A. J. C., 2010. Screening of filamentous fungi to identify biocatalysts for lupeol biotransformation. Molecules, 15(9), pp. 6140–6151. doi: 10.3390/molecules15096140.

Chikezie, P., Ibegbulem, C. and Mbagwu, F., 2015. Medicinal Potentials and Toxicity Concerns of Bioactive Principles. *Medicinal & Aromatic Plants, 4*(3), p. 202. doi: 10.4172/2167-0412.1000202.

Clarke, S. J. and Mclachlan, A. J., 2011. Interaction between complementary and alternative medicine with conventional anticancer medicine. *Cancer Forum, 35*(1), p. 6.

Constantinou, C., Papas, A. and Constantinou, A. I., 2008. Vitamin E and cancer: An insight into the anticancer activities of vitamin E isomers and analogs. *International journal of cancer, 123*, pp. 739–752. doi: 10.1002/ijc.23689.

Dande, P. R., Bonde, C. G. and Pandita, N., 2013. GC-MS analysis of bioactive unsaponifiable fraction from *Sesbania sesban* L. (Merr). *International journal of pharma and bio sciences, 4*(3), pp. 1025–1035.

Das, B., Antoon, R., Tsuchida, R., Lotfi, S., Morozova, O., Farhat, W., Malkin, D., Koren, G., Yeger, H. and Baruchel, S., 2008. Squalene selectively protects mouse bone marrow progenitors against cisplatin and carboplatin-induced cytotoxicity *in vivo* without protecting tumor growth. *Neoplasia, 10*(10), pp. 1105–1119. doi: Doi 10.1593/Neo.08466.

Díaz, K., Espinoza, L., Madrid, A., Pizarro, L. and Chamy, R., 2018. Isolation and Identification of Compounds from Bioactive Extracts of *Taraxacum officinale* Weber ex F. H. Wigg. (Dandelion) as a Potential Source of Antibacterial Agents. *Evidence-based Complementary and Alternative Medicine, 2018*. doi: 10.1155/2018/2706417.

Ding, B. J. and Löfstedt, C., 2015. Analysis of the agrotis segetum pheromone gland transcriptome in the light of Sex pheromone biosynthesis. *BMC Genomics, 16*(1), p. 711. doi: 10.1186/s12864-015-1909-2.

El-Kashef, D. F., E Hamed, A. N., Khalil, H. E., Kamel, M. S. and E Hamed Assistant Professor, A. N., 2015. Triterpenes and sterols of family Apocynaceae (2013-1955), A review. *Journal of Pharmacognosy and Phytochemistry, 21*(42), pp. 21–39.

Fernandes, F., Andrade, P. B., Ferreres, F., Gil-Izquierdo, A., Sousa-Pinto, I. and Valentão, P., 2017.The chemical composition on fingerprint of *Glandora diffusa* and its biological properties. *Arabian Journal of Chemistry, 10*(5), pp. 583–595. doi: 10.1016/j.arabjc.2015.01.012.

Fouche, G., Cragg, G. M., Pillay, P., Kolesnikova, N., Maharaj, V. J. and Senabe, J., 2008. *In vitro* anticancer screening of South African plants. *Journal of ethnopharmacology, 119*(3), pp. 455–61. doi: 10.1016/j.jep.2008.07.005.

Fox, C. B., 2009. Squalene emulsions for parenteral vaccine and drug delivery. *Molecules, 14*(9), pp. 3286–3312. doi: 10.3390/molecules14093286.

Franco, M. N., Galeano-Díaz, T., Sánchez, J., De Miguel, C. and Martín-Vertedor, D., 2014. Total Phenolic Compounds and Tocopherols Profiles of Seven Olive Oil Varieties Grown in the South-West of Spain. *Journal of Oleo Science, 63*(2), pp. 115–125. doi: 10.3989/gya.051513.

Gasca, C. A., Chaves, M., Fagg, C. W., e Magalhães, P. de O., Fonseca-Bazzo, Y. M. and Silveira, D., 2017. Pentacyclic triterpenes from leaves of Cagaita (*Eugenia dysenterica*). *Journal of Applied Pharmaceutical Science, 7*(7), pp. 051–054. doi: 10.7324/JAPS.2017.70709.

Ghasemzadeh, A. and Ghasemzadeh, N., 2011. Flavonoids and phenolic acids: Role and biochemical activity in plants and human. *Journal of Medicinal Plants Research, 5*(31),

pp. 6697–6703. doi: 10.5897/JMPR11.1404.

Gopalakrishnan, K. and Udayakumar, R., 2014. GC-MS Analysis of Phytocompounds of Leaf and Stem of *Marsilea quadrifolia* (L .). *International Journal of Biochemistry Research & Review*, *4*(6), pp. 517–526.

Grattan, B. J., 2013. Plant sterols as anticancer nutrients: Evidence for their role in breast cancer. *Nutrients*, *5*(2), pp. 359–387. doi: 10.3390/nu5020359.

Groeneveld, H. W., Steijl, H., Van Den Berg, B. and Elings, J. C., 1990. Rapid, quantitative HPLC analysis of *Asclepias fruticosa* L. 1 and *Danaus plexippus* L. 2 cardenolides. *Journal of Chemical Ecology*, *16*(12), pp. 3373–3382.

Harborne, J. B., 1998. *Phytochemical methods: a guide to modern techniques of plant analysis*. 3rd edn. London: Chapman & Hall.

Heneidak, S., Grayer, R. J., Kite, G. C. and Simmonds, M. S. J., 2006. Flavonoid glycosides from Egyptian species of the tribe Asclepiadeae (Apocynaceae, subfamily Asclepiadoideae). *Biochemical Systematics and Ecology*, *34*(7), pp. 575–584. doi: 10.1016/j.bse.2006.03.001.

Huang, M., Lu, J.-J., Huang, M.-Q., Bao, J.-L., Chen, X.-P. and Wang, Y.-T., 2012. Terpenoids: natural products for cancer therapy. *Expert opinion on investigational drugs*, *21*(12), pp. 1801–1818. doi: 10.1517/13543784.2012.727395.

Huang, Z., Lin, Y. and Fang, J., 2009. Biological and Pharmacological Activities of Squalene and Related Compounds: Potential Uses in Cosmetic Dermatology. *Molecules*, 14, pp. 540–554. doi: 10.3390/molecules14010540.

Igwe, O. U. and Okwu, D. E., 2013. GC-MS evaluation of bioactive compounds and antibacterial activity of the oil fraction from the stem bark of *Brachystegia eurycoma* harms, *International Journal of Chemical Sciences*, *11*(1), pp. 357–371.

Izzo, A. A., 2012. Interactions between herbs and conventional drugs: Overview of the clinical data. *Medical Principles and Practice*, *21*, pp. 404–428. doi: 10.1159/000334488.

Joselin, J., Brintha, T. S. S., Florence, A. R. and Jeeva, S., 2012. Screening of select ornamental flowers of the family Apocynaceae for phytochemical constituents. *Asian Pacific Journal of Tropical Disease*, pp. s260–s264. doi: 10.1016/S2222-1808(12)60162-5.

Kadhim, M. J., Sosa, A. A. and Hameed, I. H., 2016. Evaluation of anti-bacterial activity and bioactive chemical analysis of *Ocimum basilicum* using Fourier transform infrared (FT-IR) and gas chromatography- mass spectrometry (GC-MS) techniques. *Journal of Pharmacognosy and Phytotherapy*, *8*(6), pp. 127–146. doi: 10.5897/JPP2015.0366.

Kamboj, A. and Saluja, A. K., 2011. Isolation of stigmasterol and β-sitosterol from petroleum ether extract of aerial parts of *Ageratum conyzoides* (Asteraceae). *International Journal of Pharmacy and Pharmaceutical Sciences*, *3*(1), pp. 94–96.

Kaur, R. and Arora, S., 2015. Alkaloids-important therapeutic secondary metabolites of plant origin. *Journal of critical reviews*, *2*(3), pp. 1–8.

Kennedy, D. O. and Wightman, E. L., 2011. Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function. *Advances in Nutrition*, *2*, pp. 32–50. doi: 10.3945/an.110.000117.32.

von Koenen, E., 2001. *Medicinal, poisonous and edible plants of Namibia*. Windhoek: Klaus Hess Publishers/Verlag.

Komissarenko, N. F., Chernobai, V. T. and Komissarenko, A. N., 1995. New cardenolides from the leaves of *Gomphocarpus fruticosus*. *Chemistry of Natural compounds*, *31*(6), pp. 694–700.

Kostyuk, V., Potapovich, A., Stancato, A., de Luca, C., Lulli, D., Pastore, S. and Korkina, L., 2012. Photo-Oxidation Products of Skin Surface Squalene Mediate Metabolic and Inflammatory Responses to Solar UV in Human Keratinocytes. *PLoS ONE*, *7*(8), pp. 1–11. doi: 10.1371/journal.pone.0044472.

Kowalski, R., 2005. Analysis of lipophilic fraction from leaves, inflorescences and rhizomes of Silphium perfoliatum L. *Acta Societatis Botanicorum Poloniae*, *74*(1), pp. 5–10.

Kumar, S. and Pandey, A. k., 2013. Flavonoids : Reviews Chemistry and Biological Activities of Flavonoids : An Overview. *The scientific world journal*, *2013*, p. 17. doi: 10.1002/med.10033.

Liu, M. H., Zhang, Q., Zhang, Y. H., Lu, X. Y., Fu, W. M. and He, J. Y., 2016. Chemical analysis of dietary constituents in *Rosa roxburghii* and *Rosa sterilis* fruits. *Molecules*, *21*(9), pp. 1–21. doi: 10.3390/molecules21091204.

Loftsson, T., Ilievska, B., Asgrimsdottir, G. M., Ormarsson, O. T. and Stefansson, E., 2016. Fatty acids from marine lipids: Biological activity, formulation and stability. *Journal of Drug Delivery Science and Technology*, *34*, pp. 71–75. doi: 10.1016/j.jddst.2016.03.007.

López-Cobo, A., Martín-García, B., Segura-Carretero, A., Fernández-Gutiérrez, A. and Gómez-Caravaca, A. M., 2017. Comparison of two stationary phases for the determination of phytosterols and tocopherols in mango and its by-products by GC-QTOF-MS. *International Journal of Molecular Sciences*, *18*(7). doi: 10.3390/ijms18071594.

Louw, S., Burger, B. V., Le Roux, M. and Van Wyk, J. H., 2011. Lizard epidermal gland secretions. II. Chemical characterization of the generation gland secretion of the sungazer, *Cordylus giganteus*. *Journal of Natural Products*, *74*(6), pp. 1364–1369. doi: 10.1021/np1008366.

Lucero, M., Estell, R., Tellez, M. and Fredrickson, E., 2009. A retention index calculator simplifies identification of plant volatile organic compounds. *Phytochemical Analysis*, *20*(5), pp. 378–384. doi: 10.1002/pca.1137.

Marzouk, A. M., Osman, S. M. and Gohar, A. A., 2016. A new pregnane glycoside from *Gomphocarpus fruticosus* growing in Egypt. *Natural Product Research*, *30*(9), pp. 1060–1067. doi: 10.1080/14786419.2015.1107059.

Moreira, D. de L., Schaaf Teixeira, S., Helena Monteiro, M. D., Cecilia De-Oliveira, A.

A. and Paumgartten, F. J., 2014. Traditional use and safety of herbal medicines. *Revista Brasiliera Farmacognosia, 24*, pp. 248–257. doi: 10.1016/j.bjp.2014.03.006.

Mothana, R. A. A., Gruenert, R., Bednarski, P. J. and Lindequist, U., 2009. Evaluation of the in vitro anticancer, antimicrobial and antioxidant activities of some Yemeni plants used in folk medicine. *Pharmazie, 64*(4), pp. 260–268. doi: 10.1691/ph.2009.8789.

Mothana, R. A., Lindequist, U., Gruenert, R. and Bednarski, P. J., 2009. Studies of the *in vitro* anticancer, antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from the island Soqotra. *BMC Complementary and Alternative Medicine, 9*(7), pp. 1–11. doi: 10.1186/1472-6882-9-7.

Moussa, T. A. A. and Almaghrabi, O. A., 2016. Fatty acid constituents of *Peganum harmala* plant using Gas Chromatography-Mass Spectroscopy. *Saudi journal of biological sciences, 23*, pp. 397–403. doi: 10.1016/j.sjbs.2015.04.013.

Nadaf, M., Halimi, M. and Mortazavi, M., 2012. Identification of nonpolar chemical composition *Spartium junceum* flower growing in Iran by GC-MS. *Middle-East Journal of .scientific research, 11*(2), pp. 221–224. doi: 10.5829/idosi.mejsr.2013.17.05.75149.

Nirmala, M. J., Samundeeswari, A. and Sankar, P. D., 2011. Natural plant resources in anti-cancer therapy-A review. *Research in Plant Biology, 1*(3), pp. 1–14.

Novotny, L., Abdel-hamid, M. E. and Hunakova, L., 2017. Anticancer Potential Of β-Sitosterol. *international journal of clinical pharmacology and pharmacotherapy, 2*, p. 129.

Palanimuthu, D., Baskaran, N., Silvan, S., Rajasekaran, D. and Manoharan, S., 2012. Lupeol, a bioactive triterpene, prevents tumor formation during 7,12-dimethylbenz(a)anthracene induced oral carcinogenesis. *Pathology and Oncology Research, 18*(4), pp. 1029–1037. doi: 10.1007/s12253-012-9541-9.

Pédeboscq, S., Rey, C., Petit, M., Harpey, C., De Giorgi, F., Ichas, F. and Lartigue, L., 2012. Non-antioxidant properties of α-tocopherol reduce the anticancer activity of several protein Kinase inhibitors *in vitro*. *PLoS ONE, 7*(5), p. e36811. doi: 10.1371/journal.pone.0036811.

Popa, I., BĂBEANU, N. E., Nita, S. and Popa, O., 2014. Squalene-natural resources and applications. *Farmacia, 62*(5), pp. 840–862.

Rebucci, M. and Michiels, C., 2013. Molecular aspects of cancer cell resistance to chemotherapy. *Biochemical Pharmacology, 85*, pp. 1219–1226. doi: 10.1016/j.bcp.2013.02.017.

Ruiz-Matute, A. I., Hernández-Hernández, O., Rodríguez-Sánchez, S., Sanz, M. L. and Martínez-Castro, I., 2011. Derivatization of carbohydrates for GC and GC-MS analyses. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 879*(17–18), pp. 1226–1240. doi: 10.1016/j.jchromb.2010.11.013.

Saeidnia, S., Manayi, A., Gohari, A. R. and Abdollahi, M., 2014. The Story of Beta-sitosterol- A Review. *European Journal of Medicinal Plants, 4*(5), pp. 590–609. doi: 10.9734/EJMP/2014/7764.

Saha, S., Walia, S., Kundu, A., Kaur, C., Singh, J. and Sisodia, R., 2015. Capsaicinoids, tocopherol, and sterols content in chili (Capsicum sp.) by gas chromatographic-mass spectrometric determination. *International Journal of Food Properties*, *18*(7), pp. 1535–1545. doi: 10.1080/10942912.2013.833222.

Sayers, S., Ulysse, G., Xiang, Z. and He, Y., 2012. Vaxjo: A web-based vaccine adjuvant database and its application for analysis of vaccine adjuvants and their uses in vaccine development. *Journal of Biomedicine and Biotechnology*, *2012*, p. 13. doi: 10.1155/2012/831486.

Scalbert, A., Brennan, L., Fiehn, O., Hankemeier, T., Kristal, B. S., van Ommen, B., Pujos-Guillot, E., Verheij, E., Wishart, D. and Wopereis, S., 2009. Mass-spectrometry-based metabolomics: Limitations and recommendations for future progress with particular focus on nutrition research, *Metabolomics*, *5*, pp. 435–458. doi: 10.1007/s11306-009-0168-0.

Stojanović, I. Ž., Radulović, N. S., Mitrović, T. L., Stamenković, S. M. and Stojanović, G. S., 2011. Volatile constituents of selected Parmeliaceae lichens. *Journal of the Serbian Chemical Society*, *76*(7), pp. 987–994. doi: 10.2298/JSC101004087S.

Subba, B., Srivastav, C. and Kandel, R. C., 2016. Scientific validation of medicinal plants used by Yakkha community of Chanuwa VDC, Dhankuta, Nepal. *SpringerPlus*, *5*(1), pp. 1–14. doi: 10.1186/s40064-016-1821-5.

Sülsen, V. P., Lizarraga, E., Mamadalieva, N. Z. and Lago, J. H. G., 2017. Potential of Terpenoids and Flavonoids from Asteraceae as Anti-Inflammatory. *Antitumor and Antiparasitic Agents'*, *2017*, pp. 6–8.

Suttiarporn, P., Chumpolsri, W., Mahatheeranont, S., Luangkamin, S., Teepsawang, S. and Leardkamolkarn, V., 2015. Structures of phytosterols and triterpenoids with potential anti-cancer activity in bran of black non-glutinous rice. *Nutrients*, *7*(3), pp. 1672–1687. doi: 10.3390/nu7031672.

Wal, A., Rai, A., Wal, P. and Sharma, G., 2011. Biological activities of lupeol. *Systematic Reviews in Pharmacy*, *2*(2), pp. 96–103. doi: 10.4103/0975-8453.86298.

Wal, A., Srivastava, R. ., Wal, P., Rai, A. and Sharma, S., 2015. Lupeol as a magical drug', *Pharmaceutical and Biological Evaluations*, *2*(5), pp. 142–151.

Warashina, T. and Noro, T., 1994. Steroidal glycosides from *Asclepias fruticosa* L. *Chemical and pharmaceutical bulletin*, *42*(2), pp. 322–326.

WHO (2013) *WHO traditional medicine strategy 2014-2023*. Available at: http://apps.who.int/medicinedocs/documents/s21201en/s21201en.pdf (Accessed: 1 August 2017).

Zayed, M. A., Abd El-Kareem, M. S. M. and Zaky, N. H. S., 2017. Gas Chromatography-Mass Spectrometry Studies of Waste Vegetable Mixed and Pure Used Oils and Its Biodiesel Products. *Journal of pharmaceutical applied chemistry*, *3*(2), pp. 109–116. doi: 10.18576/jpac/030204.

CHAPTER 4: SELECTIVE NECROTIC CELL DEATH IN HELA CELLS TREATED WITH G. FRUTICOSUS: COLLABORATION BY ELEVATED ROS LEVELS AND LACK OF CASPASE CLEAVAGE

Florence Dushimemariya[1], Jyoti Sharma[2], Mervin Meyer[3] & Davis R. Mumbengegwi[1*]

1. Multi-disciplinary Research Center, University of Namibia

2. Hair and Skin Research Laboratory, University of Cape Town

3. Department of Biotechnology, University of Western Cape

*Corresponding author: Davis R. Mumbengegwi; email: dmumbengegwi@unam.na

4.1. Abstract

Namibia has a wealth of traditional knowledge and practices that includes the use of plants for the alleviation of a myriad of diseases. *G. fruticosus* is a plant endemic to Namibia and it used to treat a range of diseases such as stomach complaints, tuberculosis and cancer. An investigation into the biological properties of the methanolic extract of *G. fruticosus* shoots against cancerous and non-cancerous cell lines was conducted. The study evaluated the anti-proliferative activity, cytotoxicity and the apoptosis induction properties of the *G. fruticosus* extracts *in vitro*, against a panel of cells consisting of prostate (PC3), liver (HepG2), cervical (HeLa) cancer and two non-cancer cell lines skin (KMST6) and kidney (Hek293). The anticancer assay revealed potent activity of *G. fruticosus* extract on HeLa cervical cancer cells (IC_{50} 8.35^{-7} ± 0.13 g/L), and minimal cytotoxicity against a non-cancerous cell lines Hek293 (IC_{50} 3.976^{-4} ± 86.7 g/L), KMST6 (IC_{50} 4.201^{-4} ± 89.0 g/L). Cancerous cells, PC3 and HepG2 demonstrated IC_{50} 7.382^{-4} ± 6.4 and 5.035^{-4} ± 5.03 g/L respectively, indicating selectivity of extract against HeLa. Non-significant apoptosis

induction occurred in HeLa at IC_{50} concentration, indicating necrosis as possible cell death mechanism. This was further supported by significant elevation of Ros in HeLa , indicating that this mechanism may be responsible for observed reduced cell viability. Caspase 3/7 dependent apoptosis induction was not observed in neither Hek293 nor HeLa, indicating that cell death, especially at the high extract concentrations was not activated. In conclusion, this study supports the use of *G. fruticosus* within Namibia's Bergdamara/berdamara/damaqua/damaran people, with potent activity especially for cervical cancer. Mechanism of action appears to be necrosis, ROS dependent, caspase 3/7 independent. The plant needs to be pursued for additional studies to determine safety and potency against solid tumors in small mammals as these would validate its use traditionally.

Key words: Antiproliferative, caspase independent, *Gomphocarpus fruticosus*, HeLa, necrosis, selectivity.

4.2. Introduction

Traditional medicine has been used for the treatment and prevention of various illnesses, since time immemorial (Sofowora, Ogunbodede and Onayade, 2013). It is prepared in many forms such as decoction, infusions, tinctures for oral administration, ointments, poultices for topical applications or powders which are inhaled, and are often composed of different components such as plant material (flowers, roots, fruits, leaves, bark, gum), animal parts or products, soil particulate among others (Bussmann *et al.*, 2010). Plants

constitute the largest portion of traditional medicine, and have been a useful resource in finding lead compounds for treatment of many diseases, including cancer. Important clinical medicines such as vincristine, vinblastine and paclitaxol are derived in whole or in part from traditional medicines. These are Food and Drug Administration (FDA) approved drugs in clinical use for the treatment of cancers such as breast, ovarian, lung cancer and Kaposi sarcoma (paclitaxol) or rare cancers such as non-hodgkin's lymphoma, leukemia (vincristine and vinblastine) (Nirmala, Samundeeswari and Sankar, 2011). Inclusion of traditional medicine -derived drugs into mainstream use can take 14 years, from laboratory *in vitro* analysis to clinical trial phases (Schuhmacher, Gassmann and Hinder, 2016). Medicinal plants are efficacious due to their various modes of action, including apoptosis induction, sustained injury to DNA, caspase activation and antiproliferative effects by either cell cycle arrest, MAPK and NF-κβ pathway alterations (Habli *et al.*, 2017). Apoptosis has been the most commonly preferred treatment outcome, especially with chemotherapeutic drugs, because it is programmed cell death (McIlwain, Berger and Mak, 2015). However, other modes of cell death such necrosis, mitoptosis, autophagy can also occur, in an effort to regain cellular homeostasis (Chaabane *et al.*, 2013). Various caspases, are involved at various signally stages and as key players in the intrinsic (mitochondrial-dependent) and extrinsic (transmembrane activated) apoptosis induction pathways (Brentnall *et al.*, 2013).

The family Apocynaceae is reach with a variety of plants which are mostly characterized by exuding a milky latex from its fractured body parts. This family is notable for the discovery of cardenolides, which are useful for treating heart disease and are renowned

for anticancer activity. Cardenolides isolated from members of the apocynaceae family have been shown to possess cytotoxicity against a number of cancer models such as colon, breast, liver, uterus, brain, prostate, gastric, lung, breast, some which are leading causes of mortality such as breast and prostate cancer (Wen *et al.*, 2016). A recent report by Wong, Lim and Chan (2013), reviewed the activities of several members of this family, which included: *Cerbera, Catharanthus, Alstonia, Allamanda, Plumeria, Calotropis, Tabernaemontana, Vallaris* and *Nerium* species. The most notable is *Nerium oleander*, from which several cardenolides have already been isolated (Wen *et al.*, 2016). *Gomphocarpus fruticosus* or *Asclepias fruitocosa* is a milkweed erect plant also belonging to the Apocynaceae family. This plant is found in different ecological niches, in various continents and countries neighboring Namibia such as Zambia, Zimbabwe, Mozambique, Botswana, Swaziland, Lesotho and South Africa. *G. fruticosus* is used for the treatment of cancer by the bergdamara/bergdama/berdamara/damaqua people who inhabited the Grootfontein Mountains, as well as other mountains of Namibia. It is against this background that this plant is investigated for its biological activity against cancer and cytotoxicity *in vitro*. The mode of action was also explored using APOPercentage, reactive oxygen species (ROS) production and caspase 3/7 activation.

4.3. Methods and materials

4.3.1. Plant processing

Shoots, along with fruiting structures, leaves and stems of *G. fruticosus* were collected in the Otjozondjupa region of Namibia. A voucher specimen (FD14) was prepared in a plant

press, for identification and deposited with the herbarium at the National Botanical Research Institute (NBRI). Furthermore, the aerial/ above ground parts including leaves, fruiting structures and stalks were harvested and stored in brown paper bags. The plant material was sorted to remove debris before being cut into small pieces to facilitate drying (1^{-5}-2^{-5} km long). Plant material was dried on an open bench top for 3 weeks, followed by grinding into powder using an industrial miller. The ground plant material was labelled and stored at -20 °C until further needed.

4.3.2. Extract preparation

Powdered plant material 10 g derived from the shoot of *G. fruticosus* was macerated in 1^{-1} L methanol. The mixture was left at room temperature for 24 hrs, followed by gravity filtration through a Whatman filter paper. Filtration was followed by rotary evaporation, which was conducted in a water-bath set at 333.15 K, pressure was set at 6000 Pa. To further remove traces of extraction solvent, extracts were dried on a freeze dryer until extracts could easily be scrapped off the round bottom flask wall. Dry extracts were weighted and stored in air-tight containers at 253.15 K until further use.

4.3.3. Cell culture

Three cancer cell lines (HeLa, HepG2, PC3) and two non-cancer cell lines (Hek293 and KMST-6) were used in this study. Cell lines were purchased from American Type Culture Collection (ATCC). The cells were maintained in culture media (RPMI1640 or Dulbecco's Modified Eagle's Media) supplemented with 10% fetal bovine serum and 1%

penicillin/streptomycin. Cultures were grown in a humidified incubator set at 5% CO_2 and at 310.15 K. Cell culture reagents were obtained from Invitrogen and cells were cultured in ventilated 75 mm^2 culture flasks for routine maintenance and seeded in 96, 12 or 6 well plates for experimental procedures.

4.3.4. *In vitro* cell viability assay

Cell viability was investigated according to a method by (Omoyeni *et al.*, 2015) with slight modifications using the water soluble formazan salt, WST-1. The methanolic plant extract was analyzed against HeLa, HepG2, PC3 cell lines for anticancer activity and Hek293 and KMST-6 cell lines for cytotoxicity. Cells were seeded at 1×10^5 cells per well in a 96-well plate in 1^{-4} L media: DMEM (Hek293, HeLa and HepG2) or RPMI1640 (PC3). After 24 hrs, all spent media was removed by pipetting from side of the well plate and replaced with 1^{-4} L media containing various concentrations of plant extracts. *G. fruticosus* methanolic extract was dissolved in DMSO and further diluted in complete biological media, 1000 fold, such that the final DMSO concentration was less than 0.5 % (Hajighasemi and Tajik, 2017; Singh, Mckenzie and Ma, 2017). The final extract concentrations for each cell line were as follows: KMST6 (3.125^{-5}-1 g/L), Hek293 (3.125^{-5} - 1 g/L), PC3 (5^{-4}-1 g/L), HeLa (3.25^{-10}-3.25^{-5} g/L) and HepG2 (3.125^{-5}-1 g/L), followed by incubation for 24 hrs. The time of incubation was determined as the least standard time from which a significant change in cell viability can be noticed (Meyer *et al.*, 2008; Omoyeni *et al.*, 2015). A well containing cells and extract was used at each treatment concentration, to account for interference. After 24 hrs of exposure, spent media was removed and cell viability was measured using a water soluble tetrazolium reagent (WST-

1) (Roche diagnostics). The WST-1 reagent (5^{-5} L) was prepared at 1:10 dilution in complete media was added to each well and incubated for 4 hrs in a humidified incubator set at 310.15 K and 5% CO_2. Negative control wells only received cell culture media whilst positive control wells were treated with cell culture media plus 6% DMSO. The experiment was conducted in the dark by covering plates with foil. Absorbance of each plate was done in a plate reader (Omega, BMG Labtech: Germany) at 440 nm (with 630 nm as reference wavelength). Cell viability was expressed as a percentage of the absorbance obtained in untreated cells. The IC_{50}, the concentration of plant extract resulting in 50% reduction in cell viability was determined using graph pad prism version 5, using non-linear sigmoidal curve fitting. Each treatment was conducted in triplicate and no experimental repeats were conducted.

$$\text{Cell Viability \%} = (OD_{\text{treated well}} - OD_{\text{blank}}/OD_{\text{untreated well}} - OD_{\text{blank}}) * 100$$

4.3.5. Cell death mechanism

The mechanism of cell death was determined using APOPercentage assay, ROS induction and caspase 3/7 activation assay on Hek293 and HeLa cells. The cells were seeded at a density of 2×10^5 cells per well and allowed to adhere in a humidified incubator, 5% CO_2 at 310.15 K for 24 hrs, in 5^{-4} L media. Spent media was removed and replaced with 5^{-4} L fresh media containing the relevant treatment (8.35^{-7} g/L or 3.976^{-4} g/L plant extract for each of the cell lines, i.e. IC_{50} concentrations on HeLa and HeK293 cell lines respectively: based on the cell viability assay). Cells treated with 4-5% DMSO served as positive control on Hek293 and HeLa respectively, for APOPercentage and caspase activation assays. The positive control for ROS induction was 1% H_2O_2, while untreated cells served

as negative control. For all assays, treatment was followed by incubation for 24 hrs in a humidified incubator set at 5% CO_2 and 310.15 K. All spent media was removed per well and cells were harvested by gently trypsinization, pelleted by centrifugation and incubated with the appropriate dye for each of the assays.

4.3.6. APOPercentage assay

To investigate the apoptosis inducing effects of plant extracts, the APOPercentage assay was conducted on HeLa and Hek293 cell lines (Meyer *et al.*, 2008), prepared according to section above (Cell death mechanism). The cell lines were chosen because they showed the most sensitivity towards plant extract and were representative of a cancerous and non-cancerous cell lines. After cell harvesting and pelleting, APOPercentage dye was added to each cell population and incubated for 30 min, before analyzing a minimum of 10 000 cells per sample. The percentage cells positive for apoptosis at each treatment with reference to untreated and unstained cells, using flow cytometry (Becton Dickinson FACscan machine) was recorded. Graph pad prism was used to plot the data into a bar graph, and Jarque-Bera test for normality, followed by one-way ANOVA for statistical difference, $p=0.05$, in comparison to untreated cells. Each treatment was replicated a minimum of 3 times. No experimental repeats were conducted.

4.3.7. General Reactive Oxygen Species induction assay

To investigate the induction of reactive oxygen species (ROS), harvested and pelleted cells, as described in cell death mechanism section, were stained with the chloromethyl

derivative of H_2DCFDA (CM-H_2DCFDA) (Thermo Fisher Scientific) probe according to literature (Graves et al, 2009), with modifications. The experiment was incubated for 30 min at 310.15 K in the dark. After incubation, cells were washed with PBS to remove unbound dye and re-suspended in 2^{-4} L PBS before reading on a flow cytometer (Becton Dickinson FACscan machine) using FL1 filter (533/30 nm). A minimum of 10 000 cells per sample were acquired for analysis and a percentage of cells under oxidative stress were recorded with reference to untreated and unstained cells. In addition, all experiments were conducted in triplicates and no experiments were repeated. Data is presented as mean percentage ROS ± SE (minimum 3 replicates).

4.3.8. Caspase 3/7 activation assay

Caspase induction was investigated by the use of the Vybrant FAM caspase 3/7 kit (Invitrogen) and according to manufacturer's instructions (Chan *et al.*, 2012). After exposure to 3.976^{-4} g/L extract concentration as in section (Cell death mechanism), the cells were harvested, centrifuged and pellet incubated with the FLICA dye followed by incubation for 60 min in a humidified incubator set at 310.15 K and 5% CO_2. Cells were analyzed using a flow cytometer (Becton Dickinson FACscan machine) by acquiring a minimum of 10 000 cells per sample and use of the FL1 filter (533/30 nm). Data is presented as percentage caspase 3/7 activity. Graph pad prism, version 5 was used to plot the mean ± SE data (minimum of 3 replicates) into a bar graph, and conduct Jarque-Bera test for normality, followed by one-way ANOVA for statistical difference, p=0.05, in comparison to untreated cells. No experimental repeats were conducted.

4.4. Results

4.4.1. Cell viability in response to treatment with *G. fruticosus* extract.

Figure 4.1 shows the effect of different extract concentrations on non-cancer cell lines and **figure 4.2** shows the effect of increased extract concentrations on the viability of cancer cell lines. In **figure 4.1**, the decline in cell viability on two non-cancerous cell lines KMST6 (skin fibroblast) and Hek293 (embryonic kidney) shows a similar response. The IC_{50} values were $4.201^{-4} \pm 89.0$ g/L on KMST6 and $3.976^{-4} \pm 86.7$ g/L on Hek293 **(table 4.1).**

Varied IC_{50} values were obtained against cancer cell lines. The most sensitive cell line was HeLa, with an IC_{50} value of $8.35^{-7} \pm 0.13$ g/L, while higher values were observed on HepG2 ($5.035^{-4} \pm 5.03$ g/L) and PC3 ($7.382^{-4} \pm 6.4$ g/L).

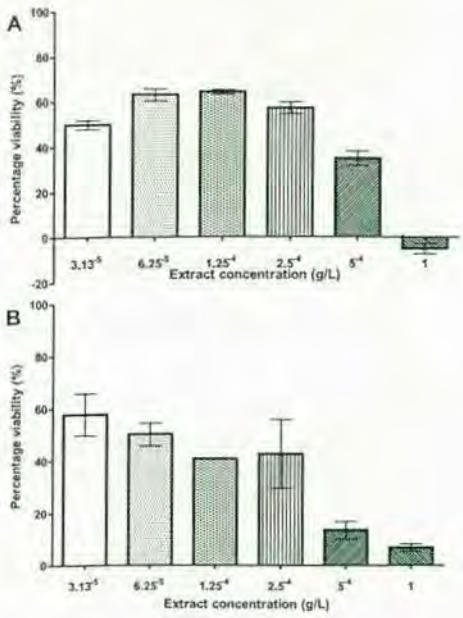


Figure 4.1 Effects of *G. fruticosus* methanolic shoot extract on two non-cancerous cell lines. Panel **A** represents KMST6 and **B** corresponds to Hek293.

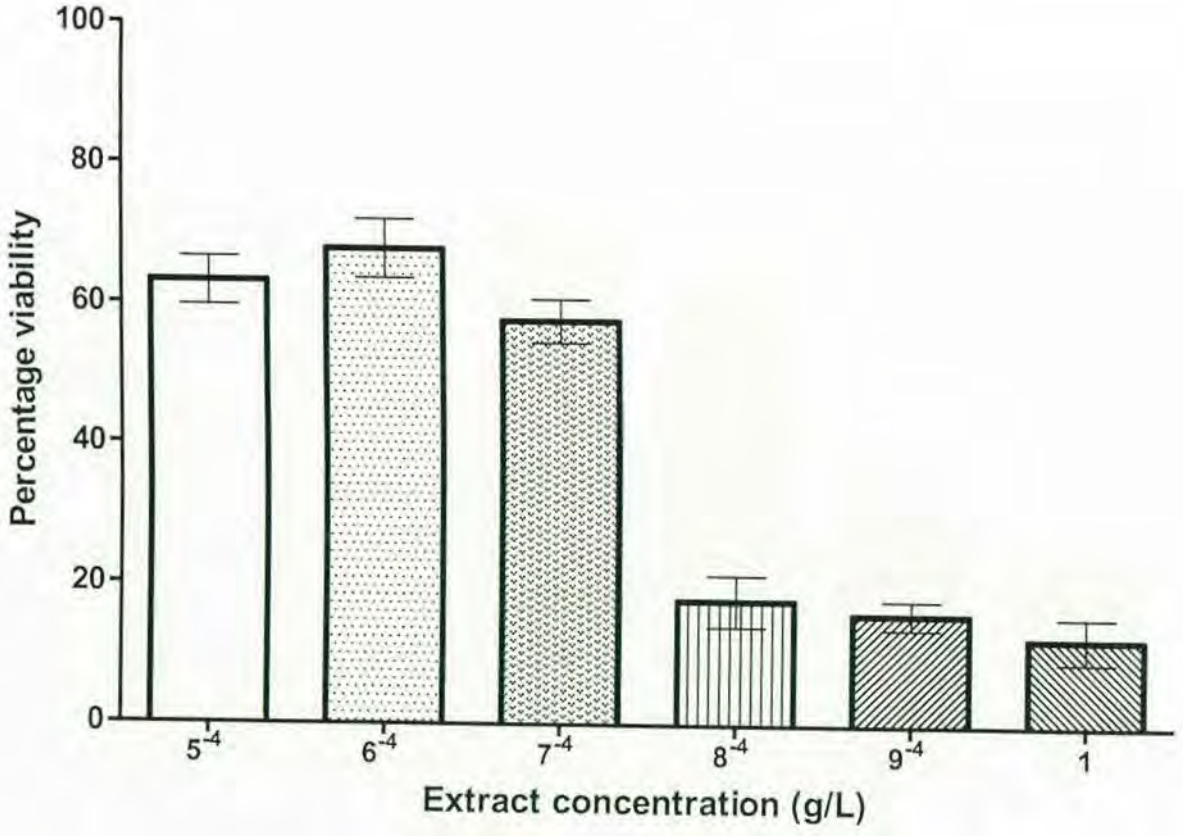


Figure 4.2. Cell viability of PC3 cell line after treatment with *G. fruticosus* methanolic extract at 5^{-4} to 1 g/L for 24 hrs.

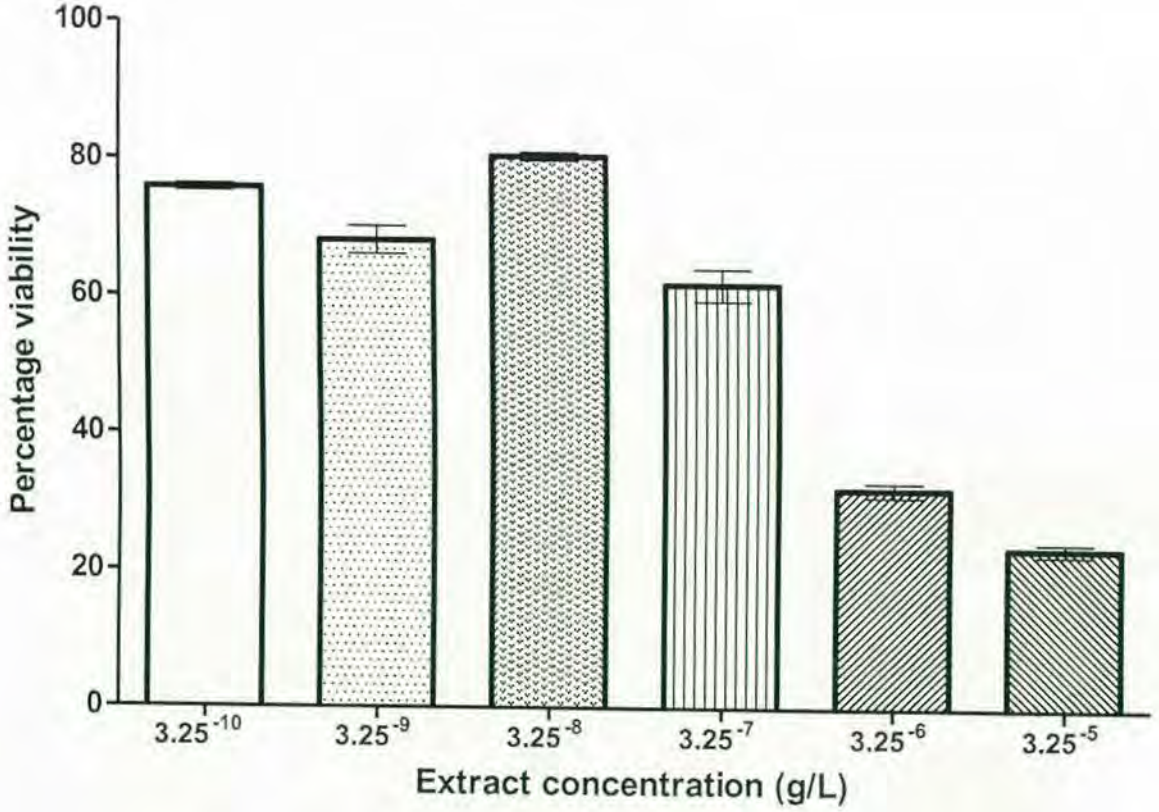


Figure 4.3. Cell viability assay on HeLa cells after treatment with *G. fruticosus* plant extract at concentrations ranging 3.13^{-10} to 3.13^{-5} g/L for a period of 24 hrs.

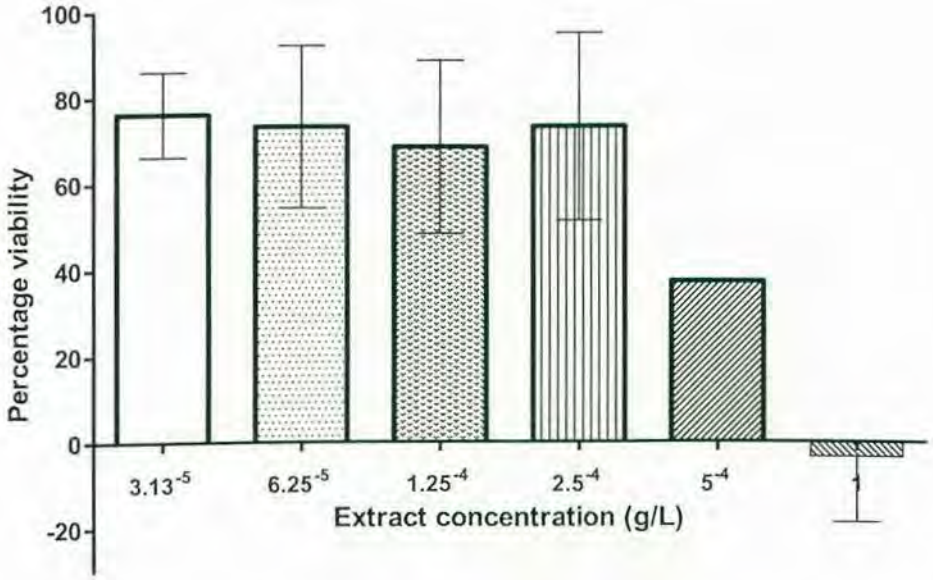


Figure 4.4. Percentage viability of HepG2 cells treated with *G. fruticosus* extract from concentrations ranging 3.13^{-5} to 1 g/L.

Table 4.1. WST-1 cell viability $IC_{50} \pm SE$ against cancerous and non-cancerous cells based on non-linear modeling.

Cell line type	Name	Cell line	$IC_{50} \pm SE$ g/L
Non-cancerous	Skin fibroblast	KMST6	$4.201^{-4} \pm 89.0$
	Embryonic kidney	Hek293	$3.976^{-4} \pm 86.7$
Cancerous	Prostate cancer	PC3	$7.382^{-4} \pm 6.4$
	Cervical cancer	HeLa	$8.35^{-7} \pm 0.13$
	Hepatocellular carcinoma	HepG2	$5.035^{-4} \pm 5.03$

4.4.2. APOPercentage assay

The cell death mechanism of *G. fruticosus* on Hek293 and HeLa cells was investigated post treatment with 3.976^{-4} and 8.35^{-7} g/l extract concentrations for 24 hrs, using a

negatively charged halogenated fluorescein dye. The results are shown in **figure 4.5**, the induction of apoptosis in Hek293 cells show high level (81.6 ±0.24%) of apoptotic cells. Whilst no significant increase in apoptosis induction was observed in Hek293 at the low extract concentration, 8.35^{-7} g/L (P=0.005) in comparison to non-treated cells. There was no significant increase in apoptosis induction in HeLa cells at the IC_{50} concentration, **figure 4.6**. Whilst, 54.8 ±2.5% of the cell population was significantly presenting with apoptotic properties after treatment with 3.976^{-4} g/L.

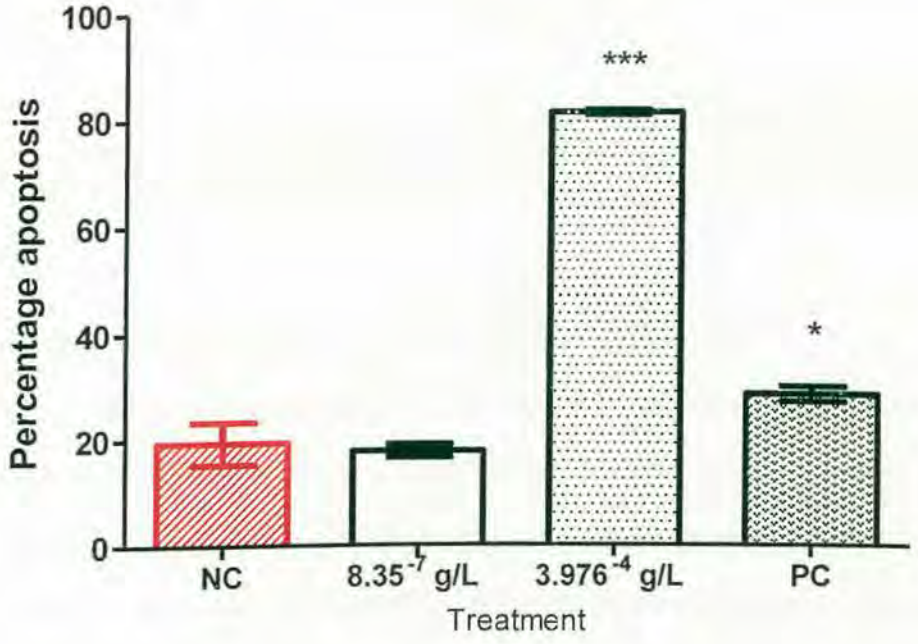


Figure 4.5. Induction of apoptosis by *G. fruticosus* extract on Hek293 cells at two concentrations after 24 hrs exposure. Asterisks denote a significant increase. NC: Negative control. PC: Positive control. Statistical significance: *P=0.05, **P=0.01 and ***P=0.001.

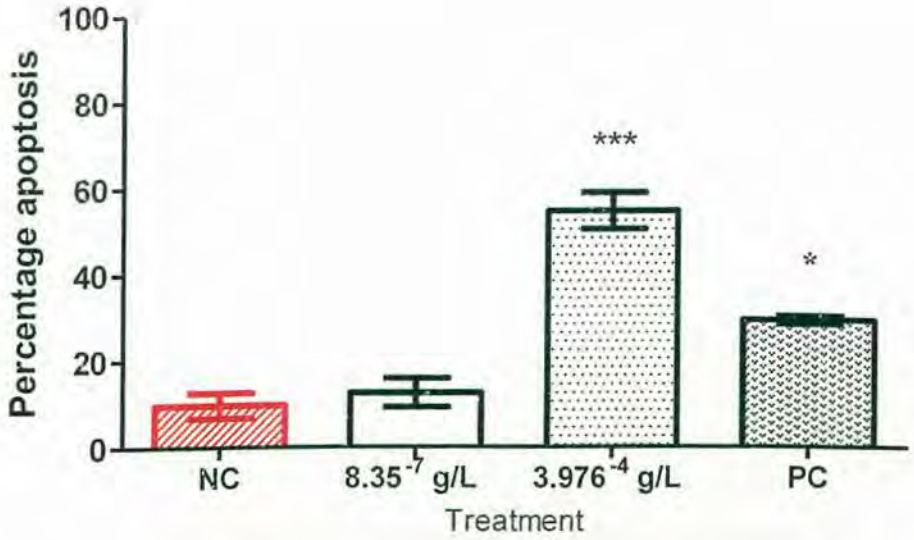


Figure 4.6. Induction of apoptosis by *G. fruticosus* extract on HeLa at two concentrations after 24 hrs exposure. Asterisks denote a significant increase. NC: Negative control. PC: Positive control. Statistical significance: *P=0.05, **P=0.01 and ***P=0.001.

4.4.3. Role of reactive oxygen species in induction of apoptosis

Figure 4.7, shows the production of reactive oxygen species in Hek293 and HeLa after treatment with plant extracts. The results obtained show no significant increase in ROS production in Hek293 cells treated with 8.35^{-7} or 3.976^{-4} g/L extract concentrations, in comparison to non-treated cells, panel A (P=0.005). However, exposure of HeLa to 8.35^{-7} or 3.976^{-4} g/L plant extract resulted in a significant increase in ROS production, 30.5± 4.7% (p=0.05) and 60.5± 4.6% (p=0.001) respectively.

The use of 1% Hydrogen peroxide as a positive control resulted in significant damage to cells, even though exposure was only for 30 min, such that cell harvest did not capture

stressed cells. The result of this severe damage are very few cells undergoing oxidative stress at seen in **figures 4.7.**

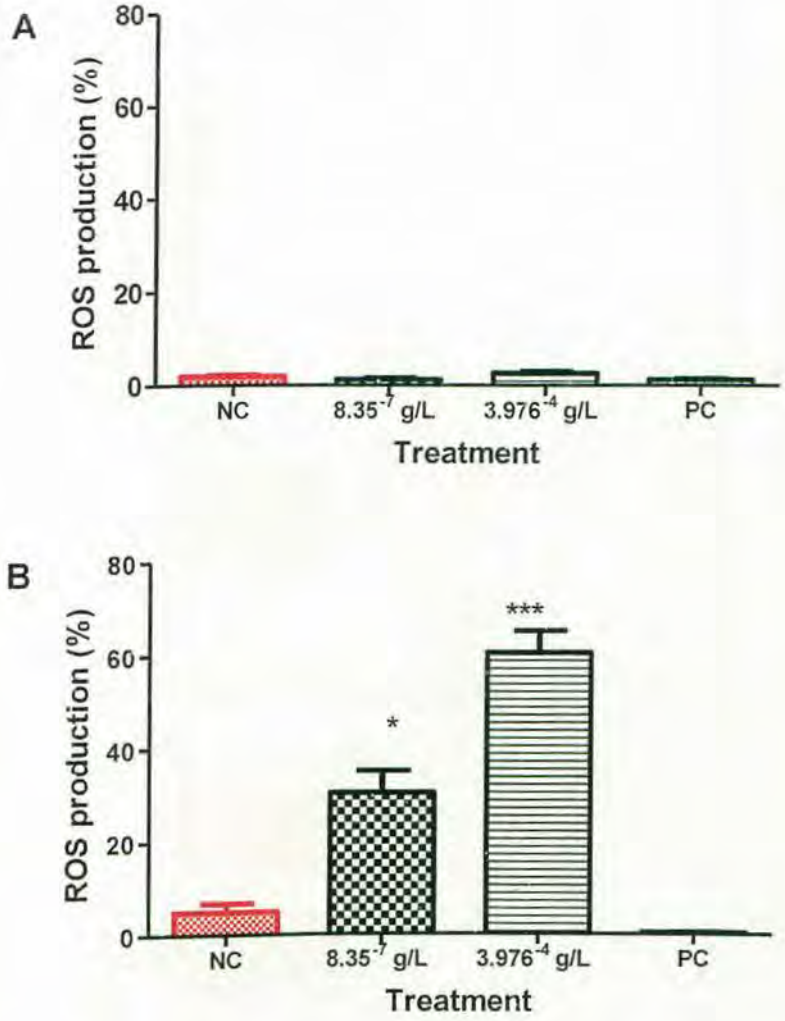


Figure 4.7. Induction of ROS in Hek293 (**A**) and HeLa (**B**) cells at two extract concentrations after 24 hrs exposure. Comparisons in each cell line were considered significant after comparison with non-treated cells, NC: Negative control. PC: Positive control. Statistical significance: *P=0.05, **P=0.01 and ***P=0.001. Asterisks denote a significant increase.

4.4.4. Effect of *G. fruticosus* on caspase 3/7 activation

Caspase 3 and 7 are very important executor caspases in the intrinsic apoptosis induction pathway. The effects of *G. fruticosus* extract on caspase 3/7 activation in Hek293 and HeLa cells are shown in **figure 4.8** below, panels A and B respectively. In both panels, there is no evidence of a significant expression of caspase 3/7 as a result of treatment with *G. fruticosus* extract.

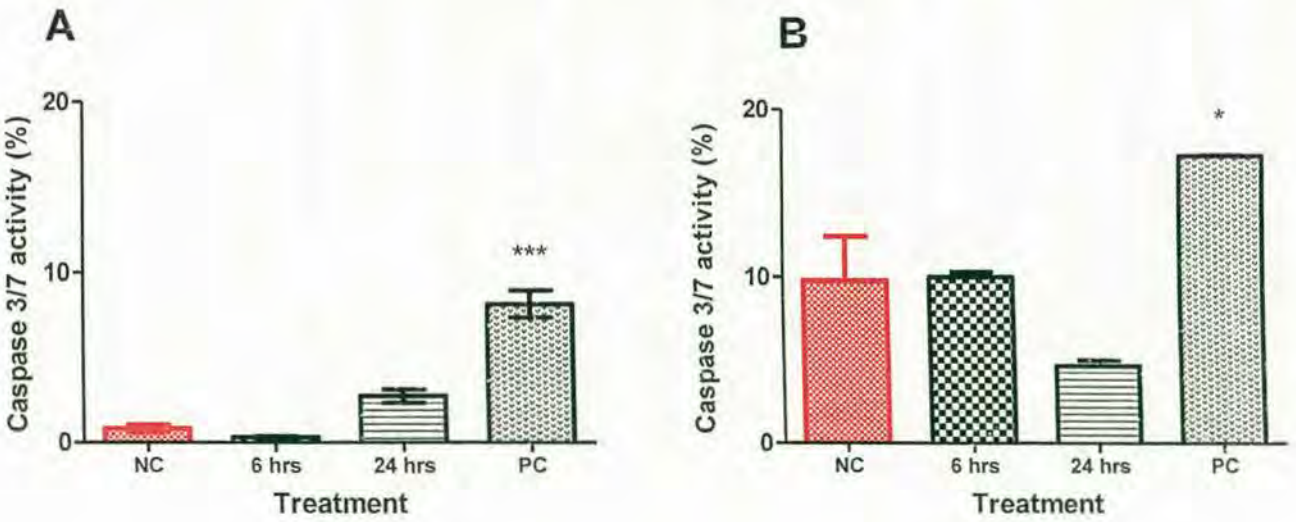


Figure 4.8. Activation of caspase 3/7 induction in HeK293 (A) and HeLa (B) cells after treatment with 3.976^{-4} g/L *G. fruticosus* extract for 6 and 24 hrs. Comparisons were made with non-treated cells, NC: Negative control. PC: Positive control Statistical significance: *P=0.05, **P=0.01 and ***P=0.001. Asterisks denote a significant increase.

4.5. Discussion

This study reports on the effect of methanolic shoot extract of *G. fruticosus* on the viability of five cell lines using the WST-1 assay which is a water soluble tetrazolium based assay. The assay is based on the premise that the tetrazolium salt permeates cell membranes and is cleaved to form formazan by mitochondrial dehydrogenases (Yin *et al.*, 2012). Only metabolically active cells are able to facilitate this transformation, therefore, formazan formed is directly proportional to viable cells. In this study, the HeLa cells were most sensitive to the methanolic extract of *G. fruticosus*, with an IC_{50} concentration $8.35^{-7} \pm 0.13$ g/L. According to the criteria set by the National Cancer Institute (NCI), an IC_{50} value below 30 µg/ml, for a crude plant extract is considered cytotoxic (Fadeyi *et al.*, 2013). Therefore, in accordance with the NCI criteria, activity of the methanolic extract of *G. fruticosus* against HeLa cell line demonstrates potent cytotoxicity, while no potent cytotoxicity was observed against HeK293, PC3, KMST6 and HepG2 cell lines since observed IC_{50} values were above the NCI criteria (Fadeyi *et al.*, 2013). This further means that *G. fruticosus* methanolic effect on these four cell lines should be considered as poor activity and does not warrant attention for either as a concern for cytotoxicity in the case of KMST6 and Hek293 or for anticancer action for PC3 and HepG2 cell lines. This observation could possibly mean that these cell lines are not as sensitive to the plant extract as HeLa cells. This may be due to resistance mechanisms expressed by these cells to the active principles contained in the extract. It could mean that these cell lines do not express transporter proteins on their cell membrane or that active efflux of the extract occurs before plant effect occurs or that these cell lines lack cellular targets on which *G. fruticosus* acts (Hanahan and Weinberg, 2011), to significantly reduce viability. Future

studies could also explore the mechanisms of this resistance while employing co-treatment with cytotoxic drugs, in a bid to improve the sensitivity of different cancer types to *G. fruticosus*, or available cancer treatments (Macedo et al, 2016), as this is a common scientific practice in recent years (Wei et al, 2015; Esfini-Farahani et al, 2017).

It's therefore noteworthy that this assay shows selectivity towards the HeLa cell line only. Cervical cancer is ranked second most diagnosed cancer in Namibia (Carrara *et al.*, 2017), and is among the top 4 most prominent cancers in women globally(Carrara *et al.*, 2017; Bray *et al.*, 2018). No report could be found of the cytotoxic activity of *G. fruticosus* extracts against cancerous cell lines used in this research through the literature with the exception of HeLa. In a study by Twilley et al, 2017, an ethanolic extract of powdered leaf material from *G. fruticosus* demonstrated activity against HeLa, with IC_{50} 51.04 ± 3.00 µg/ml. This report is contrary to observations in this study, even though its activity against Hek293 is consistent with observations in this study. The difference in the bioactivity observed in this study as compared to the study by Twilley *et al.*, (2017) may be attributed to the differences in the phytochemistry of the plant specimen investigated.(Sampaio, Edrada-Ebel and Batista Da Costa, 2016; Kumar *et al.*, 2017; Liu *et al.*, 2018). Additional reports were found of the activity of the dichloromethane/ methanol or methanol extracts prepared using either the fruiting structures, stems, leaves and roots of the plants against renal (TK10), breast (MCF7) and Melanoma (UACC62) cells lines (Fouche *et al.*, 2008). In another study, the methanolic extract of the same plant demonstrated potent activity with following IC_{50} values against urinary bladder (0.5 ±0.68 µg/ml), breast (6.2 ±1.6 µg/ml) and lung (1.2 ± 1.06 µg/ml) cancer cell lines, with IC_{50}

values all within NCI's guidelines (Mothana *et al.*, 2009).In this study, the lowest extract concentrations tested in the case of HepG2, KMST6 and Hek293 was 3.125^{-5} g/L, while 5^{-4} g/L and 3.25^{-10} g/L for PC3 and HeLa respectively all reduced cell viability by close to 50 %, demonstrating significant cytotoxicity. However, future studies need to analyze extract at lower extract concentrations to properly estimate the IC_{50} value on each cell line. Nonetheless, based on selectivity of the *G. fruticosus* extract towards HeLa, additional studies were undertaken to determine the mode of anti-proliferation. Furthermore, Hek293 was used as a control cell line for additional cell death mechanism assays. The IC_{50} value on Hek293 is over 470 times greater than that on HeLa.

The use of DMSO at 6 %, (refer to appendix B, **figure 4A**) was substantiated by the fact that DMSO at various concentrations has been shown to induce significant damage to the mitochondria and cause membrane potential deficiency, induce ROS production, leading to apoptosis and reduced cell viability even at 1 % concentration (Yuan *et al.*, 2014). This study employed DMSO concentrations of 4 to 6 % in cell lines such as HeLa and HepG2 in cell death mechanism studies, depending on the cell line and cell surface of exposure in well plates. This observation demonstrates that different cell lines and culture conditions influence the response of different cells to DMSO. While it is the proper practice to use a cytotoxic drug as positive control such as doxorubicin, ceramide, parthenolide or camptothecin, DMSO at various concentrations has been shown to significantly reduce cell viability at various concentrations, even 2 % in the case of leukemia cells (Hajighasemi and Tajik, 2017), while 5 % DMSO reduced the viability of lung adenocarcinoma cells to about 40 % (Wang *et al.*, 2012). In addition, DMSO has

also been proven to induce apoptosis in various cell lines (Koiri and Trigun, 2011). In fact, complexes containing DMSO have been proposed as viable cancer treatment options and proven efficacious *in vitro* (Jovanovic *et al.*, 2016). However, this has only been demonstrated in a limited number of cell lines and this may differ in other cell lines (Koiri and Trigun, 2011; Wang *et al.*, 2012, 2013; Yuan *et al.*, 2014; Hajighasemi and Tajik, 2017). Admittedly, it is best to use a standard control such as a drug, especially since DMSO produced not more than 40 % apoptosis induction, but standard drugs were unavailable at the time of analysis. In other studies, DMSO, which is often used to dissolve dry plant extracts, was shown to react with cytotoxic drugs such as carboplatin, cisplatin and other platinum-containing drugs, forming complexes, which result in loss of activity of the cytotoxic drug, thereby conferring protection (Hall et al, 2014).

The induction of programmed cell death was observed in Hek293 and HeLa cell lines using the APOPercentage assay which measures the phosphophatidylserine (PS) migration from the internal side of the bilayer cell membrane to the external side (Meyer *et al.*, 2008). In this study, the lower concentration (IC_{50} value on HeLa) and higher concentration (IC_{50} value on Hek293) were used as treatments on the two cells lines. The methanolic shoot extract of *G. fruticosus* positively induced apoptosis at the high extract concentration in both Hek293 and HeLa cells. Surprisingly, no significant increase in apoptosis was observed in HeLa cells treated with lower extract concentration. For HeLa cells, this observation shows that induction of apoptosis was not the cause of the observed reduction in viability, but that observed decline in cell viability may be due to other cell death mechanisms such as necrosis (Mahassni and Al-Reemi, 2013; Kwan *et al.*, 2016).

Decreased cell viability can be caused by necrosis or apoptosis. For many years, induction of apoptosis has been the desired response of anticancer treatment targets, and not necrosis. Necrosis is a cell death characterized by increased cell volume, due to uptake of water, resulting in swelling of organelles and eventual burst of the cell membrane. This end event of necrosis is not desirable as this mechanism differs from apoptosis in that, the cell products are released into the extracellular space, whereas, apoptotic cells are phagocytized and therefore the cell content isolated inside cells like macrophages (Chaabane et al., 2013). As a result, necrosis has often been considered to be uncontrolled or unprogrammed, but recent evidence has shown that necrosis may also be programmed (Chaabane et al., 2013). Several studies have reported the induction of both necrosis and apoptosis in *in vitro* bioactivity studies of drug development agents (Nirmala, Samundeeswari and Sankar, 2011; Rahmatullah, Jahan and Bashar, 2014). Necrosis is initiated due to the depletion of both ATP and NAD^+, leading to elevation of ROS and calcium within the intracellular space. Other key players in necrosis include calpains, receptor-interacting protein kinase 1 (RIPK1), tumor necrosis factor (TNF) receptor–associated factor 2 (TRAF2) and poly(ADP-ribose)polymerase (PARP) (Wu, Liu and Li, 2012). At high extract concentration, apoptosis induction of 81.6± 0.24 % was observed in Hek293 cells, while treatment at the same concentration produced an apoptosis response of 54.8 ±2.5% in HeLa cells. No significant increase in apoptosis was observed at the lower extract concentration in both cell lines. In another study, necrotic and apoptotic cells were quantified after 24 hrs of exposure to bioactive nanoparticles and the proportion of necrotic to apoptotic cells was greatest at low concentration (0.5µg/ml), while the trend was reversed at high concentration (8 µg/ml) (Kaba and Egorova, 2015). In this study, DMSO was used a positive control for apoptosis induction. Despite the

observation that apoptosis was significantly elevated in positive control when compared to untreated cells ($p=0.05$), the margin is only 5 % in comparison to untreated cells. This observation shows the need for a cytotoxic drug to be used as positive control, instead of DMSO. Koiri and Trigun,(2011) demonstrated that DMSO administered intraperitoneal to mice at approximately 7.5 g/kg per body weight was sufficient to cause apoptosis by inducing activation of the tumor necrosis factor and p53. In this study, the positive control demonstrated apoptosis induction at a level lower than the high extract concentration but this may be explained by the fact that DMSO-induced apoptosis has not been demonstrated in all cell lines. Therefore, it is recommended to use an appropriate cytotoxic control in future experiments.

The discrepancy in observations between WST-1 and APOPercentage assay may also be explained by the different mechanisms for the two assays, the WST-1 assay measures mitochondrial mediated dehydrogenase activity whilst the APOPercentage assay measures the externalization of phosphatidylserine (PS). In addition, surface areas in a 96 and 12 well plate greatly affects exposure of cells to bioactive components as orchestrated by classic volume to surface area ratio dynamics of diffusion. The production of ROS was investigated as a mechanism of apoptosis induction. The probe used in this investigation, CM-H_2DCFDA detects general oxidative stress, arising from radicals which includes H_2O_2, superoxides, hydroxyl radicals, NO. Normally, the balance of ROS is maintained by naturally occurring antioxidants in the cells. When the balance is offset, the increased amount of ROS in a cell can lead to damage to cellular macromolecules such as DNA, proteins and RNA (Stojnev, Ristić-Petrović and Janković-Veličković, 2013). In such

instances, the cell activates events leading up to programmed cell death in order to protect the integrity of the entire organism. Both extrinsic and intrinsic pathways of inducing apoptosis are possible with ROS production (Stojnev, Ristić-Petrović and Janković-Veličković, 2013).

In this study, the results demonstrate the positive induction of ROS in HeLa cells only: which was significantly elevated in cells treated with 8.35^{-7} g/L (30.47± 4.7%), and doubled to 60.5 ±4.57% at the higher extract concentration, 3.976^{-4} g/L. Therefore, general ROS generation may play an active role in inducing apoptosis and necrosis in HeLa cells (Chaabane *et al.*, 2013; Saibu *et al.*, 2014). There is evidence that the activation of ROS in cells positively induces apoptosis through several mechanisms, including checkpoint kinase 1 and 2 cell arrest, p53 and cytochrome C and other intermembrane proteins of the mitochondrial (Saibu *et al.*, 2014). Inducing ROS levels in cancerous cells above the toxicity threshold can be used as a tool to selectively target them, which would offer a selective advantage against non-cancerous cells (Panieri and Santoro, 2016), an observation seen in this study at low extract concentration (8.35^{-7} g/L). In this study, at the dose causing decline in cell viability in HeLa, ROS is significantly elevated ($p=0.001$), which is not seen at the same concentration in Hek293 cells, and incidentally coincides with high cell viability and no apoptosis induction as seen in this study. This presents an advantage, which may be exploited as a mechanism to selectively target HeLa cells. Evidence seems to suggest that ROS production at low extract concentration (8.35^{-7} g/L) favors necrosis, hence observed low apoptotic signal, while high extract concentration (3.976^{-4} g/L) favors apoptosis, since ROS thresholds dictate fate of cells (Stojnev, Ristić-

Petrović and Janković-Veličković, 2013). Normally, the production of ROS and balance with inherent antioxidants in cells seem to be the determining factor on whether cells survives or apoptosis induction ensues through the mitochondrial pathway (Marchi *et al.*, 2012). This is the first report of the ROS inducing effects of *G. fruticosus* extract in literature. In this study, 1 % H_2O_2 was used as a positive control, which significantly affected both HeLa and Hek293 cells. A study by (Xiang *et al.*, 2016) shows that different cell lines have a different H_2O_2 tolerance threshold. During the study, Xiang and colleagues exposed 293T, fibroblast and myocardial cells to a range of concentrations between 0.1-1.6 mM of hydrogen peroxide and assessed time taken by each cell line to apoptosis initiation, end or maximal level (Xiang *et al.*, 2016). A concentration dependent reversal effect was observed in all three cell lines but the response was not comparable (Xiang *et al.*, 2016). In another study, exposure of HeLa cells to concentrations of H_2O_2 levels between 50-250 μM, significantly reduced cell viability, increased dead cells and modulated the expression of caspase 3, PARP, β-actin, lactate dehydrogenase, indicating both necrosis and apoptosis as mechanisms of cell death (Park, 2014). Since cells were significantly damaged, lower concentrations of H_2O_2 should be used as positive control in future studies.

Both HeLa and Hek293 cell lines demonstrate no significant ($p=0.05$) change in caspase 3/7 cleavage, a late indicator of apoptosis, as result of treatment with *G. fruticosus* plant extract. This may mean caspase 3/7 expression is not responsible for observed apoptosis induction in either cell lines. This observation further substantiates observations of a lack of apoptosis and caspase activation in cells treated with 8.35^{-7} g/L, to indicate necrotic cell

death since necrotic cell death is devoid of caspase activity (Su *et al.*, 2016). However, since the assay only has two time points, it is possible that caspase 3/7 expression fluctuated with time (Bloemberg and Quadrilatero, 2014), especially during the earlier hours of plant extract exposure. Therefore, it is important to investigate the aspect of time, in order to properly understand the actions of *treatments* in inducing caspase 3/7 and their overall role in the intrinsic apoptosis pathway after treatment with *G. fruticosus* extract.

One common challenge faced by individuals seeking to use traditional medicine for treatment is that these are often not covered under medical insurance (Ros *et al.*, 2018) or health care products subject to government subsidies to make them more affordable to users (Antwi-baffour *et al.*, 2014). However, with science-based evidence, the acceptability of these traditional medicines can be improved, making them candidates for government subsidies and covered under health insurance, such as other conventional drugs (Antwi-baffour *et al.*, 2014). For instance, vincistine, vinblastine and paclitaxol were once isolated from traditional medicine but have gained acceptability into mainstream medicine, such that they have FDA approval (Nirmala, Samundeeswari and Sankar, 2011). Like these anticancer drugs, evidence of the anticancer action and thorough understanding of the mode of action of *G. fruticosus* may lead to its gain recognition in convectional or traditional and alternative medicine as supplement to the already existing healthcare system.

Furthermore, co-administration of herbal preparations and convectional drugs has gained popularity in research within recent years (Hashemzaei *et al.*, 2016). Other examples include the co-administration of silymarin (extracted from *Silybum marianum*) and sorafenib being investigated as a combination therapy for hepatocellular carcinoma (Cheng, Hsieh and Tsai, 2018). This is largely influenced by cancer sufferers who seek better quality of life by combining prescribed anticancer drugs and herbal traditional medicines (Cheng, Hsieh and Tsai, 2018). Unfortunately, concomitant use of traditional medicines and convectional drugs can prove dangerous in many instances, owing to negative herbal to drug interactions and may also result in treatment failure (Clarke and Mclachlan, 2011; Izzo, 2012). While this avenue has not been explored for *G. fruticosus*, co-administration of plant extract derived from the subject plant with clinically used anti-cervical cancer drugs may prove useful especially for therapy of resistant cases or other cancer types (Fridlender, Kapulnik and Koltai, 2015).This deserves full attention in subsequent studies, in order to find alternative cancer treatments, not only for cervical cancer but other cancer types too.

4.6. Conclusion

In conclusion, this paper has shown the antiproliferative effects of *G. fruticosus* methanolic extract. Anti-proliferative effect was particularly pronounced on cervical cancer cells, which shows its selectivity against prostate and liver cancer cells as well as non-cancerous skin fibroblast and embryonic kidney cells, since minimal cytotoxicity was observed on these cell lines (NCI criteria on bioactivity). Necrosis was probably the major cause of cell death in the viability investigation, instead of apoptosis, at the IC_{50} value of

HeLa. Cell death was likely caused by elevated ROS in HeLa, which shows selectivity. This creates an opportunity for treatment of cervical cancer at low extract concentration, which causes no effect in non-cancer cells. Furthermore, caspase 3/7 independent apoptosis was observed, indicating other mechanisms of cell death, especially in Hek293 cells. This is the first report of the mechanism of the anticancer action of *G. fruticosus* extract.

G. fruticosus extract to cancerous cells *in vivo* be pursued since this would be the next step in validating the plant's use for cervical cancer treatment.

4.7. References

Amaravadi, R. K. and Thompson, C. B., 2007. The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clinical Cancer Research*, *13*(24), pp. 7271–7279. doi: 10.1158/1078-0432.CCR-07-1595.

Antwi-baffour, S. S., Bello, A. I., Adjei, D. N., Mahmood, S. A. and Ayeh-kumi, P. F., 2014. The place of traditional medicine in the African society : The science , acceptance and support. *American Journal of Heath Research*, *2*(2), pp. 49–54. doi: 10.11648/j.ajhr.20140202.13.

Bloemberg, D. and Quadrilatero, J., 2014. Mitochondrial pro-apoptotic indices do not precede the transient caspase activation associated with myogenesis. *Biochimica et Biophysica Acta*, *1843*(12), pp. 2926–2936. doi: 10.1016/j.bbamcr.2014.09.002.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R., Torre, L. A. and Jemal, A., 2018. Global Cancer Statistics 2018 : GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: a cancer journal for clinicians, 68*, pp. 394–424. doi: 10.3322/caac.21492.

Brentnall, M., Rodriguez-Menocal, L., De Guevara, R. L., Cepero, E. and Boise, L. H., 2013. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biology*, *14*(1), p. 32. doi: 10.1186/1471-2121-14-32.

Bussmann, R. W., Glenn, A., Meyer, K., Kuhlman, A. and Townesmith, A., 2010. Herbal mixtures in traditional medicine in Northern Peru. *Journal of ethnobiology and ethnomedicine*, *6*, p. 10. Available at: http://www.ethnobiomed.com/content/6/1/10 (Accessed: 15 February 2018).

Carrara, H., Parken, M., Zietsman, A., Pontac, J., Lawrence, S., Horn, L. and van Schalkwyk, L., 2017. *Namibia National Cancer Registry (NNCR) Cancer incidences in Namibia 2010 - 2014*. Windhoek.

Chaabane, W., User, S. D., El-Gazzah, M., Jaksik, R., Sajjadi, E., Rzeszowska-Wolny, J. and Łos, M. J., 2013. Autophagy, apoptosis, mitoptosis and necrosis: Interdependence between those pathways and effects on cancer. *Archivum Immunologiae et Therapiae Experimentalis*, *61*, pp. 43–58. doi: 10.1007/s00005-012-0205-y.

Chan, C. K., Goh, B. H., Kamarudin, M. N. A. and Kadir, H. A., 2012. Aqueous fraphion of *Nephelium ramboutan*-ake rind induces mitochondrial-mediated apoptosis in HT-29 human coleractal adenocarcinoma cells. *Molecules*, *17*(6), pp. 6633–6657. doi: 10.3390/molecules17066633.

Cheng, Y., Hsieh, C. and Tsai, T., 2018. Concurrent administration of anticancer chemotherapy drug and herbal medicine on the perspective of pharmacokinetics, *Journal of Food and Drug Analysis*, *26*(2), pp. S88–S95. doi: 10.1016/j.jfda.2018.01.003.

Clarke, S. J. and Mclachlan, A. J., 2011. Interaction between complementary and alternative medicine with conventional anticancer medicine. *Cancer Forum*, *35*(1), p. 6. Available at: https://cancerforum.org.au/wp-content/uploads/2015/06/Mar_2011_Forum5.pdf (Accessed: 10 October 2017).

Fadeyi, S. A., Fadeyi, O. O., Adejumo, A. A., Okoro, C. and Myles, E. L., 2013. *In vitro anticancer screening of 24 locally used Nigerian medicinal plants. BMC Complementary and Alternative Medicine*. Available at: http://www.biomedcentral.com/1472-6882/13/79 (Accessed: 13 August 2018).

Fouche, G., Cragg, G. M., Pillay, P., Kolesnikova, N., Maharaj, V. J. and Senabe, J., 2008. *In vitro*

anticancer screening of South African plants. *Journal of ethnopharmacology*, *119*(3), pp. 455–61. doi: 10.1016/j.jep.2008.07.005.

Fridlender, M., Kapulnik, Y. and Koltai, H., 2015. Plant derived substances with anti-cancer activity: from folklore to practice. *Frontiers in plant science*, *6*(799), pp. 1–9. doi: 10.3389/fpls.2015.00799.

Habli, Z., Toumieh, G., Fatfat, M., Rahal, O. N. and Gali-Muhtasib, H., 2017. Emerging cytotoxic alkaloids in the battle against cancer: Overview of molecular mechanisms. *Molecules*, *22*, p. 250. doi: 10.3390/molecules22020250.

Hajighasemi, F. and Tajik, S., 2017. Assessment of Cytotoxicity of Dimethyl Sulfoxide in Human Hematopoietic Tumor Cell Lines. *Iranian journal of blood & cancer*, *9*(2), pp 48-53. Available at: www.ijbc.ir (Accessed: 14 August 2018).

Hanahan, D. and Weinberg, R. A., 2011. Hallmarks of cancer: The next generation. *Cell*, *144*(5), pp. 646–674.

Hashemzaei, M., Karami, S. P., Delaramifar, A., Sheidary, A., Tabrizian, K., Rezaee, R., Shahsavand, S., Arsene, A. L., Tsatsakis, A. M. and Taghdisi, S. M., 2016. Anticancer effects of co-administration of Daunorubicin and Resveratrol in MOLT-4 , U266 B1 AND Raji cell lines. *Farmacia, 64*(1), pp. 36–42.

Izzo, A. A., 2012. Interactions between herbs and conventional drugs: Overview of the clinical data. *Medical Principles and Practice*, *21*, pp. 404–428. doi: 10.1159/000334488.

Jovanovic, K. K., Gligorijevic, N., Gaur, R., Mishra, L. and Radulovic, S., 2016. Anticancer activity of two ruthenium(II)-DMSO-chalcone complexes: Comparison of cytotoxic, pro-apoptotic and antimetastatic potential. *Journal of B.U.ON.*, *21*(2), pp. 482–490.

Kaba, S. I. and Egorova, E. M., 2015. *In vitro* studies of the toxic effects of silver nanoparticles on HeLa and U937 cells. *Nanotechnology, science and applications*, *8*, pp. 19–29. doi: 10.2147/nsa.s78134.

Koiri, R. K. and Trigun, S. K., 2011. Dimethyl sulfoxide activates tumor necrosis factorα-p53 mediated apoptosis and down regulates d-fructose-6-phosphate-2-kinase and lactate dehydrogenase-5 in Dalton's lymphoma *in vivo*. *Leukemia Research*, *35*(7), pp. 950–956. doi: 10.1016/j.leukres.2010.12.029.

Kumar, S., Yadav, A., Yadav, M. and Parkash Yadav, J., 2017. Effect of climate change on phytochemical diversity, total phenolic content and *in vitro* antioxidant activity of Aloe vera (L.) Burm.f. *BMC Research Notes*, *10*, p. 60. doi: 10.1186/s13104-017-2385-3.

Kwan, Y. P., Saito, T., Ibrahim, D., Al-Hassan, F. M. S., Oon, C. E., Chen, Y., Jothy, S. L., Kanwar, J. R. and Sasidharan, S., 2016. Evaluation of the cytotoxicity, cell-cycle arrest, and apoptotic induction by *Euphorbia hirta* in MCF-7 breast cancer cells. *Pharmaceutical Biology*, *54*(7), pp. 1223–1236. doi: 10.3109/13880209.2015.1064451.

Liu, Y., Chen, P., Zhou, M., Wang, T., Fang, S., Shang, X., Fu, X., Cardoso, S. M. and Fazio, A., 2018. Geographic Variation in the Chemical Composition and Antioxidant Properties of Phenolic Compounds from *Cyclocarya paliurus* (Batal) Iljinskaja Leaves. *Molecules*, *23*, p. 2440. doi: 10.3390/molecules23102440.

Mahassni, S. H. and Al-Reemi, R. M., 2013. Apoptosis and necrosis of human breast cancer cells by an aqueous extract of garden cress (Lepidium sativum) seeds. *Saudi Journal of Biological Sciences*, *20*, pp. 131–139. doi: 10.1016/j.sjbs.2012.12.002.

Marchi, S., Giorgi, C., Suski, J. M., Agnoletto, C., Bononi, A., Bonora, M., De Marchi, E., Missiroli, S., Patergnani, S., Poletti, F., Rimessi, A., Duszynski, J., Wieckowski, M. R. and Pinton, P., 2012. Mitochondria-Ros Crosstalk in the Control of Cell Death and Aging. *Journal of Signal Transduction*, *2012*, pp. 1–17. doi: 10.1155/2012/329635.

McIlwain, D. R., Berger, T. and Mak, T. W., 2015. Caspase Functions in Cell Death and Disease. *Cold Spring Harbor Perspectives in Biology*, *5*, p. a026716. doi: 10.1101/cshperspect.a026716.

Meyer, M., Essack, M., Kanyanda, S. and Rees, J. G., 2008. A low-cost flow cytometric assay for the detection and quantification of apoptosis using an anionic halogenated fluorescein dye. *BioTechniques*, *45*(3), pp. 317–320. doi: 10.2144/000112908.

Mothana, R. A. A., Gruenert, R., Bednarski, P. J. and Lindequist, U., 2009. Evaluation of the *in vitro* anticancer, antimicrobial and antioxidant activities of some Yemeni plants used in folk medicine. *Pharmazie*, *64*(4), pp. 260–268. doi: 10.1691/ph.2009.8789.

Nirmala, M. J., Samundeeswari, a. and Sankar, P. D., 2011. Natural plant resources in anti-cancer therapy-A review. *Research in Plant Biology*, *1*(3), pp. 1–14. Available at: http://resplantbiol.com/article/view/243.

Omoyeni, O. A., Hussein, A., Meyer, M., Green, I. and Iwuoha, E., 2015. *Pleiocarpa pycnantha* leaves and its triterpenes induce apoptotic cell death in Caco-2 cells *in vitro*. *BMC Complementary and Alternative Medicine*, *15*, pp. 1–7. doi: 10.1186/s12906-015-0767-4.

Panieri, E. and Santoro, M. M., 2016. ROS homeostasis and metabolism: a dangerous liason in cancer cells. *Cell death & disease*, *7*(6), p. e2253. doi: 10.1038/cddis.2016.105.

Park, W. H., 2014. Anti-apoptotic effect of caspase inhibitors on H2O2-treated HeLa cells through early suppression of its oxidative stress. *Oncology Reports*, *31*(5), pp. 2413–2421. doi: 10.3892/or.2014.3084.

Ros, B., Le, G., Mcpake, B. and Fustukian, S., 2018). The commercialization of traditional medicine in modern Cambodia, *Health Policy and Planning*, *33*, pp. 9–16. doi: 10.1093/heapol/czx144.

Saibu, M., Sagar, S., Green, I., Ameer, F. and Meyer, M., 2014. Evaluating the cytotoxic effects of novel quinone compounds. *Anticancer Research*, *34*(8), pp. 4077–4086.

Sampaio, B. L., Edrada-Ebel, R. and Batista Da Costa, F., 2016. Effect of the environment on the secondary metabolic profile of *Tithonia diversifolia*: a model for environmental metabolomics of plants. *Scientific Reports*, *6*, p. 29265. doi: 10.1038/srep29265.

Schuhmacher, A., Gassmann, O. and Hinder, M., 2016. Changing R&D models in research-based pharmaceutical companies. *Journal of Translational Medicine*, *14*, p. 105. doi: 10.1186/s12967-016-0838-4.

Singh, M., Mckenzie, K. and Ma, X., 2017. Effect of dimethyl sulfoxide on *in vitro* proliferation of skin fibroblast cells. *Journal of Biotech Research*, *8*, pp. 1944–328578.

Sofowora, A., Ogunbodede, E. and Onayade, A., 2013. The role and place of medicinal plants in the strategies for disease prevention. *African journal of traditional, complementary, and alternative medicines*, *10*(5), pp. 210–229. doi: 10.4314/ajtcam.v10i5.2.

Stojnev, S., Ristić-Petrović, A. and Janković-Veličković, L., 2013. Reactive oxygen species, apoptosis and cancer. *Vojnosanitetski Pregled*, *70*(7), pp. 675–678. doi: 10.2298/VSP1307675S.

Su, Z., Yang, Z., Xie, L., Dewitt, J. P. and Chen, Y., 2016. Cancer therapy in the necroptosis era. *Cell Death and Differentiation*, *23*(5), pp. 748–756. doi: 10.1038/cdd.2016.8.

Sundquist, T., Moravec, R., Niles, A., O'Brien, M. and Riss, T., 2006. Timing your apoptosis assays. *Cell notes*, (16), pp. 18–21. Available at: www.promega.com/paguide/chap3.html.

Twilley, D., Langhansová, L., Palaniswamy, D. and Lall, N., 2017. Evaluation of traditionally used medicinal plants for anticancer , antioxidant , anti-inflammatory and anti-viral (HPV-1) activity South African Journal of Botany Evaluation of traditionally used medicinal plants for anticancer , antioxidant , anti-in f', (May). doi: 10.1016/j.sajb.2017.05.021.

Wang, C.-C., Lin, S.-Y., Lai, Y.-H., Liu, Y.-J., Hsu, Y.-L. and Chen, J. J. W., 2012. Dimethyl Sulfoxide Promotes the Multiple Functions of the Tumor Suppressor HLJ1 through Activator Protein-1 Activation in NSCLC Cells. Plos One, 7(4), p. e33772. doi: 10.1371/journal.pone.0033772.

Wang, J., Lin, D., Peng, H., Huang, Y., Huang, J. and Gu, J., 2013. Cancer-derived immunoglobulin G promotes tumor cell growth and proliferation through inducing production of reactive oxygen species. *Cell Death and Disease*, *4*, p. e945. doi: 10.1038/cddis.2013.474.

Wen, S., Chen, Y., Lu, Y., Wang, Y., Ding, L. and Jiang, M., 2016. Cardenolides from the Apocynaceae family and their anticancer activity. *Fitoterapia*, *112*, pp. 74–84. doi: 10.1016/j.fitote.2016.04.023.

Wong, S. K., Lim, Y. Y. and Chan, E. W. C., 2013. Botany, uses, phytochemistry and pharmacology of selected Apocynaceae species: A review. *Pharmacognosy Communications*, *3*(3), pp. 2–11. doi: 10.5530/pc.2013.3.2.

Wu, W., Liu, P. and Li, J., 2012. Necroptosis : An emerging form of programmed cell death. *Critical Reviews in Oncology / Hematology*, *82*(3), pp. 249–258. doi: 10.1016/j.critrevonc.2011.08.004.

Xiang, J., Wan, C., Guo, R. and Guo, D., 2016. Is Hydrogen Peroxide a Suitable Apoptosis Inducer for All Cell Types?. *BioMed Research International*, 2016. doi: 10.1155/2016/7343965.

Yin, L. M., Wei, Y., Wang, Y., Xu, Y. D. and Yang, Y. Q., 2012. Long term and standard incubations of WST-1 reagent reflect the same inhibitory trend of cell viability in rat airway smooth muscle cells. *International Journal of Medical Sciences*, *10*(1), pp. 68–72. doi: 10.7150/ijms.5256.

Yuan, C., Gao, J., Guo, J., Bai, L., Marshall, C., Cai, Z., Wang, L. and Xiao, M., 2014. Dimethyl sulfoxide damages mitochondrial integrity and membrane potential in cultured astrocytes. *PLoS ONE*, *9*(9), p. e107447. doi: 10.1371/journal.pone.0107447.

CHAPTER 5: *IN VIVO* TOXICITY EVALUATION OF THE METHANOLIC SHOOT EXTRACT OF *G. FRUTICOSUS.*

Florence Dushimemariya[1], Aloysius Lubega[2] & Davis R. Mumbengegwi[1*]

1. Multi-disciplinary Research Center, University of Namibia

2. Department of Pharmacology and Therapeutics, University of Makerere

*Corresponding author: Davis R. Mumbengegwi; email: dmumbengegwi@unam.na

5.1. Abstract

Plants have been and continue to be relevant sources of medicines, with a long history of use in Namibian communities.*Gomphocarpus fruticosus* is aused ethnomedicinaly to treat cancer-like ailments. With rising incidences of cancers and challenging health care demands, people in resource poor settings have no or limited access to adequate cancer treatment. While accessibility and affordability are key considerations under such economic conditionssafety should not be disregarded *Gomphocarpus fruticosus* has been reported to have anticancer activity in traditional settings, however, it is important for the toxicity of *G. fruticosus* extracts to be ascertained *in vivo*. The methanolic shoot extract of *G. fruticosus* was analyzed for acute toxicity in Balb C mice, administered orally and observations made for 24 hrs. Furthermore, three doses below LD_{50} (2.46^{-2}-9.84^{-2} g/kg), were administered orally over a period of 8 days with body weight, general appearance and behavior of the animals being observed. Methanolic shoot extracts of *G. fruticosus* had an acute LD_{50} of 1.967^{-1} g/kg. The obtained LD_{50} value places the methanolic extract within category 3 indicating that the shoot methanolic extract is toxic. The observed

adverse effects in experimental animals included reduced motility, hyperventilation, passing of a soft stool and a mortality rate ranging between 33.3-83.3% in treatment groups. No significant weight changes were observed in animal groups administered daily with three different extract doses below LD_{50}, when compared to untreated group. In conclusion, this study shows the toxicity of the methanolic *G. fruticosus* extract in mice, with low LD_{50} and adverse effects to the respiratory system, muscular-skeletal and gastrointestinal system. However, doses of extract below LD_{50} (2.46^{-2}-9.84^{-2} g/kg) seem to cause no overt effects, indicating the usability of the plant extract, at lower extract concentration, for possible cancer treatment, especially in a nano-particulate delivery system to improve non-specific toxicity and reduce administered dosage. Recommendations are to determine extent of toxicity of concoctions prepared similarly to methods in traditional settings (aqueous extracts), using *in vivo* models. Furthermore, additional toxicity and efficacy studies are required to clearly determine effect of extract on internal organs and biochemistry *in vivo*.

Key words: Balb C mice, category 3, diarrhea, *G. fruticosus*, GHS.

5.2. Introduction

Gomphocarpus fruticosus is a plant belonging to the Apocynaceae family. It is renowned for its uses in various traditional settings within Namibia and beyond. It is commonly used to treat stomach ailments, TB, heart conditions, pain, as an emetic, to treat nose infections, topical ailments such as itching, scabies and tumors (Fouche *et al.*, 2008; Mothana *et al.*, 2009, 2014; Ndhlala, Finnie and Van Staden, 2011; Dyubeni and Buwa, 2012; Stark, Mtui

and Balemba, 2013). The plant is known by several names in different local languages such as *|giitama||oob, ||horapob (Khoekhoegowab/Nama), Oruseppa (Otjiherero), Kapokbos, Melkbos, Tondel, Wilde kapok (Afrikaans), Wilde Baumwolle* (German) or Milkweed, Wild cotton (English). The damara community around the Grootfontein Mountains claim that a tea and powdered plant material prepared from the leaves of the plant has curative properties against cancer (Von Koenen, 2001). As a result, investigations into the *in vitro* activity of the extract revealed that methanolic shoot extract of *G. fruticosus* demonstrated both cancer growth-inhibition through necrotic cell death in HeLa (cervical cancer), in addition reactive oxygen species were significantly elevated in HeLa cells after treatment with plant extract. The plant has been studied in South Africa, Yemen, Netherlands, Portugal, Mozambique, Kenya, Egypt and Ukraine, most likely due to the presence of cardenolides (Komissarenko, Chernobai and Komissarenko, 1995; Heneidak *et al.*, 2006; Fouche *et al.*, 2008; Mothana *et al.*, 2009; Kaaria, Matiru and Ndungu, 2012; Madureira *et al.*, 2012; Marzouk, Osman and Gohar, 2016). Its cardiac glycosides are responsible for the toxic properties observed in small livestock that consume the plant, but the extent of toxicity (LD_{50}, target organs of toxicity aside from the heart) are unknown. Due to the long history of usage in man, medicinal plants are usually considered to be safer for use and also preferred because they are natural (Izzo, 2012). However, there has been a move towards the safety screening of medicinal plants. This is because herbal medicines often cause toxicity to major organs such as the liver, heart, GIT and can cause carcinogenic transformations (George, 2011; Izzo, 2012). As a result, WHO is strongly encouraging the scientific-based support for the safe use of traditional medicine, in order to supplement existing healthcare systems (WHO, 2013). This chapter will focus on acute toxicity and effect of continued exposure of plant extract

on the body weight as an indicator of safety in Balb C mice, while assessing the general well-being of experimental animals.

5.3. Methods and materials

5.3.1. Plant processing

The fresh shoot plant material was collected from the Otjozondjupa region of Namibia, in a riverbed. A voucher specimen (FD14) was deposited with the National Botanical Research Institute (NBRI) for scientific identification. The material was stored in brown paper bags for transportation to the laboratory, where the plant was cut into small pieces using a pruner. The small pieces of plant material were spread on the bench top and air dried for a period of three weeks. The plant material was further processed by grinding into powder using an industrial miller. The ground plant material was labeled and stored at 253.15 K until further use.

5.3.2. Extract preparation

Crude plant extracts were prepared by maceration of powdered plant material (10 g) in 1^{-1} L methanol at room temperature for a period of 24 hrs. After the maceration, extracts were filtered through a Whatman filter paper, using gravity filtration. Filtration was followed by rotary evaporation, which was conducted in a water-bath set at 333.15 K, pressure was set at 6000 Pa. To further remove traces of extraction solvent, extracts were dried on a freeze dryer until extracts could easily be scrapped off the round bottom flask

wall. Dry extracts were weighted and stored in air-tight containers at 253.15 K until further use.

5.3.3. *In vivo* toxicity

5.3.3.1. Animal care

Female and male Balb C mice, 28-42 days old and weighing 17.49 ± 3.01 g, locally reared and housed at the Makerere Pharmacology and Therapeutics department's animal house were used in this study. The animals were fed once a day and allowed water *ad libitum*. Animal cases made from wire mesh, glass and wood were cleaned daily. Experiment with animals was conducted in a humane manner and according to a general protocol at the Makerere University, department of pharmacology and therapeutics, based on Ghosh (2011) and Organization for Economic Cooperation and Development guidelines with slight modifications. The protocol involved a pilot study of two animals per group, treated with extracts between 2.5^{-1}-1 g/kg. The pilot study is followed by the actual study involving larger numbers of animals. Animals were fasted for 6 hrs before treatment with plant extract or saline (negative control), to maximize absorption. After determination of LD_{50}, lower extract concentrations were administered daily to access long term effects of extracts on animal behavior and other characteristics.

5.3.3.2. Acute toxicity analysis

The Acute toxicity experiment was conducted based on the procedure of Ghosh (2011) and Organization for Economic Cooperation and Development guideline 423 (OECD, 2001) with slight modifications. The animals were separated into five groups of six mice

each, which were distinctly marked, **figure 5.1.** The animals acclimatized for a minimum of 24 hrs before treatment. The animals were observed for behavioral traits before administration of plant extracts. Each group received a predetermined concentration of the plant extract calculated on weight, ranging between 1^{-1}-3.5^{-1} g/kg, following a fasting period of 12 hrs. Next, each group received the plant extract suspended in saline (Sodium Chloride Intravenous Infusion), which also served as negative control, orally administered using gastro-tube and syringe. Food was provided approximately 1 h after treatment. The animals were observed for behavior changes after administration of plant extracts, animals showing signs of toxicity or discomfort were sacrificed humanely, after 24 hrs since extract administration. The mortality rate per group were recorded and plotted against the log concentration, and resulting probit graph was used to determine the concentration resulting in 50% Death (LD_{50}). Experiments were conducted once for each concentration/treatment, per group of animals.

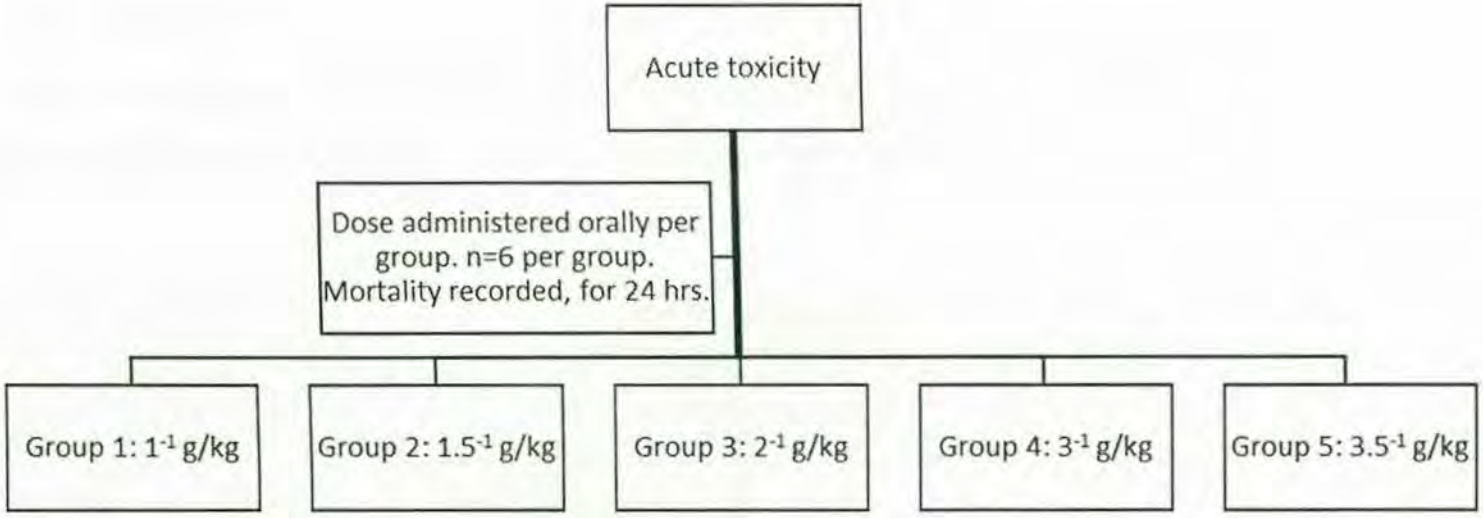


Figure 5.1. Schematic view of the acute toxicity of *G. fruticosus* extract administered as a single dose. Observations of mortality after 24 hrs per group.

5.3.3.3. Effect of prolonged exposure to G. fruticosus extract

Four groups, consisting of two animals each were used in the next experiment, **figure 5.2**. The groups received different treatments: The first group was the negative control, receiving 1.5^{-3} L saline, second group received 2.46^{-2} g/kg, and third group received 4.92^{-2} g/kg, while fourth group received 9.84^{-2} g/kg plant extract. The animals were dosed daily in accordance with

OECD guidelines (OECD, 2008). The experiment was conducted over period of 8 days and three different doses were given to the three groups of animals. The weight of each animal was taken before the start of the experiment and each day after the study commenced. Animals were allowed food and water after dosing. Animals were also observed for the duration of the experiment for behavioral changes in comparison to control group. No experimental repeats were conducted for any treatment group.

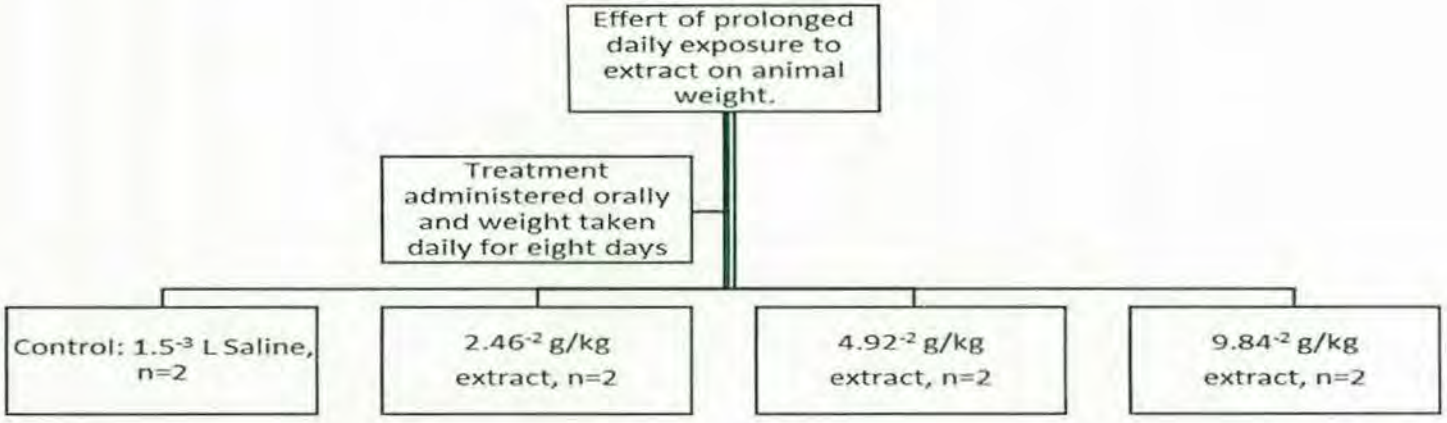


Figure 5.2. Schematic representation of sub-acute toxicity conducted for eight days by administering a daily oral dose of *G. fruticosus* methanolic extract per group, n=2.

5.4. Results

5.4.1. Acute toxicity

To evaluate the acute toxicity of Namibian methanolic *G. fruticosus* extract, six mice per group were exposed to a range of extract doses between 1^{-1}-3.5^{-1} g/kg. **Table 5.1** below shows the mortality observed at the end of the experimental duration, 24 hrs, per treatment group. In the first group treated with 1^{-1} g/kg of the plant extract, all animals survived, after 24 hrs after dosing. At 1.5^{-1} g/kg, mortality was 2 out of 6 animals, while 3 mortalities were observed in the group treated with 2^{-2} g/kg. In both group 4 and 5, the mortality rate was 83.3% respectively. No mortality was observed in animals in the control group, treated with 1.5^{-3} L saline.

Table 5.1. The toxicity effect of *G. fruticosus* shoot methanolic extracts on Balb C mice.

Group	Dose (g/kg)	Mortality/ total animals	Mortality rate %
1	1^{-1}	0/6	0.0
2	1.5^{-1}	2/6	33.3
3	2^{-1}	3/6	50
4	3^{-1}	5/6	83.3
5	3.5^{-1}	5/6	83.3

The log extract concentration and percentage of dead animals per group were used to plot a probit graph, **figure 5.3**, which was used to estimate the LD_{50} value for the acute toxicity experiment. In addition, the animals were observed for behavioral changes, by comparison with control group. The calculated LD_{50} was 1.968^{-1} ±59.16 g/kg, while a range of adverse

effects over 24 hrs, like death, disturbed digestive system (passing soft stool), heavy breathing, slow motility and in some cases, muscle spasm.

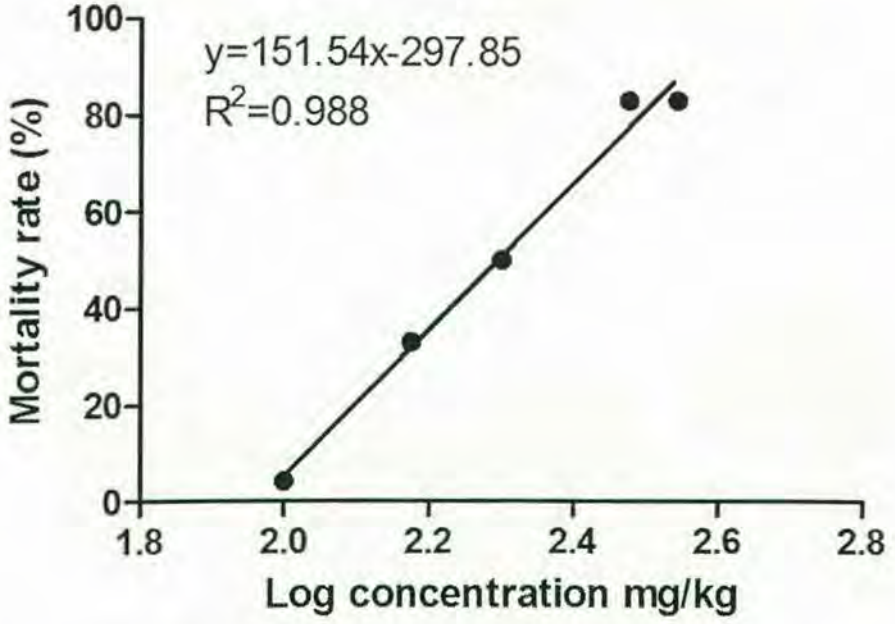


Figure 5.3. Probit plot of animal death per group treated with various extract concentrations of the methanolic shoot extract of *G. fruticosus*.

5.4.2. Effect of daily dosing of mice with fractions of *G. fruticosus* extract over 8 days

The effect of daily administration of lower fraction doses of determined LD_{50} of *G. fruticosus* over a period of 8 days further support the observed acute toxicity. Two animals per group were exposed to various plant extract doses and the change in their weight per group was compared to the control group over a period of eight days. Mean weight ±SE of the four groups are presented in **figure 5.4**, which shows the non-significant fluctuation of body weight per experimental group.

A two-way analysis of variance revealed that there was no significant difference in the change of body weight of the treated groups over the course of the experiment, p=0.05, and also in comparison to the control group. In addition, no animal death was observed among the three treated groups. However, animals in all treated groups were passing soft stool and demonstrated normal water and food intake.

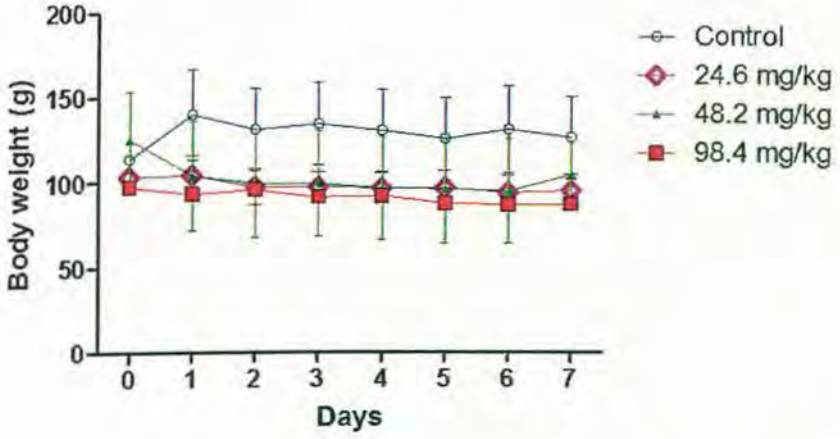


Figure 5.4. Effect of exposure to the methanolic extract of *G. fruticosus* on the weight of mice over 8 days.

5.5. Discussion

Traditionally used medicinal plants are generally perceived to be safe, especially owing to the long history of use (WHO, 2013). However, many traditional medicinal plants have been responsible for toxicity following oral use to treat various disease conditions. *G. fruticosus* is reportedly toxic (Von Koenen, 2001), owing to the presence of cardenolides and pregnane glycosides, which were not detected in this study (see previous chapter 3, despite this, the extent of toxicity is not known. According to the Globally Harmonized

Classification system of classification and labeling of chemicals (GHS), *G. fruticosus* with LD_{50} of 1.968^{-1} ±59.16 g/kg is classified as category 3, through the oral administration route (Boatman, Kelsey and Ball, 2014). This category has a range limit between 5^{-2}-3^{-1} g/kg body weight and is accompanied by a hazard warning to indicate 'toxic if swallowed.' This observation is consistent with literature, which indicated that *G. fruticosus* is considered toxic (Amiran, Lang, & Yeung, 2011, Von Koenen, 2001). This observed toxicity is probably responsible for the anticancer property the plant has demonstrated in previous sections of this study and also observed in literature (Fouche *et al.*, 2008; Mothana *et al.*, 2009). However, due to the presence of cardinolides, resulting in remarked toxicity, there is a need to develop mechanisms which allow the use of *G. fruticosus*' anticancer properties while managing its toxic effects. This mechanism is explored in a subsequent section of this study as a lipid based delivery system.

Other plants in the apocynaceae family have also been shown to be toxic. *Nerium oleander* has been found poisonous in both man and animals. It is suspected that toxicity occurs due to its actions on the Na+ K+ ATPase pumps found in cell membranes (Akhtar, Sheikh and Abbasi, 2014). In addition, cardiac effects have been demonstrated after oral exposure of 0.25 g/kg in dogs. Toxic signs included vomiting, nausea, diarrhea, tremors, pain (Camplesi *et al.*, 2017). *Calotropis procera* is a plant belonging to the apocynaceae family too, which is reportedly toxic (Ahmed *et al.*, 2016), including a citing of ocular toxicity, following accidental exposure to milky exudate from plant tissues (Waikar and Srivastava, 2015). In a report by Alonso-Castro *et al.*, (2017), eleven plants from the apocynaceae family were reported to produce a range of toxic effects, ranging from nausea, paralysis,

diarrhea, hypertension and cardiotoxicity. These plants included *Asclepias curassavica, Thevetia thevetioides, Rauvolfia tetraphylla, Plumeria rubra* (Alonso-Castro *et al.*, 2017). The findings in this report are uncommon as it is contrary to what is in literature regarding the acute toxicity of medicinally used traditional plants in recent years (Jothy *et al.*, 2011; Ping *et al.*, 2013; Kifayatullah *et al.*, 2015). Reports in literature cite that traditionally used plants are usually none toxic beyond 5000 mg/kg, which agrees with the safety expectation arising from a long history of use (WHO, 2013). Plant extracts with an LD_{50} beyond 5000 mg/kg, places these plants in category 5, the least toxic classification (Boatman, Kelsey and Ball, 2014). Besides classification and labeling of medicinal plants and chemicals, acute toxicity can help determine dosing levels and allude to modes of toxic actions resulting from plant extract use (Ping *et al.*, 2013). In addition, adverse effects such as muscle spasms, reduced motility, reduced breathing all seem to suggest that the muscular-skeletal system is affected, while a similar conclusion can be drawn about the gastrointestinal tract, due to the observed diarrhea. Further experiments need to be conducted using isolated receptor models to determine the mode of these adverse effects. For instance, exposure to chemicals acting as agonists of the Glucagon-Like Peptide 1 (GLP-1) receptors are known to result in diarrhea (Filippatos, Panagiotopoulou and Elisaf, 2014).

In this analysis, only the body weight was measured as an indicator of adverse effect to the daily exposure of the methanolic extract of *G. fruticosus*. Statistical analysis indicates that no significant difference in body weight occurred. This indicates that exposure to the various fraction doses of the extract below determined LD_{50} did not cause significant loss

of body weight, nor mortality to experimental animals. This might be due to the low extract doses used but it could also mean that body weight alone is not a sufficient indicator of toxicity. Other parameters such as histology samples prepared using major organs, biochemistry analysis to include creatinine, urea and other enzymatic markers and hematology are performed alongside this assay to further support conclusions drawn (Lakmichi *et al.*, 2011; Ghadirkhomi *et al.*, 2016; Alonso-Castro *et al.*, 2017). Animals treated with the methanolic shoot extract of *G. fruticosus* were observed to pass soft stool in both experiments. This observation seems to suggest that the alimentary canal is disturbed by the plant extract, even at lower extract concentrations.

In the traditional setting, plants for medicinal purposes are often prepared using water, which is most likely for *G. fruticosus*. This study analyzed the methanolic extract derived from the plant, which demonstrated high toxicity in this study. It is possible that residual methanol in plant extract may have affected the results observed in this study, despite that extracts had been dried to powder during preparation. Residual solvent has implications to a biological system since it has a dose and effect relationship. Methanol is classified in class 2 (solvents to be limited) with a PDE (Permitted Daily Exposure) of 30 mg per day (ICH, 2016). Its therefore necessary to determine the impact of residual solvent alone, on treated animals, in order to be confident on results obtained (Ramos, 2013). Furthermore, aqueous extracts of *G. fruticosus* should be analyzed for their toxic effects *in vivo*, in future studies.

The examination of animal organs such as liver, gut lining, lungs, heart and muscle tissue, brain, kidneys and biochemical parameters would be additional and important tools to determine the effect of *G. fruticosus* methanolic extract on experimental animals. Literature shows the importance of histological samples, hematology analysis and biochemistry, coupled with weight measurements taken for the duration of animal dosing (Kifayatullah et al., 2015). In addition, the daily dosing of animals with experimental plant was only conducted for 8 days, extension of experimental duration might give a better indication of toxic effects and extent. Observations seem to suggest adverse effects to the skeletal/muscle system, alimentary canal and the respiratory system. Additional research needs to evaluate the accumulative behavior of toxic principles found in *G. fruticosus* over a period of time.

The scientific community is currently exploring the combination of herbal and conventional drugs, in an effort to avert adverse effects brought on when using current conventional drugs for treatment (El-Sayed *et al.*, 2011) or to increase cytotoxic actions of drugs such as doxorubicin (Jeon *et al.*, 2018). It is therefore recommended that extracts derived from *G. fruticosus* be evaluated in a similar manner, especially in the search for alternative anticancer treatments. Future research efforts can focus on dose escalation and *in vivo* anticancer studies, in order to determine the therapeutic window (the doses of an active pharmaceutical agent causing therapeutic responses without any adverse effects) of *G. fruticosus*.

5.6. Conclusion

In conclusion, this study has reaffirmed the literature reports of toxicity citing of *G. fruticosus* and other members of the Apocynaceae family. *G. fruticosus* methanolic extract is toxic and is classifiable in category 3 of the Globally Harmonized Classification System. However, the results of this report, coupled with earlier reports suggest the value of the plant in treating cancer, and possibly other ailments. In view of this, it is suggested that the use of the plant be discouraged pending research, because this can result in serious adverse effects, especially since the therapeutic window has not been determined yet. Furthermore, we recommend, the development of a nanoparticulate herbal drug delivery system, which can reduce non-specific toxicity, in addition to evaluation of *G. fruticosus* in animal tumor models. This study is a step in the positive direction towards the development of an alternative and effective cancer treatment for Namibia and the global population at large.

5.7. References

Ahmed, O. M., Fahim, H, I., Boules, M. W. and Ahmed, H. Y., 2016. Cardiac and testicular toxicity effects of the latex and ethanolic leaf extract of *Calotropis procera* on male albino rats in comparison to abamectin. *SpringerPlus*, *5*(1), p. 1644. doi: 10.1186/s40064-016-3326-7.

Akhtar, T., Sheikh, N. and Abbasi, M. H., 2014. Clinical and pathological features of *Nerium oleander* extract toxicosis in wistar rats. *BMC Research Notes*, *7*(1), pp. 1–6. doi: 10.1186/1756-0500-7-947.

Alonso-Castro, A. J., Dominguez, F., Ruiz-Padilla, A. J., Campos-Xolalpa, N., Zapata-Morales, J. R., Carranza-Alvarez, C. and Maldonado-Miranda, J. J., 2017. Medicinal Plants from North and Central America and the Caribbean Considered Toxic for Humans: The Other Side of the Coin. *Evidence-based Complementary and Alternative Medicine*, *2017*, p. 28. doi: 10.1155/2017/9439868.

Amiran, M. D., Lang, Y. and Yeung, S. N., 2011. Corneal endothelial toxicity secondary to *Asclepias fruticosa*. *Eye, 25*, pp. 961–963. doi: 10.1038/eye.2011.59.

Boatman, R., Kelsey, J. and Ball, N., 2014. Acute toxicity classification for ethylene glycol mono-n-butyl ether under the Globally Harmonized System. *Regulatory Toxicology and pharmacology, 68*, pp. 41–50. doi: 10.1016/j.yrtph.2013.11.004.

Camplesi, A. C., Bellodi, C., Socha, J. J. M., Hatayde, M. R., Sobreira, M. F. da Rosa., Araujo, G. H. M. and Araujo, C. F. M., 2017. Dogs poisoned with Nerium oleander fresh leaves: clinical and electrocardiographic findings. *Ciência Rural*, *47*(6), pp. 1–6. doi: 10.1590/0103-8478cr20160970.

Dyubeni, L. and Buwa, L. V., 2012. An ethnobotanical study of plants used for the treatment of ear, nose and throat (ENT) infections in Nkonkobe Municipality, South Africa. *Journal of Medicinal Plants Research*, *6*(14), pp. 2721–2726. doi: 10.5897/JMPR10.869.

El-Sayed, E. M., El-azeem, A. S. A., Afify, A. A., Shabana, M. H. and Ahmed, A. H., 2011) 'Cardioprotective effects of *Curcuma longa* L. extracts against doxorubicin-induced cardiotoxicity in rats. *Journal of Medicinal Plants Research*, *5*(17), pp. 4049–4058. Available at: http://www.academicjournals.org/JMPR.

Filippatos, T. D., Panagiotopoulou, T. V and Elisaf, M. S., 2014. Adverse Effects of GLP-1 Receptor Agonists. *The review of diabetic studies*, *11*(3–4), pp. 202–230. doi: 10.1900/RDS.2014.11.202.

Fouche, G., Cragg, G. M., Pillay, P., Kolesnikova, N., Maharaj, V. J. and Senabe, J., 2008. *In vitro* anticancer screening of South African plants. *Journal of ethnopharmacology*, *119*(3), pp. 455–61. doi: 10.1016/j.jep.2008.07.005.

George, P., 2011. Concerns regarding the safety and toxicity of medicinal plants -An overview. *Journal of Applied Pharmaceutical Science*, *01*(6), pp. 40–44. Available at: http://www.japsonline.com/admin/php/uploads/121_pdf.pdf (Accessed: 19 January

2018).

Ghadirkhomi, A., Safaeian, L., Zolfaghari, B., Ghazvini, M. R. A. and Rezaei, P., 2016. Evaluation of acute and sub-acute toxicity of *Pinus eldarica* bark extract in Wistar rats. *Avicenna journal of phytosomedicine*, 6(5), pp. 556–566.

Ghosh, M. N., 2011. *Fundamentals of experimental pharmacology*. 5th edn. Kolkata: Hilton and Company.

Heneidak, S., Grayer, R. J., Kite, G. C. and Simmonds, M. S. J., 2006. Flavonoid glycosides from Egyptian species of the tribe Asclepiadeae (Apocynaceae, subfamily Asclepiadoideae). *Biochemical Systematics and Ecology*, *34*(7), pp. 575–584. doi: 10.1016/j.bse.2006.03.001.

ICH (2016) *Impurities: Guideline for residual solvents Q3C(R6)*. Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3C/Q3C__R6___Step_4.pdf (Accessed: 28 August 2018).

Izzo, A. A., 2012. Interactions between herbs and conventional drugs: Overview of the clinical data. *Medical Principles and Practice*, 21, pp. 404–428. doi: 10.1159/000334488.

Jeon, Y., Shin, J., Lee, S., Lee, Y. G., Kim, J. B., Kwon, H. C., Kim, S. H., Lee, K. and Han, Y. S., 2018. *Angelica gigas* Nakai has synergetic effects on doxorubicin-induced apoptosis. *BioMed Research International*, *2018*, p. 11.

Jothy, S. L., Zakaria, Z., Chen, Y., Lau, Y. L., Latha, L. Y., Sasidharan, S., 2011. Acute oral toxicity of methanolic seed extract of *Cassia fistula* in mice. *Molecules*, *16*(6), pp. 5268–5282. doi: 10.3390/molecules16065268.

Kaaria, P., Matiru, V. and Ndungu, M., 2012. Antimicrobial activities of secondary metabolites produced by endophytic bacteria from selected indigenous Kenyan plants. *African Journal of Microbiology Research*, *6*(45), pp. 7253–7258. doi: 10.5897/AJMR12.785.

Kifayatullah, M., Mustafa, S. M., Sengupta, P., Sarker, M. R. and Das, A., 2015. Evaluation of the acute and sub-acute toxicity of the ethanolic extract of *Pericampylus glaucus* (Lam.) Merr. in BALB/c mice. *Journal of Acute Disease*, *4*, pp. 309–315. doi: 10.1016/j.joad.2015.06.010.

Komissarenko, N. F., Chernobai, V. T. and Komissarenko, A. N., 1995. New cardenolides from the leaves of *Gomphocarpus fruticosus*. *Chemistry of Natural compounds*, *31*(6), pp. 694–700.

Lakmichi, H. *et al.*, 2011. Toxicity Profile of the Aqueous Ethanol Root Extract of Corrigiola telephiifolia Pourr . (Caryophyllaceae) in Rodents. 2011. doi: 10.1155/2011/317090.

Madureira, A. M. *et al.*, 2012. Antibacterial activity of some African medicinal plants used traditionally against infectious diseases. *Pharmaceutical biology*, *50*(4), pp. 481–489. Available at: http://www.tandfonline.com/doi/pdf/10.3109/13880209.2011.615841?needAccess=true (Accessed: 31 August 2017).

Marzouk, A. M., Osman, S. M. and Gohar, A. A., 2016. A new pregnane glycoside from *Gomphocarpus fruticosus* growing in Egypt. *Natural Product Research*, *30*(9), pp. 1060–1067. doi: 10.1080/14786419.2015.1107059.

Mothana, R. A. *et al.*, 2014. Evaluation of the *In Vitro* Antiplasmodial, Antileishmanial, and Antitrypanosomal Activity of Medicinal Plants Used in Saudi and Yemeni TraditionalMedicine. *Evidence-based complementary and alternative medicine*, *2014*, p. 7. doi: 10.1055/s-0033-1351928.

Mothana, R. A. A. *et al.*, 2009. Evaluation of the *in vitro* anticancer, antimicrobial and antioxidant activities of some Yemeni plants used in folk medicine. *Pharmazie*, *64*(4), pp. 260–268. doi: 10.1691/ph.2009.8789.

Ndhlala, A. R., Finnie, J. F. and Van Staden, J., 2011. Plant composition, pharmacological properties and mutagenic evaluation of a commercial Zulu herbal mixture: Imbiza ephuzwato. *Journal of Ethnopharmacology*, *133*(2), pp. 663–674. doi: 10.1016/j.jep.2010.10.053.

OECD .,2001. OECD guideline for testing of chemicals: Acute Oral Toxicity – Acute Toxic Class Method. (December), pp. 1–14. doi: 10.1787/9789264071001-en.

OECD., 2008. OECD GUIDELINES FOR THE TESTING OF CHEMICALS Repeated Dose 28-Day Oral Toxicity Study in Rodents. pp. 1–13. Available at: https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecdtg407-2008.pdf (Accessed: 21 January 2018).

Ping, K. Y. *et al.*, 2013. Acute and Subchronic Toxicity Study of *Euphorbia hirta L.* Methanol Extract in Rats. *BioMed Research International*, 14, p. 14. doi: 10.1155/2013/182064.

Ramos, C. S., 2013. Development and validation of a headspace gas chromatographic method for determination of residual solvents in five drug substances', *International Journal of Pharmaceutical Science Invention*, *2*(3), pp. 36–41. Available at: www.ijpsi.org (Accessed: 28 August 2018).

Stark, T. D., Mtui, D. J. and Balemba, O. B., 2013. Ethnopharmacological survey of plants used in the traditional treatment of gastrointestinal pain, inflammation and diarrhea in Africa: Future perspectives for integration into modern medicine. *Animals*, *3*, pp. 158–227. doi: 10.3390/ani3010158.

Waikar, S. and Srivastava, V. K., 2015. Calotropis induced ocular toxicity. *Medical Journal Armed Forces India*, 71(1), pp. 92–94. doi: 10.1016/j.mjafi.2012.08.017.

WHO., 2013. *WHO traditional medicine strategy 2014-2023*. Available at: http://apps.who.int/medicinedocs/documents/s21201en/s21201en.pdf (Accessed: 1 August 2017).

CHAPTER 6: LIPID-ENCAPSULATED *G. FRUTICOSUS* EXTRACT CAUSES ACCELERATED CELL DEATH IN HELA CELLS.

Florence Dushimemariya[1], Jyoti Sharma[2], Taahirah Boltman[3], Mervin Meyer[3] & Davis R. Mumbengegwi[1*]

1. Multi-disciplinary Research Center, University of Namibia

2. Hair and Skin Research Laboratory, University of Cape Town

3. Department of Biotechnology, University of Western Cape

*Corresponding author: Davis R. Mumbengegwi; email: dmumbengegwi@unam.na

6.1. Abstract

Plant have been used in Africa since time immemorial as sources of medicines in traditional settings, more recently they are used as sources of novel therapeutic compounds in mainstream medicine. However, poor dissolution and nonspecific toxicity often limit the use of plant compounds in the fight against diseases such as cancer globally. In this study, a lipid-based nanoparticulate delivery system was developed to improve the delivery properties of extracts of *G. fruticosus* extract, a plant used in Namibia to treat solid tumors. The lipid-based phytosomes were characterized by particle size (DLS), zeta potential and polydispersity index and the morphology was determined using scanning electron microscopy (SEM). The encapsulation efficiency of the lipid-based phytosomes was measured using spectrophotometry and their stability was evaluated. Further, the cellular uptake and apoptosis inducing effect of the lipid-based phytosomes was demonstrated in HeLa cells using flow cytometry and fluorescent microscopy following labelling with Rhodamine. The lipid-based empty nanoparticles/ phytosome were

spherically shaped and ranged from 130-150 nm using DLS after extrusion, which is within particle size range to facilitate absorption, circulation and accumulation in body organs in *in vivo* studies. The zeta potential was -0.8±1.8 to -1.6 ±0.6 mV but this was not sufficient to confer particle stability in biological media. Encapsulation of *G. fruticosus* extract in lipid vesicle was 96.51%, depicting high encapsulation efficiency (EE%). The phytosomes were taken up by HeLa cells as confirmed by flow cytometry and fluorescent microscopy. Flow cytometry further showed a shift of the cell population towards apoptosis, after treatment with phytosomes. This study demonstrated that phytosomes prepared from Namibian *G. fruticosus* extracts have potential for development as a cervical cancer treatment. Phytosomes demonstrated improved activity and acted in a short time interval. Furthermore, the encapsulation of bioactive plant extracts offers the opportunity to reduce dose required to effectively achieve cell death. Further research is required to optimize stability and shelf life of phytosomes before their evaluation in small animal disease models. The phytosomes can then be further developed for sustained release, improved bioavailability and targeted therapy, which may help meet the demand for affordable anticancer drugs in Namibia and elsewhere.

Key words: Bioavailability , *G. fruticosus*, HeLa, low confluency, phytosomes..

6.2. Introduction

Despite plants being a resource of biologically active molecules, the drug discovery and development pipeline is faced with two major challenges. The first challenge is that most drugs demonstrate poor aqueous solubility (Nirmala, Samundeeswari and Sankar, 2011)

and poor absorption. Molecular size and polarity often hinder passive uptake of bioactive molecules across membranes. This affects concentration of bioactive molecules at sites of action, contributing to low bioavailability. According to Savjani, Gajjar and Savjani (2012), over 40% of chemical leads for alternative treatments are water insoluble. In a review of medicinal plant derived compounds in clinical use or clinical trials, Nirmala, Samundeeswari and Sankar (2011) described a range of plant-derived drugs with potential therapeutic use but poor aqueous solubility. This has led to the development of synthetic derivatives such as combretastatin A-4 disodium phosphate, a derivative of combretastatin (Nirmala, Samundeeswari and Sankar, 2011), in order to mitigate against this challenge. Poor bioavailability can also lead to treatment failure and development of resistance. The second largest challenge faced in cancer therapy is the toxicity of available conventional drugs. Many drug candidates demonstrate non-selective toxicity, which leads to the occurrence of side effects, an example is Colchicine which is a potent anticancer plant-derived compound active against a number of cancers, such as leukemia. However, it's development as an anticancer treatment option is hindered by its toxicity (Nirmala, Samundeeswari and Sankar, 2011). Several derivatives of colchicine have been developed such as colchicoside, thiocolchicocide and 3-demethyl colchicine.

Nanoparticles have advanced drug delivery due to their surface to volume ratio, various types of nanoparticles such as dentrimers, nanofibers, polymeric nanoparticles, nanocrystals, liposomes and phytosomes are being developed (D'Souza, 2014). Phytosomes are a nanoparticulate delivery system, designed to encapsulate herbal material for drug delivery purposes (Awasthi, Kulkarni and Pawar, 2011). Phytosomes offer many

advantages such as improved absorption of poorly aqueous soluble herbal medicines, while targeting these to diseases sites and offering protection from damage associated with oral administration of therapeutics (Awasthi, Kulkarni and Pawar, 2011). In fact, phytosomes present other avenues for drug administration, through the parenteral route (subcutaneous, intravenous, intramuscular) (Mudshinge *et al.*, 2011; De Smet *et al.*, 2013), which improves bioavailability to 100% (Hippalgaonkar, Majumdar and Kansara, 2010), as aerosols (El-Sherbiny, El-baz and Yacoub, 2015).

In this study, phytosomes were formulated by encapsulating methanolic extracts of *G. fruticosus*, a plant used in Namibia with reported anticancer properties. The phytosomes were then characterized and evaluated for activity against HeLa cells as compared to a lipid-based formulation (nanoparticle) without plant extracts.

6.3. Methods and materials

6.3.1. Plant processing

G. fruticosus was collected in the Otjozondjupa region of Namibia, a voucher specimen (FD14) was prepared in a plant press, for identification and deposited with the herbarium at the National Botanical Research Institute (NBRI). Furthermore, the aerial/ above ground parts including leaves, fruiting structures and stalks were harvested and placed in brown paper bags. The plant material was sorted to remove debris before being cut into small pieces to facilitate drying (1^{-2}-2^{-2} m long). Plant material was dried on an open bench

top for 21 days, followed by grinding into powder using an industrial miller. The ground plant material was labelled and stored at 253.15 K until further needed.

6.3.2. Extract preparation

The powdered plant material (10 g) derived from the shoot of *G. fruticosus* was macerated in methanol (1^{-1} L). The mixture was left at room temperature for 24 hrs, followed by gravity filtration through a Whatman filter paper. Filtration was followed by rotary evaporation, which was conducted in a water-bath set at 333.15 K, pressure was set at 6000 Pa. To further remove traces of extraction solvent, extracts were dried on a freeze dryer until extracts could easily be scrapped off the round bottom flask wall. Dry extracts were weighted and stored in air-tight containers at 253.15 K until further use.

6.3.3. Formulation of nanoparticles/ phytosomes and their characterization.

To improve solubility of plant extracts, the methanolic extract of *G. fruticosus* was encapsulated into nanoparticles using a mixture of L-α-phosphatidylcholine (50 mg) (Sigma Aldrich) and cholesterol (0.2 mg) (Sigma Aldrich), using a method called thin layer hydration (Rasaie *et al.*, 2014). Firstly, 3 different phytosomes formulations containing varying quantities (0.5, 1 and 2 mg) of plant extract were formulated to optimize the encapsulation efficiency. A calibration curve was developed using spectrophotometry was used to determine that phytosomes prepared using 2^{-3} g extract had better encapsulation efficiency (refer to appendix C, **table 6A**), in comparison to those prepared using 5^{-4} or 1^{-3} g plant extract. The size, polydispersity index and zeta potential

of the phytosomes were measured before and after manual extrusion (refer to appendix D, **figure 6A-C**).

Following this, empty nanoparticles and phytosome (plant extract loaded): both labeled with rhodamine B (Sigma Aldrich) were prepared with different formulations according to **table 6.1,** the ratio of phosphatidylcholine and cholesterol was maintained as 250:1 (W/W), which was similar to phytosomes formulated in previous section. Both nanoparticles and phytosomes were extruded manually to a range of 130-150 nm, using an extruder (Avanti Polar Lipids), through a 200 and 100 nm membrane filters. The extruded formulations were compared to unextruded formulations using dynamic light scattering (DLS), Scanning Electron Microscopy (SEM), Zeta potential (ZP), encapsulation efficiency (EE) and polydispersity index (PDI). Particle size analysis using DLS was conducted on a suspension of nanoparticles and phytosomes in a Malvern Zetasizer NanoSeries. Light was refracted at 90 ° angle at 298.15 K. Approximately 8^{-4} L of the homogenous lipid suspension was analyzed in a standard disposable cuvette. A folded capillary zeta cell was used for the ZP analysis, by injecting 1^{-3} L of a homogenous nanoparticulate suspension into the tube. For each sample, three measurements were taken and presented as average ± standard error.

Table 6.1. Constitution of lipid-based nanoparticles/ phytosome.

Particle description	Cholesterol :PC(W:W)	Plant extract (g)	Rhodamine (g)	Methanol (L)	Chloroform (L)

Nano-particle		-	2^{-5}	1^{-3}	9^{-3}
	250:1				
phytosome		2^{-3}	2^{-5}	1^{-3}	9^{-3}

Key: PC= Phosphatidylcholine, W:W=Weight:Weight

The two formulations (nanoparticles and phytosomes) were also analyzed for surface morphology, before and after extrusion, for shape and size using Scanning Electron Microscopy (SEM). Samples were prepared by adding $\approx 5^{-5}$ L of a homogenous suspension of the formulations onto a metal stub mounted with a carbon coated surface. The samples were left to air dry in a fumehood for 48 hrs then coated with a carbon coater for 5 min before analysis using Auriga Gemina SEM instrument. Resulting micrographs were further analyzed using ImageJ, to average a minimum of ten distinct nanoparticles per sample. Size using SEM is presented as mean ± SE.

Two formulations (nanoparticles and phytosomes) containing rhodamine were analyzed for encapsulation efficiency (EE%) using the indirect method (Omwoyo *et al.*, 2014), in preparation for their analysis on cell lines. To determine the encapsulation efficiency, a calibration curve was developed by initially doing a scan to determine the extract absorption maximum between 200-500 nm, at various extract concentrations dissolved in PBS. The wavelength with the highest absorption was determined as 269.5 nm. Following this, extract concentrations between 5^{-2}-1.5^{-1} g/L were measured in triplicate to develop

calibration curve in accordance with Beer Lambert's law of linearity. This curve was used to determine concentration of extract suspended in supernatant obtained from nanoparticluate solution after pelleting samples. Therefore, the samples were spun down at 16 400 rpm for 2 hrs in a centrifuge. The supernatant was aspirated and diluted 5 fold with PBS before reading at 269.5 nm using a UV-Vis (GBC Cintra 2020). The experiment was conducted in triplicate and the average absorbance readings were used to determine the encapsulation efficiency (EE%) according to the formula as follows (Yi *et al.*, 2014).

EE% = (Initial extract weight - Weight of free extract/ Initial extract weight)* 100

6.3.4. Nanoparticulate stability under experimental conditions

To measure the stability of nanoparticles and phytosomes in cell culture media, 1^{-3} L suspension of each of the rhodamine labelled formulations were spun down at 16 400 rpm to collect the pellet and remove any free extract not encapsulated. The pellet was re-suspended in 1^{-3} L of either DMEM or RPMI1640 containing 10% fetal bovine serum, L-glutamine and 1 % streptomycin and penicillin. Incubation followed for a period of 24 hrs in a humidified environment set at 310.15 K and 5 % CO_2. After 24 hrs, the media was aspirated after centrifuging and resuspension of pellet in PBS. The nanoparticles and phytosomes were then analyzed for size (DLS), polydispersity index and zeta potential using a Malvern Zetasizer instrument (Moore et al., 2015).

6.3.5. Rhodamine-labelled phytosome uptake in HeLa cells

To observe uptake of rhodamine labelled phytosomes in HeLa cells, the cells were exposed to phytosomes for 4 hrs, following this, the treated cells were seeded in 6 well plate containing a sterile glass coverslip at a density of 3×10^8 cells per L. The cells were then observed for morphological changes in comparison to control untreated cells and images were captured using a fluorescent microscope (Axioplan-2 Imaging Fluorescent Microscope) under a rhodamine filter for red emission and in bright field. Photomicrographs were taken under both exposures and the images were overlayed to reveal the localization of the fluorescent spots.

6.3.6. Apoptosis inducing effects of rhodamine-labeled phytosomes in HeLa cells

The pro-apoptotic activity of nanoparticles/phytosomes was evaluated using HeLa cells. The cells were seeded at a density of 2×10^5 cells per well and allowed to adhere in a humidified incubator, 5% CO_2 at 310.15 K for 24 hrs, in 5^{-4} L media. Spent media was removed and replaced with 500 µl fresh media containing nanoparticles, phytosomes (equivalent to 3.976^{-4} g/L plant extract) and media only. Incubation was allowed for a period of 24 hrs and cells were observed under a microscope at end of experiment. Spent media was removed before cell harvesting by gentle trypsinization, centrifugation and pellet incubated in APOPercentage dye (Meyer et al., 2008). Cells were analyzed using flow cytometry (Becton Dickinson FACscan machine). Filters FL1 (533/30 nm) and FL4 (675/25 nm) were used to capture fluorescent signals produced by Rhodamine B and APOPercentage dye on an x and y plot respectively.

6.4. Results

Table 6.2 presents physical characteristics of lipid-based formulations such as size (DLS and SEM), polydispersity index (PDI) and zeta potential of the formulations before and after it was extruded. The diameter of particles ranged between 1^{-6}-2^{-6} m before extrusion but was reduced to a range of 1.3^{-7}-1.5^{-7} m after extrusion by DLS method. On the other hand, size ranged between 9.5^{-8}-1.1^{-7} m and 8^{-8}-9.5^{-8} m before and after extrusion, using SEM respectively. The results show a remarkable difference in particle size as determined by the two methods. The PDI significantly reduced from a range of 0.5-1 before extrusion and 0.19-0.2 after manual extrusion. However, no significant change in the zeta potential ($p=0.05$) was observed before and after extrusion. **Table 6.**2 shows that both nanoparticles and phytosomes were negatively charged. Furthermore, the zeta potential ranged between -0.87 and -1.5 before and it was -0.62 to -1.6 after extrusion. **Figures 6A-C**, appendix D, shows the physical parameters of phytosomes and nanoparticles whilst table 6A, shows encapsulation efficiencies of phytosomes formulated using different extract quantities. This allowed for optimization of phytosomes for carrying capacity (refer to appendix C, **table 6A**).

Table 6.2. Physical characteristics of nanoparticles/ phytosomes.

Measured parameter		**Formulation**	
		Nanopartticles	**Phytosomes**
Particle size[1] (nm)	Un-extruded	1158.4 ± 149.1	1964.9 ± 171.2
	Extruded	139.3 ± 2.1	143.2 ± 2.2
Particle size[2] (nm)	Un-extruded	98.6 ± 1.4	107.8 ± 1.8
	Extruded	92 ± 9.1	82.4 ± 3.7
PDI	Un-extruded	1.0 ± 0	0.48 ± 0.03

	Extruded	0.194 ± 0.004	0.197 ± 0.003
ZP (mV)	Un-extruded	-0.87 ± 1.1	-1.5 ± 1.0
	Extruded	-0.82 ± 1.8	-1.6 ± 0.6

Key. Particle size[1] by DLS and Particle size[2] by SEM. PDI: Polydispersity index, ZP: Zeta potential. Data presented as Mean ± SE.

Table 6.2 and **figure 6.1** show the morphology of the lipid based formulations before and after extrusion. SEM analysis shows that particle diameter ranged 108 ±1.8 nm to 82.4 ± 3.7 nm. The micrographs showed spherical structures with uneven surface morphology, **figure 6.1**. Generally, average diameter of the nanoparticles and phytosomes show a reduction after extrusion.

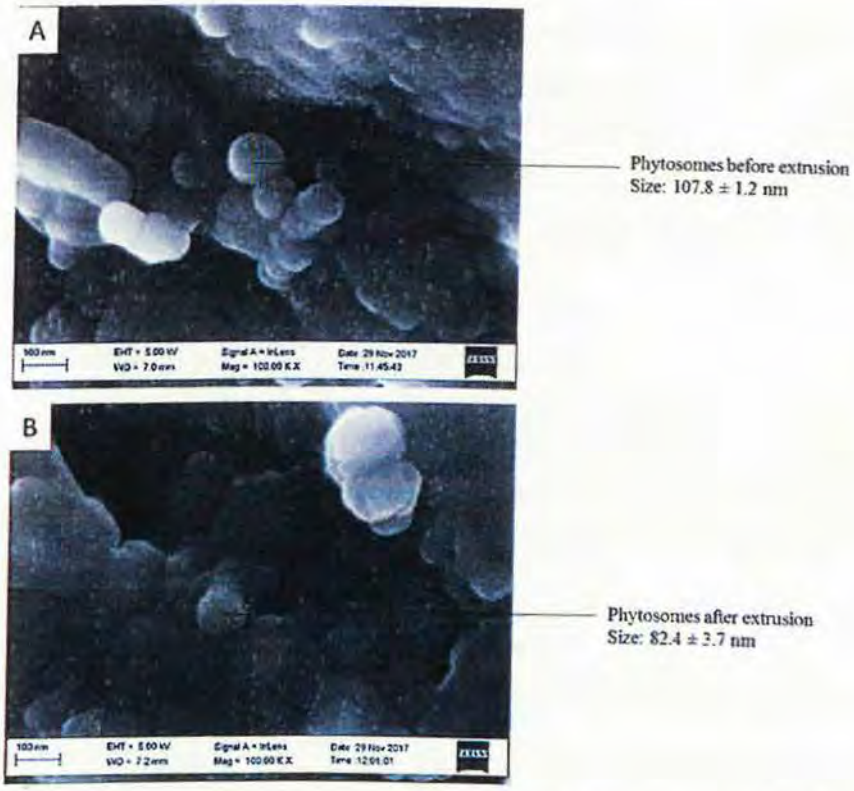


Figure 6.1. Micrographs of phytosomes before and after extrusion using SEM. Micrographs A represents before extrusion, while B shows after extrusion.

Previously, a calibration curve (refer to appendix E, **figure 6D**) developed using different plant extract concentrations was established and used in this analysis. The variance in absorbance between supernatant obtained after ultra-centrifugation of nanoparticles and phytosomes was statistically significant, p=0.01, **table 6.3**. This difference in absorbance was attributed to the presence of plant extract, since this was the only distinction between the two formulations. This calculated variance, was further used to calculate encapsulation efficiency (EE) as a percentage of the initial plant extract incorporated during formulation of the phytosome, **table 6.4.**

Table 6.3. Average absorbance of the formulated nanoparticles and phytosomes.

Phytosome description	Mean absorbance ±SE	Difference in absorbance	Statistical difference
Nanoparticles	0.36 ±0.0018	0.0341	**
Phytosomes	0.39 ±0.0028		

Key: SE: Standard error, **: significant difference, p=0.01.

Table 6.4. Encapsulation efficiency of phytosomes.

A (g)	B (x value)	C (g)	D (g)	E (g)	EE %
2^{-3}	0.034	1.39^{-5}	6.97^{-5}	1.9303^{-3}	96.51

Key: A: Initial extract weight, **B**: Absorbance value from table 4.4, **C**: Extrapolated extract using y=0.0065x-0.0585, **D**: Free extract per 5^{-3} L formulation (**C** ×5), **E**:(encapsulated extract per 5^{-3} L): **A-D**.

Figure 6.2 shows the physical characteristics of the two formulations before and after exposure to biological media containing 10% FBS. Measurements taken of each formulation before incubation served as a comparison tool after incubation with media, for each individual formulation. The effect of incubation in two different media on the particle size is shown in **figure 6.2A**. Before incubation, particle diameters ranged from

138.1 ±1.7 nm to 183±1.7 nm. However, after incubation in RPMI1640 and DMEN media, diameters of the nanoparticle significantly increased to 2.07^{-7} ± 2.6 m and 1.64^{-7} ±1.2 m respectively. No significant change in diameter was observed in phytosomes, which were incubated in RPMI1640 media, while diameter significantly decreased in those incubated in DMEM, 1.58^{-7} ± 2.3 m.

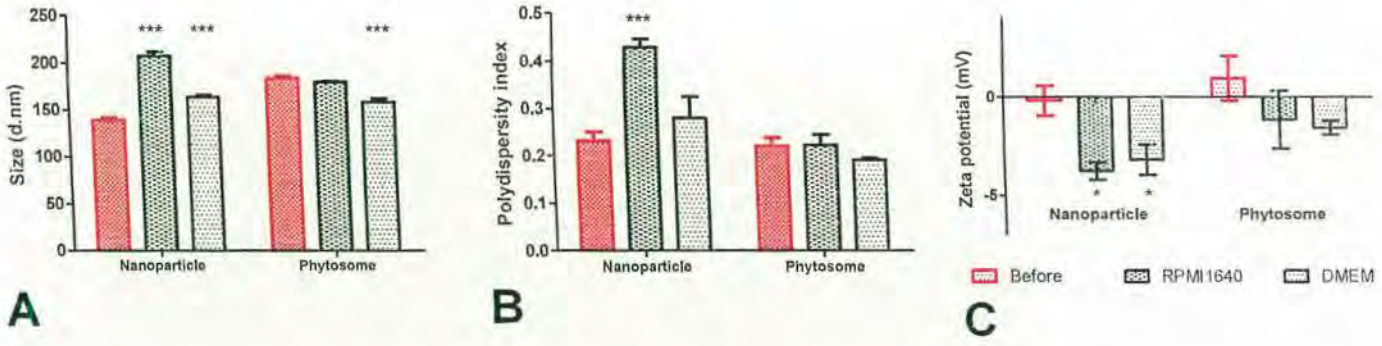


Figure 6.2. Stability of nanoparticle and phytosome formulations in media (RPMI1640 and DMEM). Comparisons before and after incubation for 24 hrs. Analysis in three panels for of size (A), PDI (B) and ZP(C). Data expressed as mean ±SE (n=3). (* or ** or *** was considered significantly different in comparison to measurement before, for each formulation, p=0.05 or 0.01 or 0.001 respectively). Asterisks denote a significant difference.

The effect of media on particle polydispersity is presented in **figure 6.2B**. PDI of nanoparticles incubated in RPMI1640 media significantly increased, in comparison to the PDI before incubation in media 0.43 ± 0.08. Interestingly, PDI did not change in phytosomes, **figure 6.2B**.

The effect of incubation of the nanoparticle and phytosomes in different media on the zeta potential is shown in **figure 6.2C**. Both nanoparticles and phytosomes remained negatively charged after incubation in either DMEM or RPMI1640. Nanoparticles incubated in

DMEM and RPMI1640 showed a significant increase in negativity. There was no significant change in zeta potential observed in the phytosomes.

Figure 6.3 shows the picture taken by overlay of two images of the same field, one under bright field and the other by exciting the sample with fluorescent light. The resulting image shows fluorescent areas outside the cells but also within cells, **figure 6.3.**

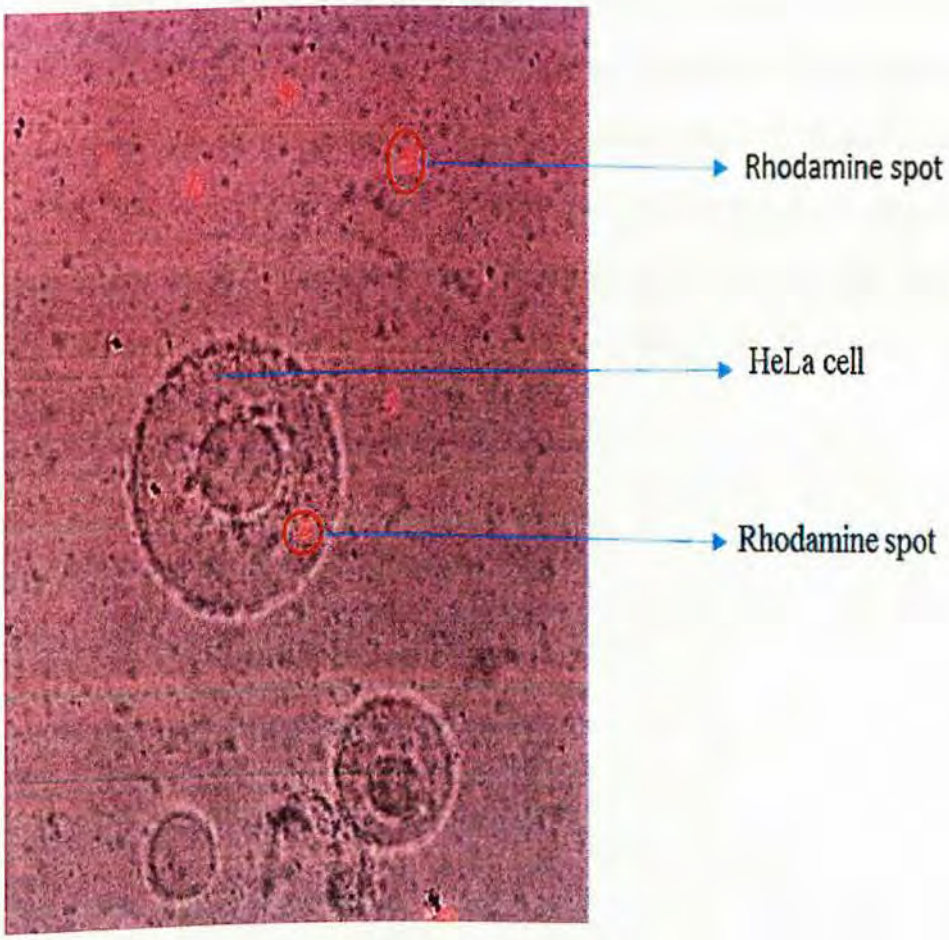


Figure 6.3. Live cell fluorescent imagery of HeLa cells exposed to phytosomes after 4 hrs, showing localization of rhodamine inside Hela cells.

The apoptosis inducing effects of rhodamine-labeled nanoparticles and phytosomes in HeLa cells are shown in **figure 6.4**. Cells receiving no treatment were compared to cells treated with nanoparticles and phytosomes. Less than 1% of these HeLa cells were undergoing apoptosis (upper and lower right quadrants). Cells treated with nanoparticles showed an increase in rhodamine signal in upper left quadrant, 11.8%, and about 0.7% cells undergoing apoptosis (seen as cells in the upper and lower right quadrants). Cells treated with phytosomes show an increase in rhodamine stained cells (11.4%) and 5.4% cells undergoing active apoptosis. In addition, cells treated with phytosomes displayed reduced confluency in comparison to cells treated with nanoparticles only and the negative control. Cells treated with phytosomes also showed classic evidence of apoptosis, such as nuclei condensation and rounded detached morphology. The bottom left quadrants in A-C demonstrated cells which were neither positive for rhodamine signal nor APOPercentage dye.

Treatment:	Negative control	Nanoparticles	Phytosomes
Confluency:	≈70%	≈60%	≈40%
Description:	Distinct nuclei	Distinct Nuclei	Rounded mass/ no nuclei

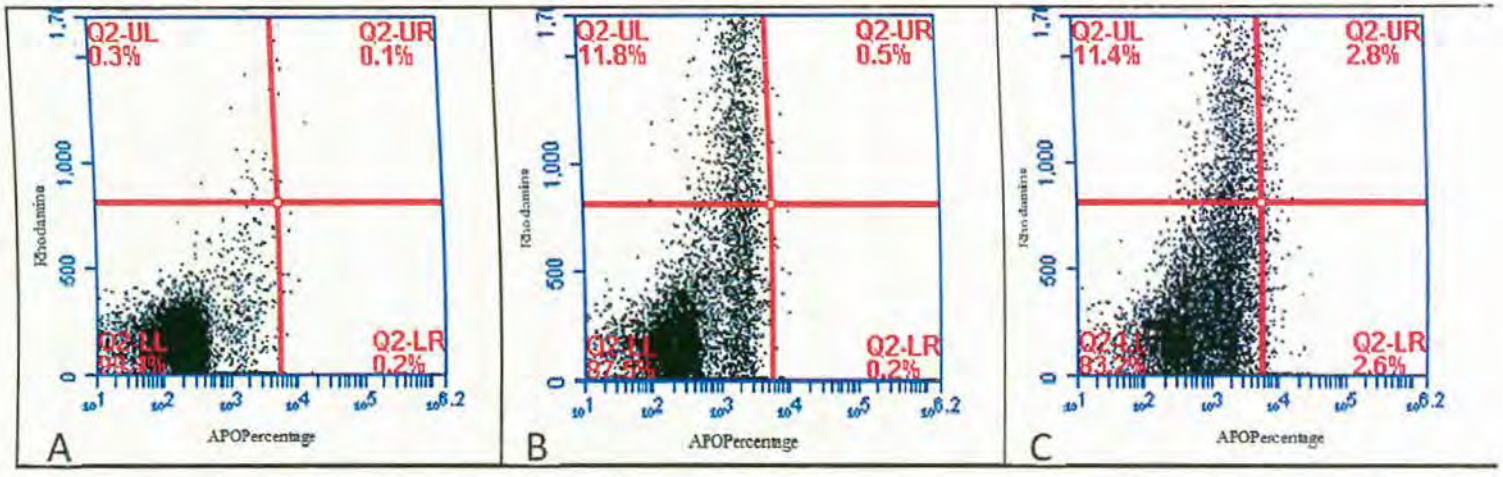


Figure 6.4. APOPercentage assay on HeLa cells using nanoparticles and phytosomes. Results show a shift of the cell population to on the x-axis, coupled with y-axis, in comparison to negative control cells. Rhodamine signal on y-axis shows uptake of nanoparticles and phytosomes in cells. Treatment with phytosomes resulted in significant reduction in confluence.

6.5. Discussion

To characterize the nano-formulations, physical characteristics were investigated. Manual extrusion significantly reduced the diameter of all formulations from 1000-2000 nm to 130-150 nm. The reduction in size of the phytosomes was necessary to make uptake into cells possible (Salatin, Dizaj and Khosroushahi, 2015). A small size also prevents nanoparticles/phytosomes from being eliminated in an *in vivo* system by the hepatic first-pass effect, and permits crossing of the blood-brain barrier (BBB) through fenestrations found in vascular tissues (Gaumet *et al.*, 2008). The phytosomes also demonstrated a significant decrease in particle size spread, as measured by the PDI, after manual extrusion from broadly dispersed to a narrowly spread suspension after extrusion, as demonstrated by DLS. The zeta potential (ZP) which is a measure of the repulsive forces between similarly charged particles (Mpofu, Msagati and Krause, 2014) did not change after manual extrusion. The ZP influences the stability of the particles in the suspension. The more negatively charged the zeta potential, the more stable the particles are. A ZP range between -30 to -60 mV indicates good stability of particles in suspension (Salopek, Krasic and Filipovic, 1992). Lipid based formulations were negatively charged but not sufficiently negative to confer stability or long shelf life. This observation is further supported by agglomeration which is evident in stability studies (Salopek, Krasic and Filipovic, 1992) and generally presents as an increase in particle size. The results generally show an increase in phytosome size, PDI and alteration of charge around particles, after the incubation period for all formulations in media. Furthermore, increase in the particle diameter of the formulations may be due to the attraction of proteins found in media, onto the particle surface area, forming a corona (Hansen & Thünemann 2015). Incubation in

media also affected the ZP of formulations. The initial ZP of each formulation was in the range -5 to +5 mV. Future studies should also ascertain the shelf life of phytosomes stored in solutions such as phosphate buffered saline, especially at various pH values and temperatures.

Other reports show that physical characteristics of the particles in solution such as particle size, zeta potential and polydispersity index can be used as an indicators of stability (Moore et al. 2015; Shah et al. 2014; Pavlin & Bregar 2012; Argentiere et al. 2016). However, it is recommended to use additional methods to establish stability, including shelf life and half-life of formulations. The formulation of the phytosomes should be optimized by varying the composition of cholesterol, the plant extract and phosphatidylcholine ratios. Rasaie et al., (2014) demonstrated that the addition of cholesterol improved stability of quercetin phytosomes by 3 weeks, which can significantly increase shelf life. In addition, the use of a negatively charged phospholipid such as phosphatidylserine (Li *et al.*, 2015), may also contribute to the negative charge on lipid formulations, therefore to the stability of resulting lipid particles. Other methods can employ surface modifications of the phospholipid head or tail region, with structures that may increase stability and shelf life (Pavlin and Bregar, 2012). The general findings show a corresponding decrease in phytosome size as observed in DLS analysis of the same samples. However, SEM sizes seem particularly smaller in comparison with results obtained using DLS as given by the Malvern instrument. This difference can be explained by difference of physical state of nanoparticulate samples at time of analysis. Samples analyzed for particle size using DLS are prepared in solution, while SEM samples are

prepared as dry smears on stubs (Eaton *et al.*, 2017). Both these measurements are important and demonstrate the particle size in solution and its true size respectively. Analysis of particle morphology showed that formulations were spherically shaped. Research has found that the shape of the nanoparticles affects the ease and rate of nanoparticle uptake in cells (Salatin, Dizaj and Khosroushahi, 2015; Xie *et al.*, 2017). Spherically shaped nanoparticles are easily taken up into cells due to their symmetry (Toy *et al.*, 2014).

The encapsulated efficiency (EE) of the 2mg plant extract per 50mg PC was calculated to be 96.51 %, which is equivalent to 3.86^{-1} g/L. The observed EE% is high and reports seem to indicate varied values of EE depending on the components used during phytosome construction (Sahu and Bothara, 2015). The optimization of the formulation, especially for quantity of cholesterol used, may increase EE and therefore lead to increased stability of the resulting phytosome. This study used an indirect method to determine EE %, using UV-Vis,. The direct method is similar to the methods employed in this assay, except that phytosomes are washed to remove unencapsulated extract, followed by lysis of nanoparticles to yield extract encapsulated within the nanoparticulate structure. This is followed by quantification of extract using methods such as UV-vis, HPLC or other (Branquinho *et al.*, 2014; Li *et al.*, 2016). In future, additional methods such as HPLC (after quantification of indicator compounds in *G. fruticosus* extract) or dialysis can be used to determine EE % more accurately.

The live image taken during the analysis of rhodamine labeled phytosomes showed localization of rhodamine outside and within a cell. This study confirmed the physical uptake of phytosomes across bi-membrane surrounding cells. Nanoparticles are taken up across cell membranes by two major endocytotic mechanisms: pinocytosis or phagocytosis. Phagocytosis is not selective and involves uptake of particles that are larger than 200 nm, while pinocytosis is selective in comparison, involving particles smaller than 200 nm (Xie *et al.*, 2017). This indicates that pinocytosis is the most probable mechanism of phytosome internalization, based on the size of the particles. However, this analysis can further be improved by confocal microscopy using of Z-stack analysis. This analysis can be used to locate the fluorescent spot in a 3D image of the HeLa cell (Blumenfeld *et al.*, 2014).

This study shows an increase in rhodamine stained cells, in cells treated with nanoparticles and phytosomes (11.8 and 11.4%) respectively. Higher apoptosis was also observed in HeLa cells treated with phytosomes, in comparison to those treated with media or nanoparticles although it was low in comparison to free extract (chapter 4). It has been reported that encapsulation of phytoproducts increases their uptake across the cell membrane leading to increased bioavailability (Awasthi, Kulkarni and Pawar, 2011) and improved bioactivity. In a study by Shalini, Kumar and Birendra, (2015), encapsulation of *T. arjuna* improved its bioactivity in MCF7 cells, as compared to free extract alone, reducing the IC_{50} from 2.5^{-2} g/L to 1.5^{-2} g/L. In another study, encapsulation of rutin improved its absorption across rat abdominal skin by almost three fold (33 ± 1.33%) in comparison to un-encapsulated rutin (13 ± 0.87%) (Das and Kalita, 2014). This improved

bioactivity may explain the observed results in this study, because cells treated with phytosomes depicted reduced confluence, because of accelerated cell death, occurring approximately 6 hrs after the start of the experiment. In previous chapter 4, treatment of HeLa cells at a similar extract concentration, demonstrated 54.8 ±2.5 % apoptosis after 24 hrs of exposure. This could mean that a subpopulation of cells left were not undergoing apoptosis, which would explain the low observed apoptosis. Signs of apoptosis were also observed in wells treated with phytosomes, such as nucleus condensation and cell shrinkage. These observations, coupled with the evidence of phytosome uptake and apoptosis mechanism of action demonstrated by flow cytometry data, supports the theory that treatment with phytosomes demonstrated improved bioactivity. In addition, observation that experimental endpoint (6 hrs for phytosomes and 24 hrs for plant extract) to achieve observed apoptotic signs further confound this theory. However, no direct comparison for efficacy could be made due to the time difference in which apoptotic signs were observed. To improve this study in future, the experiments need to be optimized by reducing extract concentration and experimental exposure time. To make the study more robust, analysis of the apoptosis-inducing effects of phytosomes containing *G. fruticosus* on non-cancer cell lines or employing another method of analysis such as cell viability in order to compare IC_{50}s, could be considered. Future studies can focus on the efficacy of the phytosomes in an *in vivo* disease model, in comparison to free extract.

Phospholipids as the encapsulation vehicle used in this study is ideal because it is biodegradable and also biocompatible.(Gnananath, Nataraj and Rao, 2017). As a result, it improves the absorption of the herbal bioactive components across the cell membrane,

while presenting other modes of administration such as topical and intravenous introduction. The encapsulation of herbal bioactives using phospholipids creates an opportunity for both passive and active targeting to sites of action (Gnananath, Nataraj and Rao, 2017). Advances in lipid based delivery systems have seen the utilization of various surface modification/functionalization techniques to accumulate encapsulated bioactive at tumor sites through endocytosis via overexpressed receptors on tumor cells (Riaz *et al.*, 2018). The extracellular space surrounding tumor cells is often different as compared to normal cells, with poor oxygen levels and acidic pH, which have been demonstrated as physiological triggers for payload release (Kulkarni *et al.*, 2016; Kim *et al.*, 2018) These novel functionalization techniques (Riaz *et al.*, 2018) can be explored in future studies to improve the pharmacokinetic and pharmacokinetic properties of *G. fruticosus* phytosomes for cervical cancer treatment.

6.7. Conclusion

G. fruticosus extracts encapsulated in phytosome demonstrated uptake and caused significant reduction in cell viability presented as reduced confluency in HeLa cells, therefore, phytosomes demonstrated increased bioactivity in HeLa cells, in comparison to extract alone. However, the phytosomes were not stable when incubated with media containing 10% FBS even though stability in storage at different pH or temperatures is necessary to determine shelf life. Phytosome's optimization and investigations in cancerous tumors *in vivo* should be considered as future research activities. This plant demonstrates potential as herbal medicine and its encapsulation adds value to naturally derived plant extract and offers an avenue to reduce doses, while achieving bioactivity.

This will lead to improved bioavailability and stability of plant extracts in animal studies as well as alternative herbal administration routes such as parenteral or as aerosols.

6.8. Reference

Awasthi, R., Kulkarni, G. T. and Pawar, V. K., 2011. Phytosomes: An approach to increase the bioavailability of plant extracts. *International Journal of Pharmacy and Pharmaceutical Sciences*, 3(2), pp. 1–3.

Blumenfeld, C. M. *et al.*, 2014. Cellular Uptake and Cytotoxicity of a Near-IR Fluorescent Corrole-TiO2 Nanoconjugate. *Journal of Inorganic Biochemistry*, *140*, pp. 39–44. doi: 10.1126/science.1249098.Sleep.

Branquinho, R. T. *et al.*, 2014. HPLC–DAD and UV–spectrophotometry for the determination of lychnopholide in nanocapsule dosage form: validation and application to release kinetic study. *Journal of chromatographic science*, *52*, pp. 19–26.

D'Souza, S., 2014. A Review of *In Vitro* Drug Release Test Methods for Nano-Sized Dosage Forms. *Advances in Pharmaceutics*, *2014*, pp. 1–12. doi: 10.1155/2014/304757.

Das, M. K. and Kalita, B., 2014. Design and Evaluation of Phyto-Phospholipid Complexes (Phytosomes) of Rutin for Transdermal Application. *Journal of Applied Pharmaceutical Science*, *4*(10), pp. 51–57. doi: 10.7324/JAPS.2014.40110.

Eaton, P. *et al.*, 2017. A direct comparison of experimental methods to measure dimensions of synthetic nanoparticles. *Ultramicroscopy*, *182*, pp. 179–190. doi: 10.1016/j.ultramic.2017.07.001.

El-Sherbiny, I. M., El-baz, N. M. and Yacoub, M. H., 2015. Inhaled nano- and microparticles for drug delivery. *Global Cardiology Science and Practice*, *2*, pp. 1–14. doi: 10.5339/gcsp.2015.2.

Gaumet, M. *et al.*, 2008. Nanoparticles for drug delivery: The need for precision in reporting particle size parameters. *European Journal of Pharmaceutics and Biopharmaceutics*, *69*(1), pp. 1–9. doi: 10.1016/j.ejpb.2007.08.001.

Gnananath, K., Nataraj, K. S. and Rao, B. G., 2017. Phospholipid complex technique for superior bioavailability of phytoconstituents. *Advanced Pharmaceutical Bulletin*, *7*(1), pp. 35–42. doi: 10.15171/apb.2017.005.

Hippalgaonkar, K., Majumdar, S. and Kansara, V., 2010. Injectable Lipid Emulsions—Advancements, Opportunities and Challenges. *AAPS PharmaSciTech*, *11*(4), pp. 1526–1540. doi: 10.1208/s12249-010-9526-5.

Kim, M. W. *et al.*, 2018. A Promising Biocompatible Platform : Lipid-Based and Bio-Inspired Smart Drug Delivery Systems for Cancer Therapy. *International journal of molecular sciences*, *19*, p. 3859. doi: 10.3390/ijms19123859.

Kulkarni, P. *et al.*, 2016. Hypoxia Responsive, Tumor Penetrating Lipid Nanoparticles for Delivery of Chemotherapeutics to Pancreatic Cancer Cell Spheroids. *Bioconjugate chemistry*, *27*(8), pp. 1830–1838. doi: 10.1021/acs.bioconjchem.6b00241.MALDI.

Li, C. *et al.*, 2016. Development and validation of a method for determination of encapsulation efficiency of CPT-11/DSPE-mPEG2000 nanoparticles. *Medicinal*

chemistry, *6*(5), pp. 345–348. doi: 10.4172/2161-0444.1000368.

Li, J. *et al.*, 2015. A review on phospholipids and their main applications in drug delivery systems. *Asian Journal of Pharmaceutical Sciences*, *10*, pp. 81–98. doi: 10.1016/j.ajps.2014.09.004.

Mpofu, S. J., Msagati, T. a M. and Krause, R. W. M., 2014. Synthesis of liposomes using α-phosphotidycholine and metabolites obtained from *Elephantorrhiza elephantina* and *Pentanisia prunelloides*. *African Journal of Biotechnology*, *13*(12), pp. 1402–1412. doi: 10.5897/AJB2013.13330.

Mudshinge, S. R. *et al.*, 2011. Nanoparticles: Emerging carriers for drug delivery. *Saudi Pharmaceutical Journal*, *19*, pp. 129–141. doi: 10.1016/j.jsps.2011.04.001.

Nirmala, M. J., Samundeeswari, a. and Sankar, P. D., 2011. Natural plant resources in anti-cancer therapy-A review. *Research in Plant Biology*, *1*(3), pp. 1–14. Available at: http://resplantbiol.com/article/view/243.

Omwoyo, W. N. *et al.*, 2014. Preparation, characterization, and optimization of primaquine-loaded solid lipid nanoparticles. *International journal of nanomedicine*, *9*, pp. 3865–3874. doi: 10.2147/IJN.S62630.

Pavlin, M. and Bregar, V. B., 2012. Stability of nanoparticle suspensions in different biologically relevant media. *Digest Journal of Nanomaterials and Biostructures*, *7*(4), pp. 1389–1400.

Rasaie, S. *et al.*, 2014. Nano Phytosomes of Quercetin: A Promising Formulation for Fortification of Food Products with Antioxidants. *Pharmaceutical sciences*, *20*, pp. 96–101. Available at: http://pharm-sci.tbzmed.ac.ir (Accessed: 20 February 2017).

Riaz, M. K. *et al.*, 2018. Surface Functionalization and Targeting Strategies of Liposomes in Solid Tumor Therapy : A Review. *International journal of molecular sciences*, *19*, p. 195. doi: 10.3390/ijms19010195.

Sahu, A. R. and Bothara, S. B., 2015. Formulation and Evaluation of Phytosome Drug Delivery System of Boswellia Serrata Extract. *International journal of rsearch in medical sciences*, *4*(2), pp. 94–99. Available at: http://www.ijorim.com/siteadmin/article_issue/143746657719_Asit Sahu .pdf.pdf (Accessed: 20 September 2017).

Salatin, S., Dizaj, S. M. and Khosroushahi, A. Y., 2015. Effect of the surface modification, size, and shape on cellular uptake of nanoparticles. *Cell Biology International*, *39*, pp. 881–890. doi: 10.1002/cbin.10459.

Salopek, B., Krasic, D. and Filipovic, S., 1992. Measurement and application of zeta-potential. *Rudarsko-geolosko-naftni zbornik*, **4**, pp. 147–151.

Savjani, K. T., Gajjar, A. K. and Savjani, J. K., 2012. Drug Solubility: Importance and Enhancement Techniques. *Pharmaceutics*, *2012*, pp. 1–10. doi: 10.5402/2012/195727.

Shalini, S., Kumar, R. R. and Birendra, S., 2015. Antiproliferative effect of Phytosome complex of Met hanolic extact of *Terminalia Arjuna* bark on Human Breast Cancer Cell

Lines (MCF-7). *International journal of drug development and research,* 7(1), pp. 173–182.

De Smet, L. *et al.*, 2013. Optimization of drug delivery systems for intraperitoneal therapy to extend the residence time of the chemotherapeutic agent. *The Scientific World Journal, 2013*, p. 7. doi: 10.1155/2013/720858.

Toy, R. *et al.*, 2014. Shaping cancer nanomedicine: The effect of particle shape on the *in vivo* journey of nanoparticles. *Nanomedicine (Lond)*, 9(1), pp. 121–134. doi: 10.2217/nnm.13.191.

Xie, X. *et al.*, 2017. The Effect of shape on Cellular Uptake of Gold Nanoparticles in the forms of Stars, Rods, and Triangles. *Scientific Reports*, 7(1), pp. 1–9. doi: 10.1038/s41598-017-04229-z.

Yi, Y. *et al.*, 2014. Single-step assembly of polymer-lipid hybrid nanoparticles for mitomycin C delivery. *Nanoscale Research letters*, *9*, p. 560. doi: 10.1186/1556-276X-9-560.

CHAPTER 7: OVERALL CONCLUSIONS AND RECOMMENDATIONS

7.1. Conclusions

Access to effective and safe cancer treatment continues to be a challenge in developed and developing countries. Whilst the search for alternative treatments continue to be a constant occupation of scientist, lead compounds are often rendered not useful owing to poor bioavailability and nonspecific toxicities, resulting in treatment failure and side effects such as diarrhea, vomiting, alopecia and fatigue. *G. fruticosus* is used for treating tumors in Namibia and has demonstrated potential in in vitro assays against several cell lines. However, it is also described as toxic with cardenolides suspected to cause cardiotoxicity in animals, hence why the plant has not been developed or pursued further for its anticancer properties. This study Therefore, this study was conducted, 1) the phytochemical profile of *G. fruticosus* harvested in Namibia was determined using TLC and GCMS techniques. 2) the anti-proliferative, cytotoxicity activity of *Gomphocarpus fruticosus* extract and its mechanism of action *in vitro*. 3) the extent of toxicity and toxic symptoms associated with *G. fruticosus* was determined *in vivo* using mice. And 4) the plant extract was formulated into a lipid based nanoparticle delivery system, which was characterized and evaluated *in vitro* as an anticancer treatment.

Firstly, the phytochemistry investigation using thin layer chromatography revealed the presence alkaloids, flavonoids, anthraquinone, terpenoids and steroids in the methanol, chloroform and dichloromethane extracts. The aqueous extract, used commonly in

traditional settings, only showed the presence of anthraquinones and flavonoids. The methanolic extract was further analyzed using GC-MS revealing the presence of a number of 16-20 carbon chain unsaturated fatty acids and triterpenols. Triterpenols included α-amyrin, lupeol and their esters and also steroidal compound β-sitosterol, all reported to possess anti-inflammatory, anticancer and analgesic properties validating the traditional medicinal use of this plant. Furthermore, the presence of fatty acids and other phytocomponents rationalize the medicinal use of *G. fruticosus* and explain its mode of preparation and administration, in Namibia's traditional setting.

Secondly, the methanolic shoot extract showed antiproliferative properties against the cervical cancer cell line HeLa (IC_{50} 8.35^{-7} g/L) with low cytotoxicity as determined against Hek293 cell line (IC_{50} 3.976^{-4} g/L). The mechanism of cell death leading to the antiproliferative effect of the plant extract on HeLa cells at such a low concentration was not the apoptotic pathway but other cell death mechanisms such as necrosis through a ROS related pathway and lack of caspase 3&7 cleavage.

Thirdly, the *in vivo* toxicity analysis in Balb C mice revealed an LD_{50} value of 1.968^{-1} g/kg, which puts the extract in category 3 as toxic according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Adverse effects included diarrhea, slow motility, muscle spasms and heavy breathing, which were observed in treated animals. However, daily administration of doses lower than determined LD_{50} did

not significantly affect the weight of the experimental animals nor cause any deaths, in comparison to untreated group, alluding to the usefulness of extract as lower doses.

Finally, formulation of the methanolic shoot extract into a rhodamine-labelled lipid-based nanoparticulate herbal (phytosome) delivery system (1.3^{-7}-1.5^{-7} m diameter) using phosphatidyl choline and cholesterol resulted in uptake of the plant extracts (encapsulation efficiency of phytosomes, 96 %) as visualized by flow cytometry and fluorescent microscopy. The uptake of the phytosomes resulted in increased apoptosis in HeLa cells at concentration comparable to the free extract in a shorter time frame. It is speculated that the reduced confluence observed after a short time framein this experiment was a result of accelerated apoptosis. We therefore recommend that further studies be conducted to determine biological effects of extracts and phytosomes against tumors *in vivo*, while developing a stable nano-particulate system to improve delivery, solubility and improve nonspecific toxicity.

7.2. Contribution to new knowledge

The study is the first of its kind, involving a Namibian plant. It has significance in the on-going search for efficacious anticancer drug candidates, which demonstrate selectivity for cancer cells as seen in this study and not nonspecific cytotoxicity. The study also shades new light on the mechanism of action to possibly necrosis. Necrosis is becoming an acceptable outcome of chemotherapy, whereas, apoptosis was previously expected.

Furthermore, encapsulation into phytosomes adds value and is a step towards the possible adoption of *G. fruticosus* as herbal treatment for cancer, especially cervical cancer.

7.3. Recommendations

It is recommended that future research efforts strengthen the identity and isolation of components found in Namibian plant, *G fruticosus*, so that the phytochemicals responsible for the observed bioactivity maybe determined This could include the use of bioactivity guided-fractionation, on a panel of cancerous cell lines. Compound identification can be improved by using Liquid Chromatography Mass Spectroscopy (LCMS) or High Pressure Liquid Chromatography (HPLC) to uncover non-volatile compounds. The sub-acute effect of crude extracts need to be ascertained using histology *in vivo*, over a period of 90 days, while also determining organ distribution of both crude and encapsulated extract *in vivo,* so that the findings can add weight to observations in this study and show organ targets for toxicity. In addition, the phytosome formulation should be optimized to improve its physical stability, while maintaining its encapsulation efficiently in order to improve its shelf life, while ensuring sustained release of active herbal components in living systems. Furthermore, it is recommended that IC_{50} determination for phytocome be compared under similar experimental conditions as free extract, so results are comparable. Finally, it is recommended that the phytosomes and crude extract be compared for its effect on cancerous tumors in an *in vivo* model whilst also determining the pharmacokinetics of the phytosomes and their herbal component levels in blood. Since aqueous extracts are generally used traditionally, it is recommended that it be investigated for bioactivity in future.

CHAPTER 8. APPENDICES

Appendix A. Plant collection form

NBRI: DATA COLLECTION FORM

Collect.No..... Date.....
Collector/s..... GPS.....S
Locality (please describe properly, not just GPS) ....E
.....Grid.....
.....Altitude.....
Aspect..... Slope..... Exposure.....
Photo no's..... Voucher for.....

DESCRIPTION OF PLANT Please give as much information as possible
Species..... Perennial/Annual
Habit (e.g.shrub).....Height.....cm
Occurrence (e.g.common).....
Indigenous names and languages.....
.....
.....
Flower/Inflorescence (colour,shape,size,smell,texture).....
.....
.....
Leaves (simple/compund,margin,shape,colour,texture,stipules).....
.....
.....
.....
Stems and bark (colour,texture,habit - e.g. erect stem with yellow papery peeling bark - presence or absence and smell of sap or latex - e.g. milky latex with unpleasant smell).....
.....
.....
Fruits and seed (presence/absence,shape,colour,edibility and taste if edible, single or clustered,maturity).....
.....
.....
.....
Roots and underground organs (shape,texture,colour,size).....
.....
.....
Other (e.g.: uses,ecology).....
.....
.....

Habitat			Vegetation	Substrate	Soil type	Lithology	Moisture	Biotic effect
cliff/ace	plain	lake/pond	desert	soil	sand		well-drained	road/rail side
mountain/ hillslope	plateau	marsh/ swamp/ wetland	shrubland open/closed	stony/rock y soil	sandy loam	mica schist	moist/damp	cultivated land
mountain/ hill peak	valley	river/stream	forest	gravel	loam	dolomite	seasonally waterlogged	abandoned land
talus/scree	donga/ gulley/ ditch	river/stream bank	grassland	bare rock	loamy clay	granite	permanently waterlogged	heavily grazed
					gravel			
dune slope	depression/ pan	seepage area	karroo	water	clay	lime	free standing water	garden
dune crest	dam	waterfall/ rapids	thornbush savanna	roots	calcrete	quartzite	running water	plantation
interdunal street	dry river/ stream bed	unknown		termite mound	black turf	other	mist/fog	recently burnt
ravine/kloof/ gorge	floodplain			other	humus rich		tidal	disturbed – other
ridge	estuary/ lagoon				salt/brack			other

Figure 3A. Plant collection form deposited with voucher specimen at *NBRI*

Appendix B: Cell viability response of cell lines to 6% DMSO.

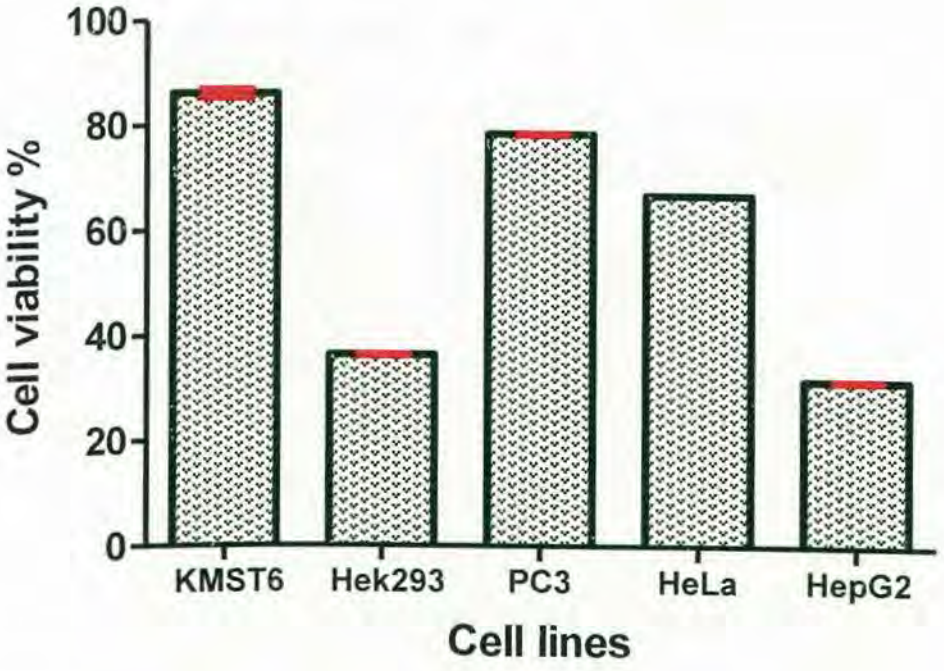


Figure 4A. Response of different cell lines to 6% DMSO, used as positive control in cell viability assay. Response varied depending on susceptibility of cell line under investigation, with Hek293 and HepG2 being most sensitive.

Appendix C: Optimization of nanoparticles with varying extract concentrations for EE%. Chapter 6

Table 6A. Encapsulation efficiency of 3 batches of phytosomes constructed with varying extract concentrations.

Phytosome	Extract mass (g)	Absorbance	Extrapolated free extract (g)	Free extract in 5^{-3} L batch (g)	EE%
1	5^{-4}	0.1382	3.03^{-5}	1.51^{-4}	69.7
2	1^{-3}	0.4164	7.31^{-5}	3.65^{-4}	63.5
3	2^{-3}	0.1891	3.81^{-5}	1.91^{-4}	90.5

Appendix D: Physical characteristics of empty nanoparticles and phytosomes.

Chapter 6.

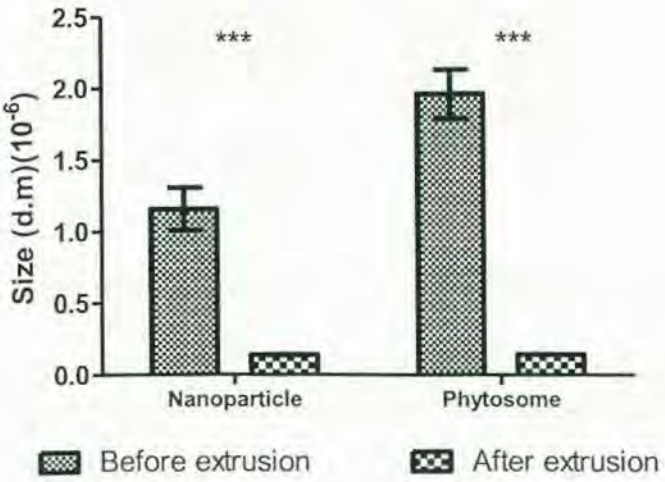


Figure 6A. Particle size comparison of nanoparticles/ phytosomes before and after extrusion. Asterics denote significant difference. P=0.001 of both formulations before and after extrusion.

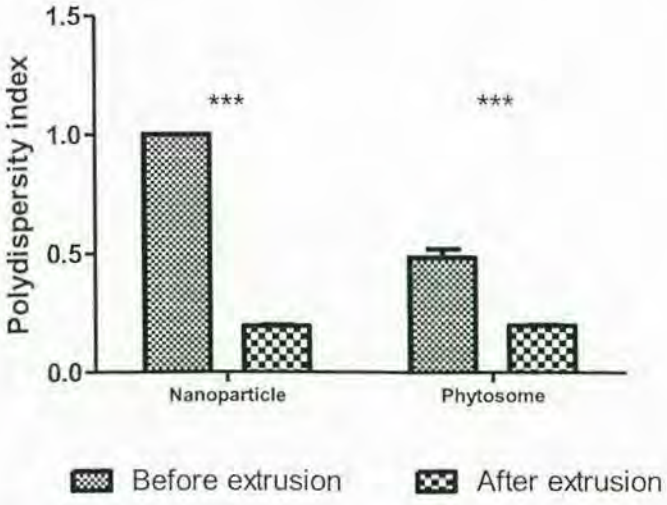


Figure 6B. Polydispersity index of formulated nanoparticles/phytosomes before and after extrusion. Asterics denote significant difference. P=0.001 in the particle spread after extrusion.

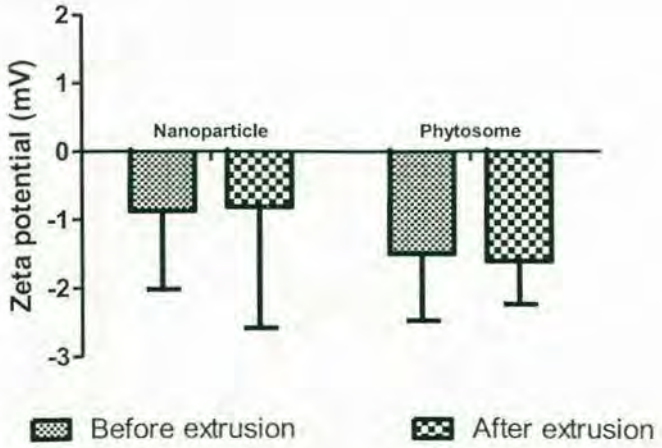


Figure 6C. Zeta potential of nanoparticles/ phytosomes before and after manual extrusion. No significant change in zeta potential was observed in both formulations.

Appendix E. Calibration curved of increasing extract preparation and absorbance on UV Vis mchaine.

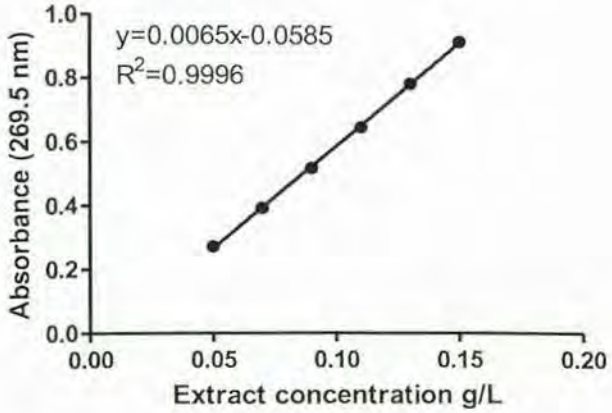


Figure 6D. Calibration curve developed with *G. fruticosus* plant extract, concentrations ranging between 50-150 µg/ml with absorbance values in obedience to Beer-Lambert law.