AN INVESTIGATION OF THE *IN VITRO* ANTI-CANCER PROPERTIES OF

ACANTHOSICYOS NAUDINIANUS,FOCKEAANGUSTIFOLIA, cf SALVADORA

PERSIA AND *NYMANIA CAPENSIS* FROM HARDAP AND //KARAS REGIONS OF

NAMIBIA

A THESIS SUBMITTED IN FULFILMENT

OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE

OF

THE UNIVERSITY OF NAMIBIA

BY

HATAGO ≠AIBATE STUURMANN

200926900

OCTOBER 2016

MAIN SUPERVISOR: DR DAVIS R MUMBENGEGWI

CO-SUPERVISOR: DR MARTHA KANDAWA-SCHULTZ

DR DALU MANCAMA

ABSTRACT

Cancer has become a global health burden accounting for about 8.2 million deaths every year worldwide. Cancer patients or people, who experience symptoms similar to that of cancer in Namibia's rural settings, may endure suffering for months and may even die without receiving medical attention, due to the lengthy distance between the rural areas and the nearest medical centre and the long referral process. It may even take more time to get the correct medical attention for t particular cancers. In such circumstances, patients make use of traditional medicinal plants, as an alternative and primary option for treating cancer-related ailments. However the majority of knowledge on plant based medicine in Namibia lacks scientific support and documentation in terms of safety and efficacy. Therefore this study aimed at contributing to the generation of such supporting scientific data. Plants were collected from the Hardap and the //Karas regions of Namibia. The *Acanthosicyos naudinianus* root *Fockea angustifolia* whole plant and the tuber, *cf Salvadora persia* root and *Nymania capensis* root were used to prepare aqueous (water) and organic (ethanol) plant extracts. The extracts were screened for *in vitro* anticancer properties using Renal cancer cell line (TK10), Breast cancer cell line (MCF-7), and the Human Melanoma cell line (UACC-62). For the cytotoxicity test, the Fibroblast cell line (WI38) was used and the Sulforhodamine B assay was employed to determine cell viability. Furthermore, Thin Layer Chromatography and the Diphenyl Picryl Hydroxyl (DPPH) radical scavenging assay were utilized for phytochemicals screening, and determination of antioxidant activity of the plant extracts respectively. The phytochemical screening results showed alkaloids, flavonoids, anthraquinones,

coumarins, and saponins were present in all plant parts that were screened for. Furthermore two of the aqueous plant extracts and four of the ethanol plant extracts of the investigated 10 plant part extracts exhibited *in vitro* anticancer activity. The aqueous root extract of *Acanthosicyos naudinianus* exhibited potent activity; IC_{50} (6.25 µg/ml, 16.41 µg/ml and 48.85 µg/ml) against MCF-7, UACC-62 and TK-10 respectively with moderate cytotoxicity against fibroblast cell lines. However the ethanol root extract of *Acanthosicyos naudinianus* root exhibited low *in vitro* anticancer activity and weak cytotoxicity effect of (IC_{50} 83.34 µg/ml).Most significantly mechanism of action of the most potent, aqueous *Acanthosicyos naudinianus* plant extracts against the renal cancer cell lines was through the apoptotic pathway of the nuclear nucleosome enrichment with the up-regulation of the Histone proteins in the chromatin. The anticancer activities of the plant extracts may be attributed to the presence of phytochemicals in these plant extracts. The bioactivity observed in these extracts may be used as a baseline for anticancer drug development. Finally, the scientific findings of these plants serve as a rationalization for the use of these plants to treat cancer within the traditional settings as primary alternative medicine. Further studies are required to assess the extent of toxicity before the extracts can be recommended for mainstream usage.

Key words: Hardap region, //Karas region, Anticancer, *Acanthosicyos naudinianus*

Table Of Content

List of figures

List of Tables

LIST OF PUBLICATIONS

1. Hatago Stuurmann[1] Davis Mumbengegwi[1] (2015), An Investigation Of The *In Vitro* Anti– Cancer Properties Of *Acanthosicyos naudinianus.* (Poster presentation)

LIST OF CONFERENCES AND PRESENTATIONS

1. Stuurmann, H.A., Mumbengegwi, D.R, Kandwa- Schultz, M., Mancama (2016) Identification of the active components and antitumor screens of Namibian plant extracts 8-10 September (2016) University Of Western Cape, Cape Town South Africa.

2. Stuurmann, H.A., Mumbengegwi, D.R, Kandwa- Schultz, M., Mancama (2016) Proteomic Analysis of Treated Renal Cancer Cell Lines by SWATH Mass Spectrometry,   28 August to 1 September Kruger National park, South Africa

3. Stuurmann, H.A., Mumbengegwi, D.R, Kandwa- Schultz, M., Mancama, D. (2014) An investigation of the in vitro anticancer properties of indigenous plant from Hardap and //Karas regions, 27-29 October, White Sand Hotel, Da es Sallaam Tanzania.

4. Stuurmann, H.A., Mumbengegwi, D.R, Kandwa- Schultz, M., Mancama, D. (2014) An investigation of the in vitro anticancer properties of indigenous plant from Hardap and //Karas regions (2014), University OF Namibia 2nd Science conference, University OF Namibia Library auditorium, Windhoek Namibia.

5. Stuurmann, H.A., Mumbengegwi, D.R, Kandwa- Schultz, M., Mancama, D. (2015) Valorization of medicinal plants used by the Nama tribe to treat cancer, 30 July ,Green Symposium, University OF Namibia Library auditorium, Windhoek Namibia.

6. Stuurmann H. A [1], Mumbengegwi D.R. [2], Böck R.A.[2] (2015) *In vitro* investigation of anticancer properties of *Sclerocarya birrea* (marula plant) and *Adansonia digitata* (baobab plant) extracts, 23-25 September, National Commission for Research Science and Technology (NCRST), Hilton Hotel, Windhoek Namibia

7. Ideas that work (Theme), 8-9 October 2015 Council for Scientific and Industrial Research (CSIR) Conference , Pretoria South Africa

8. Hatago Stuurmann[1] Davis Mumbengegwi[1] (2015), An Investigation Of The *In Vitro* Anti– Cancer Properties Of *Acanthosicyos naudinianus* 18-22 November, African Organization for Research and Training in Cancer (AORTIC) Conference, Marrakesh Morrocco.

9. Stuurmann, H.A., Mumbengegwi, D.R, Kandwa- Schultz, M., Mancama, D. (2015) Valorization of medicinal plants used by the Nama to treat cancer 29 May,Umhlanga , Durban South Africa

ACKNOWLEDGEMENT

I want to take this chance to thank the Heavenly Father for the opportunity that he blessed me with a the chance to do my masters under his guidance during workshops, training, conferences, experiments, write up of this thesis, and his protection, during the lonely, quite most challenging times of this journey.

My profound gratitude goes to my Supervisor Dr D.R. Mumbengegwi, for his tireless support, and guidance throughout the study. Thank you for your dedication, mentorship and guidance which has helped shape and contributed toward me completing this research study and towards my professional and technical growth. I want to express appreciation Dr. M. Kandwa- Schultz, and Dr D Mancama, D. for the patience, encouragement and expertise. Thank you for the opportunity to carry out this research, in a well-equipped scientific and technological research environment with expert guidance.

I want to thank Mrs Natasha Kolesnikova and Dr Stoyanfrom the Council for Scientific and Industrial Research (CSIR), for the training guidance and assistance in cell culture, anticancer and cytotoxicity screening and the SWATH-MS, and gene ontology screens respectively. My thanks go to National Herbarium of Namibia under the Ministry of Agriculture, water and forestry, and Dr E. Kwembeya for the scientific identification of the plants used in this study.

I dedicate this thesis to the people who gave me the timeless support my family particularly my parents Petrus Boois and Dorothea Boois. I want to thank you Pappy

and Mammy who tirelessly work hard to put me through school, send me to University and also supported me when I told them my plans for doing a Masters in Science. Pappy, Mammy you are my rock, there were times when I thought of giving up and going home but you encouraged me to the very last.

My thanks goes to my Siblings , Damaros Stuurmann , Dantagob Boois and Buruxa Boois ,for the peace of mind they rendered to me with the joy, peace and happiness they brought to life particularly my nieces and nephew Charmain, Fabian, Condelizza, Kalahari, Memes , Xavi (Stuurmann)

My hard felt gratitude goes to my sponsors the SABINA is funded by The Carnegie Corporation of New York as a Regional Initiative in Science and Education (RISE) for the African Continent for the financial and academic / professional growth they have granted me. This has allowed me to grow as an individual and as a young women scientist in the field. The workshops, seminars and conference which were organised by the SABINA network helped shaped my academic communication and presentation skills. My academic supervisors and advisors that help me make stepwise decisions, on experiment and what was suitable for the project.

Lastly I want to thank the Biomedical Research Laboratory, University of Namibia Windhoek and Council for Scientific and Industrial Research (CSIR), Pretoria for support in completing my duties and work in the respective laboratories during my study.

DEDICATION

I dedicate this thesis to Madawab (Father) and Laitagos (Mother) for being my best supporters during this journey. " *Hatagos ao ≠gasen aos, ta xu aos* !!!" that's what you guys would say. Love you guys' a lot.

DECLARATIONS

I, [Stuurmann Hatago ≠Aibate], declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education. No part of this report may be reproduced, stored in any retrieval system, or transmitted in any form, or by means (e.g. electronic, mechanical, photocopying, recording or otherwise) without the prior permission of the author, or The University of Namibia in that behalf. I, [Stuurmann Hatago ≠Aibate], grant The University of Namibia the right to reproduce this thesis in whole or in part, in any manner or format, which The University of Namibia may deem fit, for any person or institution requiring it for study and research; providing that The University of Namibia shall waive this right if the whole thesis has been or is being published in a manner satisfactory to the University.

………………………………. [Signature] Date……………………………..

Stuurmann Hatago ≠Aibate

ABBREVIATIONS

BMP - Bone Morphogenetic Protein

CBC - Complete Blood Count

CT - Computed Tomography

DCIS- Ductal Carcinoma *In Situ*

DMSO-Dimethyl Sulphoxide

DNA Deoxyribonucleic acid

DPPH –2, 2 Diphenyl Picryl Hydroxyl

ER -Oestrogen Receptor

BHD - birt-hogg-dube

HER2/neu receptor- Human Epidermal Growth Factor Receptor

IC_{50}-50% of Cell Growth Inhibition

ILC -Invasive Lobular Carcinoma

IVP - Intravenous Pyelogram

LC-MS - Liquid Chromatography - Mass Spectra

MRI - Magnetic Resonance Imaging

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide

PET - Positron Emission Tomography

PR -Progesterone Receptor

PTM - Post-translational modifications

RCC - Renal cell carcinoma

SAC - Spindle Assembly Checkpoint

SRB - Sulforhodamine B

SWATH-MS -Sequential Window Acquisition of all Theoretical Mass Spectra

TLC - Thin Layer Chromatography

UV - Ultra-Violet rays

VHL - Von hippel-Lindau

WHO – World Health Organization.

XTT - Sodium 3′-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6-nitrobenzene) sulfonic acid hydrate

CHAPTER ONE: INTRODUCTION

1.1 Orientation of the study

In spite of the presence of various treatment options cancer is still a global health burden (Alawode, 2013), accounting for about 8.2 million deaths, with a predicted increase to 13 million deaths per year in the coming twenty years. More than half of these deaths occur in developing countries (World Cancer Report, 2014).The Cancer registry of Namibia has also confirmed the increase in cancer related deaths in Namibia. The most prevalent cancers in Namibia are breast cancer (27.6 %), and Kaposi sarcoma in men (22.1 %) prostate cancer (19.2%) cervical cancer (17.1%) Kaposi sarcoma in women 10.3% (Dushimemaria & Mumbengegwi, 2015).These statistics show that the cancer mortality and morbidity is on the increase despite the presence and availability of conventional medicine, and treatment for cancer such as the chemotherapy , radiotherapy and surgery (Jardines *et al*., 2015; Rasool Hassan, 2012)

This study draws interest to the use of medicinal plants in traditional settings for treatment of cancer-related ailments as an alternative treatment. Cancer patients in rural traditional settings endure extended medical appointments for treatment, due to the lack of appropriate services and facilities in these areas. Furthermore, the high cost associated with modern health care services are prohibitive for such cancer patients, hence the use of medicinal plants for primary health care. However, the majority of knowledge on plant based medicine in Namibia does not have scientific support, in terms of characterization, efficacy and safety. To date there are only few studies

conducted to valorize traditionally used medicinal plants in Namibia. Therefore to contribute to the generation of supporting scientific data in this field, this study investigated the anticancer properties of indigenous plants such as *Acanthosicyos naudinianus* root *Fockea angustifolia* root *and whole* plant and, *cf Salvadora persia* root and *Nymania capensis* root found in Hardap and //Karas regions used to treat cancer,. Their efficacy and cytotoxicity profile were determined whilst also considering the mechanisms involved, to understand various pathways and proteins that are up regulated and down regulated due to treatment with particular plant extracts.

Consequently recognizing the activated cell death, whether the mechanism of action is though the induction of apoptosis or necrosis (Graidist, Martla, & Sukpondma, 2015; Hiruma *et al.*, 2015; Reed, Kutasovic, Lakhani, & Simpson, 2015). Lastly the screening for the presence or absence of phytochemicals classes in these traditionally used plants. Some of these phytochemicals such as alkaloid, flavonoids, tannins, and steroids are present in a wide variety of plants, and are well reported (Peteros & Uy2010). Recent studies have shown that phytochemical compounds (secondary metabolites) produced by plants have potential therapeutic /medicinal properties, such as anti-inflammatory, antioxidants, and anticancer properties (Aberoumand, 2012; Alawode, 2013; Murugesan & Deviponnuswamy, 2014).

1.2 Statement of the problem

The World Health Organisation (WHO) has reported increase in mortality and morbidity of various cancer worldwide over the past decade, with cancer accounting for about 8% of all deaths (World Cancer Report, 2014), second to HIV/AIDS, This trend is also observed in Namibia. Numerous communities in the southern regions of Namibia utilizes ethnomedicinal plants to treat cancer associated symptoms, more specifically plant such as *Acanthosicyos naudinianus*, *Fockea angustifolia, cf Salvadora persia* and *Nymania capensis*. Namibia's wealth in medicinal plants and the knowledge thereof can be use to develop alternative or complimentary medicine to treat conditions associated to cancer particularly in the rural areas of Namibia. However, there is a need for scientific evidence on the properties, the of effectiveness and the safety, of *Acanthosicyos naudinianus*, F*ockeaangustifolia, cf Salvadora persia* and *Nymania capensis* to treat symptoms similar to that of cancer through screening for plant phytochemical compounds and testing of these traditionally use medicines using bioassays.

1.3 Objectives

The study generally aimed at investigating the *in vitro* anti-cancer activity of crude extracts of *Acanthosicyos naudinianus , Fockea angustifolia, cf Salvadora persia* and *Nymania capensis* found in Hardap and the //Karas regions of Namibia. The specific objectives were

- To screen for phytochemical classes associated with anticancer activity namely alkaloids, flavonoids, anthraquinones, coumarins, and saponins in both aqueous and organic plant extracts.

- To screen for antioxidant activity associated with anticancer activity in both aqueous and organic plant extracts

- To evaluate the potential anti-cancer activities of both aqueous and organic plants extracts on MCF-7, UACC-62, TK-10 cancer cell lines and the cytotoxicity of these extracts on non-cancerous cell lines WI-38 cell line.

- To determine the mechanisms of action involved in the anticancer activity of the plant extracts.

1.4 Significance of the study

The study will play an essential role in generating and adding knowledge on plants used to treat cancer that exists orally amongst the local traditional Nama people of the Hardap and the //Karas regions. Furthermore the study will also serve as a foundation for future studies on possible plant derived cancer drug discovery and the process of policy making. Finally the study will be a scientific validation for the use of the plants investigated in this study in traditional settings.

1.5 Limitations of the study

The investigation of *in vitro* anticancer properties of plant extracts was a laboratory based study where both the ethanol and aqueous plant extracts of were screened against the immortalized cell lines breast cancer MCF-7 and the melanoma cancer UACC-62 and lastly the renal cancer cell line TK-10.The immortalized cancer cell lines were used hence the results obtained from this work may not reflect the actual clinical setting. The cancer cell lines (breast, melanoma, and renal cancers) were linked to the traditional uses of the selected plants for this study were used, which may not be a representative

of all cancers cell lines, as there are various cancer and cancer subtypes. The crude plant extracts preparation methods in this study may differ from that of the traditionally used methods, normally the solvent system that is used in the traditionally preparation is water, while this study used water and ethanol. Water was used in this study see activity of these plants extracts as it is in the traditional settings by the Nama communities. While ethanol was used in this study, since there are various reports that demonstrate organic solvents to be more effective in dissolving plant phytochemical compound than water which is used in traditional setting as a solvent system. Consequently the use of the two solvent systems also illustrated the wide-range spectrum of plant phytochemical constituents.

The differences in solvent system used for extraction, plays a vital role in the in the compound extraction. Thus the extraction solvents that were used in the study were ethanol and water, which may not have targeted all polar to medium polar compounds, and the non-polar compounds of interest. Consequently not all the phytochemicals were screened for; only the phytochemicals associated with anticancer properties such as flavonoid, saponins and steroids that were screen for against the breast, renal and melanoma cancer (Fernando & Rupasinghe2013).

CHAPTER TWO: LITERATURE REVIEW

2.1 Cancer establishment

Research into cancer has a long standing history thus there is a better understanding of what the cancer is, and its causes and treatments. Cancer is a multifaceted genetic disease; which involves quantitative and qualitative changes in the gene's role, and expression involving physiological alterations leading to the loss of normal cell growth (Mcinerney *et al.*, 2014). Literature has emphasized the association of two gene types with the establishment of cancer (Rice, Bryant, Handley, & Hall, 2014; K. Zhu et al., 2015), these are the proto-oncogenes and the tumor suppressor genes that play critical part in prompting the activation of cancer in case of mutations (Rice *et al.*, 2014).

Different theories exist in literature regarding the role of the two gene types in cell cycle and the development of cancer but they all reach a common consensus. Which is that cells with mutations in the two gene types mentioned above compromise the fidelity of DNA replication which in turn leads to mutations in other genes, such as the genes that control apoptosis(Alison & College, 2001; Mahoney, Arfuso, Millward, & Dharmarajan, 2014; Rice *et al.*, 2014; Society, 2014). A great deal of previous research (Chen, Williams, Filippova, Filippov, & Duerksen-hughes, 2014; Rice et al., 2014; K. Zhu et al., 2015) into carcinogenesis has focused on the change in the function of the two genes as a result of mutation in the cellular processes. Hence the proto-oncogenes are implicated in enabling unregulated cell growth and splitting, through the signalling of growth factors and their specific receptors (Rice et al., 2014). While the tumor-

suppressor genes plays apart in managing check point, pauses or arrest during the cell cycle, this activity is to fix DNA and make certain the veracity of the cell's genetic material (K. Zhu et al., 2015) Contrary to the normal function of the cell cycle, where the development and the fatality of cells are kept in balance, by apoptosis a programmed cell death mechanism, to remove unwanted or damaged cells (Rice *et al*., 2014); in cancerous cells the alternation in one of the two genes or both causes an in balance thus there is a disruption. This leads to bypassing of the apoptosis, and rapid uncontrollable cell growth known as cancer (Mcinerney *et al.*, 2014).

2.2   Cancer as a disease

Unregulated cell growth causes various types of hematologic cancer cells and tumors of different sizes at different locations of the human. There are two tumors types the benign and the malignant (Mcinerney *et al*., 2014).Numerous studies suggest that benign growth of cell are characterized by the dawdling growth, non-invasiveness and non-metastasis, these tumors typically grow locally in an unreserved manner and squeeze on the surrounding organs (American cancer society, 2015; Hyland Katherine, 2008; Komen, 2014). Malignant growth of cells is characterized by rapid uncontrollable growth, associated with invasiveness or metastasis of the cancer. During the translocation of malignant cancer cells from their original site to secondary sites, the cells divide further forming more malignant tumor cells (Alison & College, 2001). These translocation is achieved by using the circulatory system and the lymphatic system of the body as a means of transport and finally the malignant nature of the tumor, destroys primary and secondary invaded tissues, organs and in turn the entire

body thus malignant tumors are life threatening. Consequently the knowledge on how the cell cycle is regulated under normal conditions is important, as it is the key in understanding the how cancer comes about, its development and how the cancer drugs are designed to treat these complex disease (Lu *et al.*, 2014). .

2.3 Cancer Biology and treatment

2.3.1 Cell cycle regulation

A large and growing body of literature (Ben-shlomo, 2014; Mahoney *et al.*, 2014; R. Y. L. Tsai & Pederson, 2014) has investigated the significance in the regulation of the cell cycle under normal conditions and whence it's under attacked by cancer. The cell cycle has a pivotal role in the growth, and development in all living organism, through a series of events. Typically it is expected of the cell to undergo DNA replication for cell growth, however there is a need to ensure that these cells undergoes correct well defined division (Hackermüller *et al.*, 2014). Thus there are mechanism in place to guarantee that the cell progeny which are as a result of cell division have intact genetic material (Ben-shlomo, 2014) Much of the current literature on the process by which cells duplicate and synthesize genetic material to produce new cell material focuses on signal pathways to complete the production (Hackermüller *et al.*, 2014; Pinheiro & Sunkel, 2014). Cell division is divided into two main phases the Interphase and the M phase (Pinheiro & Sunkel, 2014). For many years the details of this phenomenon was surprisingly neglected by various studies prior to (Collins, Jacks, & Pavletich, 1997). The interphase is the stage between mitotic cell divisions; where the cell divided into

G1, S, and G2 (figure 1).This type of cell division is where the cell nucleus divides, forming two daughter cells containing the identical quantity of genetic material as the parent cell (Pinheiro & Sunkel, 2014; G. Wang, Jiang, & Zhang, 2014). Firstly the G1 phase of the cell cycle is between the last mitosis and the start of DNA replication, the S phase is the restricted to the production of DNA and centrosome duplication (G. Wang *et al.*, 2014). Lastly, the G2 phase initiates the separation and replication of a cell's chromosomes duplicated in the S phase for the following mitosis M phase as see in figure1 (Heuvel, General, & Cancer, 2005; Malumbres & Barbacid, 2009).
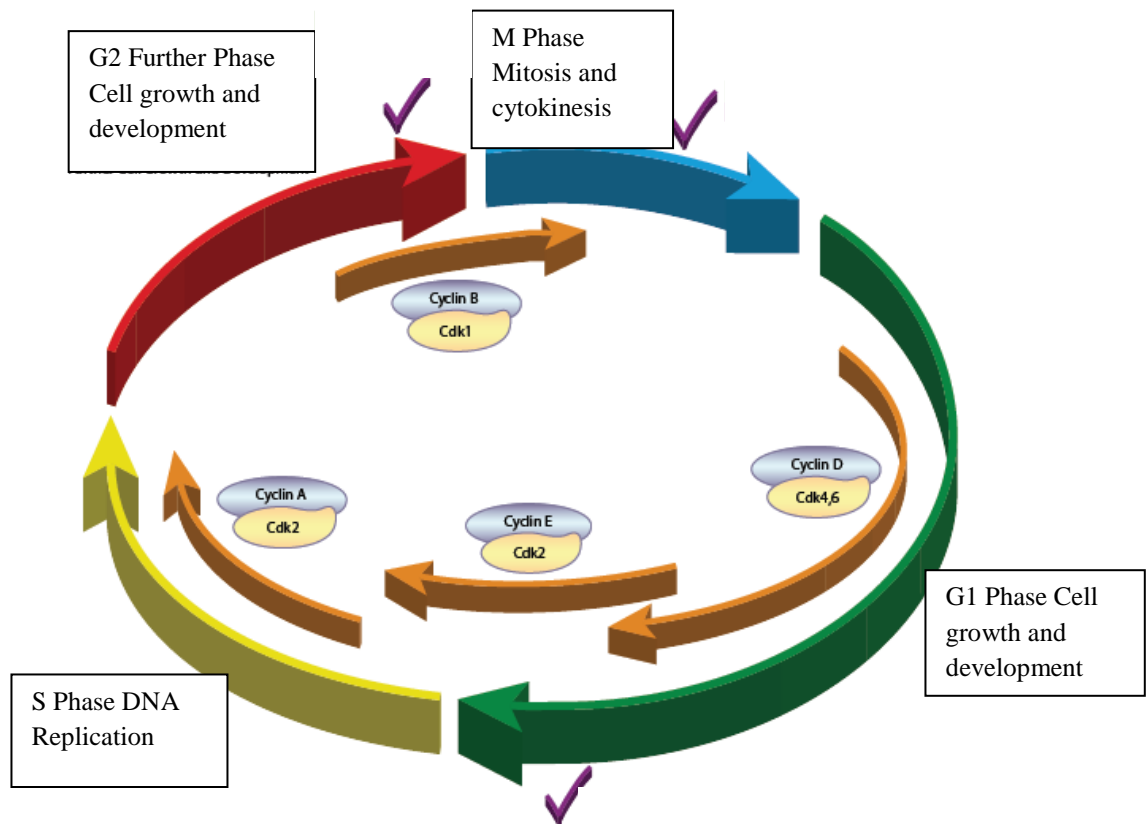
Figure 1 Events of the cell cycle:

The interphase of the cell cycle involves events of DNA replication takes place during the S phase. The G_1 is the gap between M phase and S phase, while G_2 is the gap between S phase and M phase. During the M phase, the nucleus and the cytoplasm divide. There is a large volume of published studies describing the role of the different phases of the cell cycle each involves complex series of events (Bertoli, Skotheim, & Bruin, 2013; Lee, Davies, & Mishima, 2012; Mathew *et al.*, 2014). The available evidence seems to suggest that the G_1 phase is the time of the cell cycle between the last mitosis and the start of DNA replication; as a result cytokinesis follows this particular phase (Mahoney *et al*., 2014). Hence the G_1 stage is where most of the cells spend a greater part of the cell cycle in this phase. Also cells which have gone through the cell cycle and finished dividing spend time in this phase until they die (Urrego *et al.*, 2014). Furthermore the time spent in this phase is constant with the same cell types, however there is a difference observed with cell types, under regular circumstance (Heuvel et al., 2005). According to Bertoli *et al*., (2013) the cell cycle regulators that are chiefly present through the transitions from the (Gap Phase) G_1 to S (Synthesis phase) are the cyclins D and E, and cdk 2, 4, and 6. Consequently the cell ensures that the DNA is complete, has no damages and also whether the conditions outside the cell are favourable for the progression of cell division. This is however one of the important stage of the cell cycle which also known also the DNA check point (Urrego *et al.*, 2014).

Over the past decade, most research on the cell cycle emphasizes the function of the regulators involved in the control of the cell cycle, at each phase. These are referred to as the cell cycle checkpoints. This spot plays an significant role in the management of

the cell division in the correct manner, otherwise elevated incidence of mutations will be found (Ben-shlomo, 2014; Bristow *et al.*, 2014).There seems to be compelling reasoning that the cell cycle checkpoints, are stages during which the cell cycle is detained in the G1, S phase, G2 or even mitosis in case of DNA injury through mutation (Mahoney et al., 2014). The DNA duplication checkpoint, and the DNA replication checkpoint makes certain that mitosis is not commenced in anticipation for complete accurate DNA duplication, in addition to ensuring no dual DNA is replicated (Bertoli*et al*., 2013) to avoid conditions such as genetic chimera. Another is the Spindle Assembly Checkpoint (SAC), which holds-up the starting of the anaphase in anticipation of all chromosomes to be correctly affixed to the mitotic spindle (Mombach, Bugs, & Chaouiya, 2014). Consequently these checkpoints are made up of a sensor that identifies the error, which triggers a signal through a signal transduction passageway by the elements. Finally (C. Behl and C. Ziegler, 2014; Mombach et al., 2014) suggest that, the response by the cell cycle to the above mentioned obstruction of the cell cycle progression, results in the activation of :

1. Cell cycle arrest for a temporary period during which DNA repair is taking place or

 2. The cell may undergo permanent arrest known as senescence phase.

3. Lastly apoptosis also known as programmed cell death.

However, in the case of any fault or disturbances at the restricted points during the above mentioned three processes of DNA repair, Senescence programmed and apoptosis the cell cycle may be led into DNA mutation which may in turn cause cancer (Mahoney *et al*., 2014). In recent years there have been increasing amount of literature supporting

that the causes of cancer or carcinogenesis may be influenced either by endogenous or exogenous dynamics (Poon, Mcpherson, Tan, Teh, & Rozen, 2014).The combination of the two types of domains entails the possibility of complex synergistic or individual harmful impact on the DNA material of the human cell lines (Chen *et al*., 2014). Consequently the endogenous dynamic is more virus oriented focusing more on the intra faulty activities on the DNA material within the human body cells, such as the activation of mutation in genes and mismatch repair deficiencies. The complexity of the cancer disease is also based on the on the extent to which the disease progress and the severity thereof which is categorized into different cancer stages.

2.3.2. Cancer Stages

There is a growing body of literature that supports and recognizes the importance of knowing the stage to which the cancer has developed for the selection of the appropriate treatment option (Rakha *et al*., 2010; Sagalowsky, 2011). Therefore it is important to note that various cancer diseases may have different classification, groupings and staging systems, with regards to the extent to which the disease severity has developed at the time of diagnosis. Furthermore the diversity and the specificity of the cancer cell type and the extent to which other organs have been damage also plays a vital role for proper classification of cancer into stage groupings. Hence the classification of cancer into grouping helps with determining the type of treatment that is appropriate for the particular cancer stage. Consequently this is also influential on the establishment of the patient survival rate. Moreover some staging systems may be associated with a vast number of cancers while others are more for specific cancer types.

Firstly there is the TNM system which is the T (Tumour) N (Node) M (Metastasis) system (Alison & College, 2001; Escudier et al., 2012; Perera, Gnaneswaran, Jennens, & Sinclair, 2013). This classification system is based on the magnitude of the tumor and the extent to which the particular cancer has multiplied to the Lymph nodes and the distance of metastasis. Then there is the Roman numeral staging I-IV system (National cancer institue, 2012) which is governed on the following category I - Small localized meaning that the tumor is still close to the site where the cancers was created, usually these tumor are invasive and has not grown deeply into the nearby tissues thus no spread to either lymph nodes and other parts of the body and these tumor are usually curable. While II and III –are for classification for a locally advanced cancer and the cancer that involves invasion of the lymph nodes where the tumors size are larger in size than stage 1 tumors and that have developed more intensely into close by tissue and multiplied to lymph nodes although not to other parts of the body. Although the cancer stage II indicates less complexity than cancer stage III the stage is still detrimental to the human body.

Finally the cancer stage IV – where the cancer has reached an inoperable and metastasis level (American Cancer Society, 2014; American cancer society, 2015). This means that the cancer has spread to other organs or parts of the body and is hence "metastatic" .Nevertheless each of the three cancers has their staging and grouping accordingly, and based on this the treatment option is determined whether it is chemotherapy, radiation or surgery.. Hence to address the ill of cancer to the body, many different treatment options are presented being it, conventional or traditional methods.

As mentioned earlier there are various factors that influence the availability and the choice of treatment. Factor such as what type of cancer it is, the location and the stage to which the cancer has developed, also the availability of the treatment and its effectiveness in treating the disease. Despite the presence of this treatment options there is still an increase in the cancer mortality and morbidity, worldwide. Consequently the presence of conventional cancer treatment options has to a certain extent addressed the disease. Treatment for cancer patient also has lightened the burden on the world health sectors. The most common conventional treatment options are: surgical, radiation, chemotherapy, hormonal therapy, and biological therapy. These treatment options in place focus on the cancerous cells that are actively replicating, aiming at directly damaging DNA which will be interference to the cell cycle progression and activating programmed cell death (apoptosis). Otherwise anticancer agents also act indirectly hindering the process of tumor cell mitosis through crippling the molecules necessary to DNA synthesis (Gavhane *et al.*, 2011).

2.3.3.Cancer treatments, and disadvantages

The above mentioned treatments are used solely or in combination (Alawode, 2013), depending on the nature of the cancer. In addition each of these conventional methods will be discussed, starting with the surgery treatment during which the cancerous tissue is excised, this is normally after adjuvant therapy, this is frequently used to minimize the tumor in size adequately to make surgical removal less extensive surgery (Escudier*et al*., 2012), the adjuvant therapy is also use to making certain that the any remaining cancerous tissue is destroyed. Then there is chemotherapy which the use of drug

regiments, this may be used alongside the other treatment option, to kill the cancer (Solowey *et al*., 2014).

Lastly the use of radiotherapy to treat cancer, the treatment employs the use of high ionizing energy, of electromagnetic x- ray, gamma ray and particle beam radiation of electrons protons and alpha particles. Consequently radiation to destroy cancerous tissue cells, through interfering with molecules such as DNA, distorting the replication during the process of cell cycle (R Dummer, Hauschild, Guggenheim, Keilholz, & Pentheroudakis, 2012; Perera *et al*., 2013). However since the study's focus will be on breast, human melanoma, and renal cancer, their individual treatment options will be discussed below. Breast cancer is normally treated by surgery to the remove of the cancerous mass and chemotherapy (tamoxifen, anthracycline doxorubicin) drugs targeting the process of cell division (Dolle *et al.*, 2009; Nelson *et al*., 2009; Partridge*et al.*, 2014). Several studies reports the use of chemotherapy using ipilimumab (Yervoy) and vemurafenib (Zelboraf), immunotherapy using cytokines and radiation to treat melanoma cancer (R Dummer *et al*., 2012; Halpern Allan, Perry Robins, 2011; Perera *et al*., 2013).

Finally the renal cancer is treated using chemotherapy (vinblastine, floxuridine, 5-fluorouracil (5-FU), capecitabine, and gemcitabine) immunotherapy using cytokines and radiation (American cancer society, 2013). The conventional methods mentioned above have draw backs which will be addressed as follows. There is evidence that the limitation associated with treatment of cancer has an impact on the increasing mortality

and morbidity of cancer. In view of the fact that one of the main obstacles, reported with regards to conventional cancer is the drawbacks of such conventional cancer treatments options (Ahmed, 2013; Mcinerney *et al.*, 2014) For the reason it is of importance to outline the disadvantages that are associated, with these particular treatments, as they are known to have detrimental effect on the human body.

 Several studies has documented , that surgery increases the chances of normal tissues in the vicinity of the cancerous tissue may experience major tissues deformities and dysfunction in the long run (Gavhane *et al*., 2011). Also cancer patients with compromised immune systems in the presence of other diseases may not endure the surgery treatment. The (American cancer society, 2014) states that the key concern with the use of chemotherapy is some of the draw backs of this drugs , one of which is the resistance of the cancer to the drug, since the cancer mutates continuously the drugs lose their specificity to the particular malignancy. Secondly chemotherapy toxicity targets tissues that contain actively dividing cells e.g. hair follicles, gonads, bone marrow, and the gastrointestinal tract (Abdul & Hassan, 2012). That's why cancer patients experience side effect of Alopecia (hair loss), next is myelo suppression which is the suppression of white blood cells and platelet production this is associated with infections and bleeding (Macmillan cancer support, 2012). Then there is the occurrence of   painful mouth sores (stomatitis) , cancer patients also experience diarrhoea which also causes dehydration (Dennert & Horneber, 2006). There is also a possibility of permanent infertility due to reduced spermatogenesis and ovarian follicle formation. Increased chances of premature menopause may be stimulate due to the use of chemotherapy.

 Lastly the cancer causing nature of some of the therapies such as alkylating agents like cytoxan and nitrogen mustard, which may stimulate secondary malignant cancer, while the toxicity of chemotherapy may cause damage to various organs such as the heart, kidney, liver, lung, and even the nervous system of the body. Lastly the ineffective penetration of anticancer agent into solid malignant cancer has been a weakness of chemotherapy (Gavhane *et al.*, 2011; Rasool Hassan, 2012; Remy, Marret, & Bonnet, 2005).Previous studies have reported that ionizing radiation has no distinction between normal tissues and malignant tumors thus both type are damaged. Hence the damage of normal tissue and organs of the body may result into: Firstly bone marrow suppression, dryness of mouth (xerostomia) if salivary glands are irradiated (Tolentino *et al.*, 2011).

Then there is the inflammation of various organs of the body such as the esophagus may cause painful dysphasia the mucosal lining of the gastrointestinal tract can cause diarrhea, nausea, or vomiting. Next painful mouth sores may also occur. Radiation also damages the lung which may lead to pneumonitis and pulmonary fibrosis causing hypoxia and dyspnea. Cataracts (if eyes are irradiated) Infertility (if gonads are irradiated). In instances of radiation treated rectal cancer problems are bowel obstructions; bowel dysfunction presented as fecal incontinence to gas, loose or solid stools, evacuation problems or urgency; sexual dysfunction, skin changes such as alopecia, erythema, pruritis, increased pigmentation, or desquamation. Since radiation is itself capable of causing cancer, secondary malignancies (e.g., leukemia, thyroid cancer) may be induced by radiation therapy. Lastly radiation therapy also causes: spinal cord

dysfunction (myelopathy), pericardial or myocardial damage, bone necrosis, and necrosis of the bowel (Gavhane *et al.*, 2011).

 Despite these disadvantages the conventional cancer treatment provides the reduction of various cancers within the human body to a certain extent. The preventative and curative properties associated with plants are because they are considered to be rich in resources that exhibit particular therapeutic values (Abdul & Hassan, 2012; Enyiukwu, Awurum, Ononuju, & Nwaneri, 2014). These rich resources are secondary metabolites that are present in the plants (Agnieszka Jamio1kowska, 2014). Naturally plants produce primary and secondary metabolites. The primary metabolites are found in various medicinal plant species they are linked to broad phylogenetic classes (Ramasubramania Raja. R Sreenivasulu M, 2015). Secondary metabolites are made as a result of adverse ecological circumstances, producing an immense library of chemical compounds (Agnieszka Jamio1kowska, 2014).

It is however important to note the possibility of plants being either wild or domestic species determines the range of secondary metabolites. The wild species naturally grow in their untamed habitat where it sustain itself. On the contrary the domesticated species are cultivated through selective breeding by human beings in gardens at home, or on a large agriculture plot where the plantation or garden is cared for on a regular basis. Hence this different in environmental condition, (temperature, soil composition and availability of water) have an influence on the metabolites produced by such plants (Radušienė, Karpavičienė, & Stanius, 2012; Ramakrishna & Ravishankar, 2011).

However the function and the benefits of these compounds to the plant is not well defined (Ramasubramania Raja. R Sreenivasulu M, 2015).The latter has no apparent implication in the normal growth, development and reproduction of the plants. According to (Agnieszka Jamio1kowska, 2014; Alamgir, Rahman, & Rahman, 2014; Enyiukwu et al., 2014; Makkar, Norvsambuu, Lkhagvatseren, & Becker, 2009) There is however a general inference that secondary metabolite produced by plants confer survival properties . Secondary metabolites play an important role in the protection against predators such as parasites, herbivores, mammals and diseases, for interspecies competition, and to facilitate the reproductive processes coloring agents, attractive smells, etc (Ramasubramania Raja. R Sreenivasulu M, 2015; Taylor et al., 2014) Furthermore the survival properties exhibited by plants are linked to the plants medicinal properties observed. Therefore some of these medicinal plants are used traditionally to treat various ailments in the human body (Bhat, 2014; Naidu, Bahadur, & Kanungo, 2014; Savithramma, Rao, & Suhrulatha, 2011) .

The uses of plant for medicinal purposes has found firm ground not only in the traditional settings but also in contemporary, modern allopathic medicine (Prasad S, 2015). There are several research and development steps before the stage where plant medicine or herbal medicine can be considered to be medicinal drugs. To demonstrate this, various studies will be discussed, beginning with identifying the use of medicinal plants in different parts of the world for diseases and ailments (Alsarhan, 2014; Hassan, Rahman, Deeba, & Mahmud, 2009; Khare, 2007; Verma & Singh, 2008)., The use of

medicinal plants is still prominent in many countries despite the presence of modern and contemporary medicine (Coady & Boylan, 2014). The key aspects about the above claim may be linked to the availability herbal remedies, and its historic and cultural associations of medicinal plants. Further medicinal plants are used by almost 80% of the world's population for various therapeutic purposes whether it is to treat disease or ailment of some sort (Adnan, Bibi, Mussarat, Tariq, & Shinwari, 2014; Bhat, 2014; Mohanty & Pradhan, 2015).Numerous studies have attempted to address a general concern on the efforts to perpetuate knowledge on ethnobotany that exist amongst population orally (Allen & Hatfield, 2004; Coady & Boylan, 2014; Maroyi, 2013; Shosan, Fawibe, Ajiboye, Abeegunrin, & Agboola, 2014).

Research is conducted in various fields to preserve the medicinal plant knowledge that exists amongst the knowledge holders and an interest in linking ethnobotany and possible pharmacological value of plants (Gavhane et al., 2011; INwachukwu, Umeh, I., Slyvester, & Magnus, 2010; Silva & Fernandes Júnior, 2010). Primary metabolites such as the carbohydrate, protein, triglycerides (fats and oil) are known to be the core to the nutritional bases of most food crops which is essential to human beings (INwachukwu *et al.,* 2010). However the important role played by the secondary metabolites the interest of this particular study. The major plant compounds such as alkaloids, terpenoids and flavonoids are the backbone of the medicinal properties observed in plant and their derivative products (Adnan *et al.*, 2014). Medicinal plants have been used and will continue to be used for physical and spiritual ailments of diseases in humans focusing

on the wellbeing of human through curative and preventative means (INwachukwu *et al.*, 2010).

2.3.4. Anticancer medicinal plants

Medicinal plants and their derivatives have shown potential biological activity against the various cancers, in various *in vitro, in vivo* and human clinical trials to the extent that they are used in conventional chemotherapy (Alam & Khan, 2014; Hiruma *et al.*, 2015; Prakash, Kumar, Kumar, & Ajeet, 2013; Solowey*et al.*, 2014). Despite the demonstration of anticancer bio-activity by these plants and their derivatives there is still a long way to go in terms of address the constrain exerted by the complexity of the cancer disease in terms of treatment, such as the drug resistance, side effects, which may arise due to non sensitivity and specificity of drugs .

However the gap in this field can only be tackled through knowing the progress in this particular field of interest with reference to what has been done to date on cancer drug discovery and development. Thus the investigation for anti-cancer plant sources dates back to 1950s with the breakthrough and improvement of the vinca alkaloids (vinblastin and vincristine), the Paclitaxal or taxol isolates and then the *campothecin* derivative (Prakash *et al.*, 2013). The three anticancer plant derivatives will be outlined, to provide an understanding on the development of different anticancer plant and plant derived sources to date.

(Khan, Aliabbas, Kumar, & Rajkumar, 2009; Ode, Asuzu, & Ajayi, 2011; Rasool Hassan, 2012; Veeresham & Chitti, 2013). The *Catharanthus roseus* G. Don. (*Apocynaceae*), seen in figure 2 is a medicinal plant endemic to Madagascar from which vinca alkaloids (vincristine and vinblastine) as can be seen in figure 3 were derived, played asignificant role in the discovery of anticancer plant source (Khan et al., 2009; Ode et al., 2011; Rasool Hassan, 2012; Veeresham & Chitti, 2013). These plant extracts were fractionated, which resulted in the seclusion of the vinca alkaloids that are the vincristine (VCR) and the vinblastine (VLB).

Furthermore these two were the first agents launched in clinical applications. The vincristine (VCR) exhibits bioactivity against severe lymphocytic leukemia in children, while the vinblastine (VLB) is used to treat breast cancer, lymphomas, leukemias, testicular cancer, lung cancers, and Kaposi's sarcoma (Mu *et al.*, 2012). Additional improvement on the vinca alkaloids was the development of synthetic analogues vinorelbine (VRLB) and vindesine (VDS) also for the use in cancer treatment as demonstrated by (American cancer society, 2014; R Dummer *et al.*, 2012; Partridge *et al.*, 2014; Prakash *et al.*, 2013).

Figure 2 Catharanthus roseus (L.) G. Don (Madagascar periwinkle) Apocynaceae Family. (Morebise, 2015) Contains over 130 compounds, and many of which have cytotoxicity .from which Vincristine and Vinblastine id derived
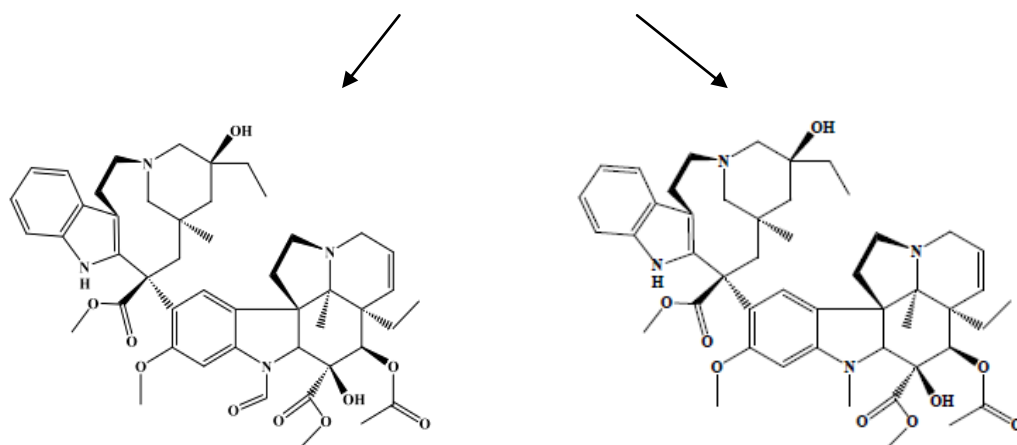
Figure 3 Chemical structure of Vincristine and Vinblastine respectively (Mu et al., 2012)

Another plant that is also useful in treating cancer is the *Taxus brevifolia* as can be seen in figure 4 and many other Taxus plant species which are used by native Americans to treat various ailments (Alawode, 2013; Julsing, Koulman, Woerdenbag, Quax, & Kayser, 2006; Prakash *et al*., 2013; Prasad S, 2015; Ramasubramania Raja. R Sreenivasulu M, 2015). Existing research recognizes the critical role played by the bioactive molecules paclitaxel or taxol that was first secluded from the bark of *Taxus* as

well as active paclitaxel analogs, docetaxel (Taxotere) the chemical structure are seen in figure 5 ,(Khan *et al*., 2009) in the treatment of wide variety of cancers including breast, ovarian and non-small-cell lung cancer, and has also shown efficacy against Kaposi sarcoma (Prakash *et al*., 2013).

Figure 4: *Taxus brevifolia* from which paclitaxel and docetaxel are derived
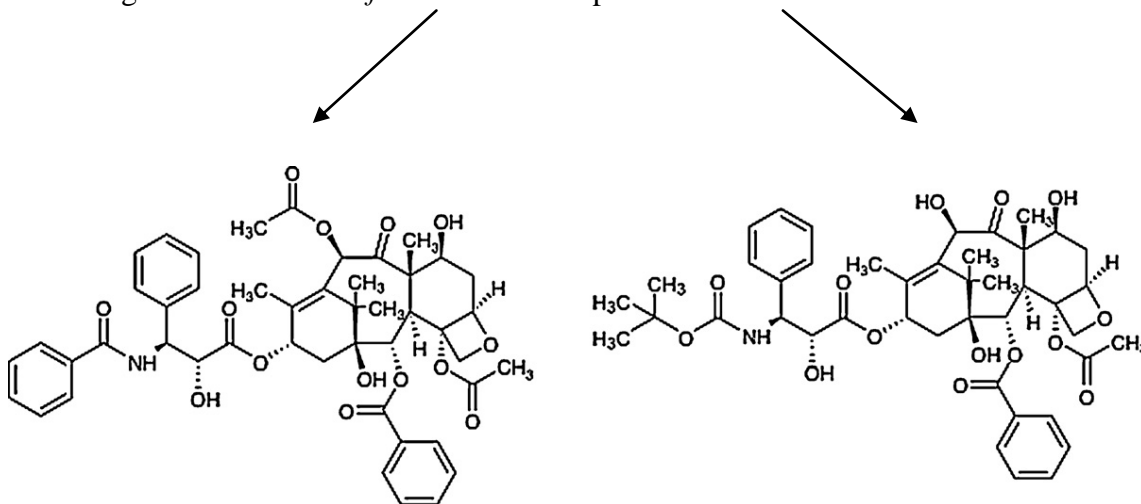
Figure 5: Chemical structures of paclitaxel and docetaxel respectively (Watchueng & Gao, 2016)

Lastly the Chinese ornamental tree, known as *Camptotheca acuminata,* as can be seen in figure 6 whose plant extract has been reported to have anticancer active compounds

(Prasad S, 2015). Campothecin was the first isolated from the *Camptotheca acuminate.* The plant isolate was used in clinical trials for the first time in 1970s, but failed as a result of toxicity. Henceforth the camptothecin derivatives underwent further studies from which and Topotecan seen in figure 7 (Hycamtin) used for ovarian and small- cell lung cancers and Irinotecan used for colorectal cancer were developed *(Prakash et al.,* 2013; Veeresham & Chitti, 2013).

Figure 6:*Camptotheca acuminata* from which Topotecan and Irinotecan are derived
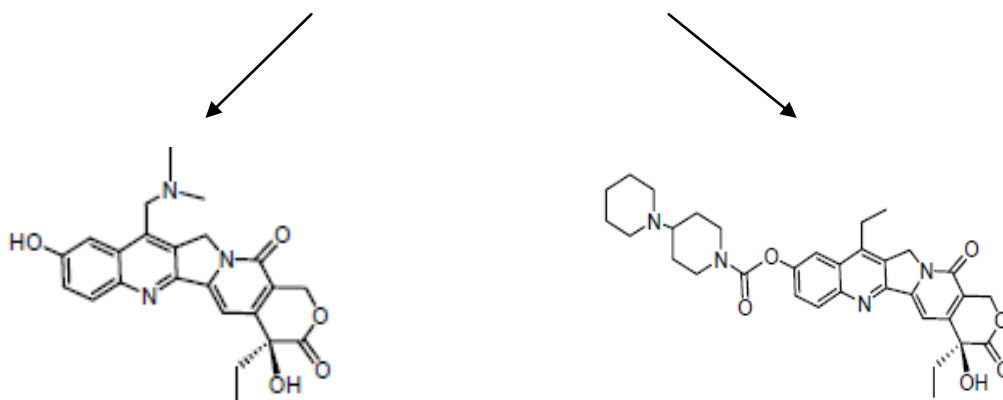
Figure 7: Chemical structures of Topotecan and Irinotecan respectively(Wu et al., 2015)

Medicinal plants also exhibit antioxidants are compounds that stops, delay or prevent oxidative stress. These compounds are found in plant and are also know for their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Dai & Mumper, 2010). These properties are beneficial to diseases such as cancer and coronary heart disease (Mediani, Abas, Tan, & Khatib, 2014).

Therefore it would be beneficial to this study to carryout test to understanding the link between the antioxidant compounds and their radical scavenging abilities   According to reports several aromatic compounds found in plants have been reported as having anti-inflammatory, antiallergic, antimutagenic, antiviral, antithrombotic, and vasodilatory actions (Oliveira et al., 2012). These bioactive antioxidants compounds comprises of the classes phenols, flavonoids, terpenes, tocopherols, nitrogen compounds, vitamins, terpenoids, carotenoids and other endogenous metabolites, which have been identified in previous research to have possible bioactivity properties. Plant medicinal abilities may be as a result of synergistic of individual effect of some of these plant compounds.

The phenol classes of antioxidants are also commonly from the Lamiaceae family (Chinsembu & Hedimbi, 2010). While the most common flavonoids present in medicinal plants include quercetin, kaempferol, luteolin and apigenin (Oliveira et al., 2012; Ozkan, Kamiloglu, Ozdal, & Boyacioglu, 2016; Phosrithong & Ungwitayatorn, 2010; Sen & Chakraborty, 2011). The flavonoids class of compounds exhibits it medicinal abilities against various disease by the act of reducing the multiplying of the free radical, and the formation of the radicals.

Medicinal plant terpenes come mostly in two form the mono- and diterpenes. Monoterpenes classes of compounds are such as carvacrol, menthol, myrcene and thymol know to be the most common in medicinal plant (Ozkan et al., 2016; Silva & Fernandes Júnior, 2010). While the carnosic class of compounds are the most common diterpene in medicinal plants (Ozkan et al., 2016; Park & National, 2012).

The buildup of reactive oxygen species, which are not unfavorable for the normal functioning of the human body in plastids, which plays a significant task in controlling singlet oxygen levels within thylakoid membranes, is controlled by the presence of tocopherol class of compound, commonly present in the form of fennel cumin and the carotenoids class of compounds are commonly found in plants in the form of β-carotene, lutein, and zeaxanthin (Ozkan et al., 2016; Sen & Chakraborty, 2011). Furthermore the anticancer and cytotoxicity of these plant extracts have certain criteria it need to meet to be considered as anticancer or toxicity levels.

The American National Cancer Institute (NCI) guidelines defines the limit for the criteria of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation of <30 µg /mL after the exposure time of 72 hours. While the crude extract with (IC_{50}) of <20 µg/mL is considered highly cytotoxic (Vijayarathna & Sasidharan, 2012). In other word its reduced dose and higher inhibition level are more suitable concentration used. The basis of the endogenous and the exogenous cancer causing factors, the carcinogens, and the risk factors of each of the three focal cancers of this study is outlined in detail below.

2.4 Cancer Epidemiology and risk factor

2.4.1 Cancer risk factor

Several recent studies (Jardines *et al.*, 2015; Mcinerney *et al.,* 2014) on risk factors of cancer have fast becoming a key instrument in understanding the causation of all cancer subtypes. Historically, research investigating the risk factors associated with cancer are known to increase the probability of a person developing cancer through compromising the normal functioning of the body however the focus is to a larger extent on the exogenous risk factors in contrast to the endogenous factors (Bertrand *et al.*, 2013; Dolle *et al.*, 2009; Samet *et al.*, 2009).Additionally risk factors maybe general, nonetheless there are specific unique associations towards each type of cancers. By far the best known general risk factors for cancer are chemicals, radiation, and viruses (WHO, 2004).

Incidentally some of the risk factors can be managed but others not. Recent evidence suggest that breast cancer has some of the very common factors which are age, family and clinical history, late pregnancy, prolonged exposure to hormones and life style factor (Bertrand *et al.*, 2013).   Women over the age of 40 are regarded as probable candidate for breast cancer also if there is a personal or relatives history about breast cancer previously the individual is at amplified risk (Alieldin *et al.*, 2014; American cancer society, 2012). While women who have their first pregnancy after the age of 35 are most likely to developing breast cancer (Jardines *et al.*, 2015). Consequently family history of breast cancer increases the likelihood of getting cancer as it is passed on

from one generation to another particularly in the case of first and second relative (Dossus & Benusiglio, 2015).

Inflammatory breast cancer and the locally advanced, invasive breast cancers are most common in this case (Schairer *et al*., 2013; Thomson *et al.*, 2014). Hence these cancer types are commonly influenced by genetic factors such as the two genes most often found in hereditary breast cancer called the BRCA1 and BRCA2. Hormonal behaviors and exposure to exogenous hormones is also another factor that may contribute to the increase of breast cancer. Several studies suggest that hormone replacement therapy (HRT) or postmenopausal hormone use, increases the risk of invasive breast cancer by 26 % (Jardines *et al*., 2015).

Consequently life style choices such a physical inactivity which may result in overweight or obesity, diet and alcohol consumption may also engage in the triggering of cancer development (WHO, 2004). There might be similarities amongst the risk factors of breast melanoma and renal cancer. Hence in a similar way the risk factors for renal cancer will each be highlighted next. The main risk factor for nearly every melanoma cancer is the exposure to ultraviolet rays, being it the sun which supplies small fractions of UV rays (Ahmed, 2013) this damages the DNA of the skin cells, and hence the sun is the core source of UV resulting into the cause of this particular cancer.

There is consensus amongst researchers that fair skin, freckling, and light haired individuals with a weakened immune system, are all prone to melanoma cancer

(Gawkrodger, 2004; Narayanan, Saladi, & Fox, 2010) with factors such as personal and family history of the disease the increasing the likelihood of an individual getting skin cancer (Dorothy A Shead, Laura J Hanisch , Lacey Marlow, Maria Ho, Nicole Mcmillian , Susan Kidney, 2014). Lastly the risk factors that increases the chances of an individual getting renal cancer are smoking which is a growing trend in developing countries increases the likelihood of Renal cancer carcinoma (RCC) with about 20% and 50% in female and the male on the smoking individuals in the population (Chow, Dong, & Devesa, 2010), while (Protzel, Maruschke, & Hakenberg, 2012) reports that frequent smoking is associate with higher risk of getting renal cancer.

Furthermore some studies suggest that that cigarette smoking increases the risk of renal cancer by a chronic condition known as tissue hypoxia which is caused by carbon monoxide and increased levels of DNA damage in peripheral blood lymphocytes (Qayyum, Oades, Horgan, Aitchison, & Edwards, 2012). Obesity has also been reported to increase the renal cancer incidence, not only in third world countries but also in middle and low income countries with an estimated 24% in the male population and 34% in the female population worldwide (Markevičius & Jankevičius, 2015). Long term exposure to chemicals such as cadmium metal herbicides, and organic solvents (trichloroethylene) is known to be carcinogenic to humans (American Cancer Society, 2014; Chow *et al.*, 2010; Markevičius & Jankevičius, 2015).

However there are also some endogenous types of inherited diseases that elevates the chance of getting (RCC) for example von hippel-Lindau (VHL) disease, (Losonczy *et al.*, 2013) hereditary papillar renal cell carcinoma (Hansen *et al.*, 2015), hereditary

leiomyoma-renal cell carcinoma, (Henrion *et al*., 2013) birt-hogg-dube (BHD) syndrome, hereditary renal oncocytoma, and familial renal cancer family history, high blood pressure, and finally advanced kidney diseases (Shuch *et al*., 2014).

Conversely the presence or absence of risk factors may not be conclusive whether an individual will get cancer or not. In addition there is also an uncertainty of the extent to which the cancers may be influenced by various risk factors. Depending on the exposure of the specific cell type or tissue to a certain risk factor being it exogenous or endogenous, the site of origin of the particular cancer determines to what form of - cancer belongs.

2.4.2 Incidence of Breast, Renal and Melanoma cancer

The different forms of cancers depend on the types of cells the cancer originate from, categorizing them into the sarcomas, lymphomas, carcinomas and leukemia (Oncology group, 2014). Sarcomas are tumors that grow from connective tissues in the body, such as muscles, fat, bones, and joint cartilage (Latif, Zaiden, Pham, & Rana, 2010) while the cancers that originate in the lymphatic system and wherever lymphocytes are present in the bone marrow, lymph nodes, the spleen, the intestines, are referred to as lymphomas (Feature, 2008).

Conversely carcinomas are the cancer that originates in the lining layer of the tissue such as the epithelial and glandular cell tissue (Gavhane*et al*., 2011). While the hematological cancers such as leukemia is as a result of immature blood cells that accumulate in the blood stream (Feature, 2008). Nevertheless the three cancers that are

of interest to this study namely breast, melanoma, and renal cancers are further summarized below to depict a clear understanding on where each of the originates from.

2.4.2. (a) Breast Cancer

The cancer of the breast is a common malignant or benign condition of the breast organ distinguished by the uncontrollable growth of abnormal cell in the milk producing gland and or the duct through which milk reaches the nipples (Macmillan cancer support, 2012). Over the past decade breast cancer has been on the rise. The cancer is most frequent amongst women worldwide, in both the developed and developing countries; it is by far the second most frequent cancer in the world(Boyle, 2012). There were about 1.67 million new cancer cases diagnosed in 2012. Breast cancer deaths are rated to be 522,000 per year (Xiangming *et al*., 2014). The cancer is known to affect females more than males with a ratio of approximately 100:1 for women to men (Ferlay *et al*., 2013; Jardines *et al*., 2015) In 2008 breast cancer in Africa reported about 92,000 new breast cancer cases, while accounting for approximately 50,000 deaths (Evan *et al*., 2011). In Namibia breast cancer is amongst the most prevalent cancer at about 28% (Dushimemaria & Mumbengegwi, 2015).

The spread of malignant tumor is facilitated through blood stream and the lymphatic system also according to (Alitalo & Detmar, 2012) the formation of new blood vessels by angiogenesis to feed on the nutrients to promote its out of control growth is observed (American cancer society, 2014; Komen, 2014). Furthermore it is important to note

cancers are named after their site of origin as mentioned before, for instance Breast cancer has its starting site at the breast organ but eventually through metastasis the cancer migrates to other surrounding tissues such as the liver and cause infection and causes damage in those tissues at the secondary site, this cancer will still be referred to as Breast cancer (Komen, 2014).

Central to the entire discipline of cancer,it is important to know that each of the three cancer of interest to this study have their subtypes which can either be benign or malignant. For this reason it is important to note that there are different forms of breast cancer the benign breast lumps such as fibrocystic transformation of the breast that results from fibrosis which is the development of scar- like tissue and or cysts which is a watery packed sac in the breast. These benign breast lumps are experienced by numerous women at some point in their lives. Benign breast tumors such as *fibroadenomas* or *intraductal papillomas* are irregular growths, but are non cancerous and neither are they malignant (Joanna, 2015; Solak, Yalaz, & Sivrikoz, 2013).

On the contrary there are also malignant breast tumors such as the ductal carcinoma in situ (DCIS) which is formed at the duct (the milk transport vessel), but does not invade or harm other tissues (Siziopikou, 2013). Than there is the invasive ductal carcinoma (IDC) which is capable of infiltrating and causing harm to the neighboring tissue (Dossus & Benusiglio, 2015). There is also the invasive lobular carcinoma (ILC) which is formed at the glands where milks is produced (Christgen & Derksen, 2015). Other less

frequent cancers are those such as the paget disease of the nipples, angiosarcoma and phyllodes tumor (American cancer society, 2014).

Breast cancer is also classified based on the condition of three specific cell exterior receptors, they are the oestrogen receptor (ER), the progesterone receptor (PR) and the Human Epidermal Growth Factor Receptor (HER)2/neu receptor (Viale, 2012). (Hefti *et al*., 2013) There are claims that the hormone receptor- positive breast cancer is controlled by the estrogen and the progesterone hormones (Hefti *et al*., 2013).

2.4.2 (b) Melanoma Cancer

Melanoma cancer is another whose incidence and mortality have increases in the past 50 years globally. The cancer has the highest incidence in Australia and New Zealand (Sneyd & Cox, 2013; Williams & Dienes, 2014). In 2007 about 2,173 melanoma cases were reported of which 292 died from the disease, whereas Australia reported 10,342 melanoma cancer cases and 1.279 deaths. The cancer particularly affects fair skinned individuals (Fabbrocini *et al.*, 2010; Godar, 2011) with up to six percent, cases reported, particularly in the Europe, United States, Australia and New Zealand (Reinhard Dummer *et al*., 2011; Sneyd & Cox, 2013; Williams & Dienes, 2014).

In Namibia the annual crude incidence of about 1.5 per 100,000 Namibia, Female between the age of 15−44 years, with about 150 deaths annually ("Human Papillomavirus and Related Diseases Report," 2015).   The understanding of melanoma cancer is based on appreciating the function of the epidermis which is the outer layer of the largest organ of the body the skin, the epidermis is made out of three main types of

cells, the flat squamous cells, the basal cells and the melanocytes. The latter type of cells types is dependable for generating a protective dark pigment called melanin, when these cells are harmed or damaged they become melanoma (American cancer society, 2015).

The *malignant melanoma* and *cutaneous melanoma* are some of the common harmful forms of melanoma cancers (Bichakjian et al., 2011; Perera et al., 2013). On the other hand the benign form of melanoma includes the appearance of tan, brown, or black raised spots with a "waxy" texture which is referred to as Seborrheic keratoses and others such as hemangiomas, lipomas, and wart (Perera *et al*., 2013) hence most of the benign tumors infrequently turn into cancer.

2.4.2. (c) Renal Cancer

Renal cancer is not as common as the breast cancer and the melanoma cancer. This type of cancer accounts for 110,000 to 160, 000 deaths, annually (Protzel *et al*., 2012).Which approximately 2% of all cancers worldwide, with about 270 000 new cases reported yearly (Qayyum *et al.*, 2012). The general incidence of renal cancer in Africa is low compared to the other third world countries (Evan *et al*., 2011; Protzel *et al*., 2012) Lastly the renal cell carcinomas (RCC) also known as the cancer of the kidney. The chief function of the kidney is to sieve your blood consequently removing excess water, salt, and waste products from the body (American Cancer Society, 2014). However the presence of cancer the function of the organ is compromised. Furthermore there are different types of renal cancers, depending on the microscopic morphology of the kidney cancer cell., For instance the clear cell papillary, chromophobe, and finally the collecting duct, renal cell carcinomas (RCC) (Shuch *et al*., 2014; Vera-Badillo, Conde,

& Duran, 2012),these are the common malignant forms of this cancer (Shuch et al., 2014; Vera-Badillo et al., 2012). Then there is the benign form of the diseases such as the renal adenoma, oncocytoma, and the angiomyolipoma (Wood & Sandler, 2009).

2.5 Plant diversity in Namibia

Namibia is a country with diverse assortment of plants this is particularly due to the many climatic ecological zones in the country including the desert which is characterized by dry temperatures and low rain, where vegetation is mainly grasses that grow yearly and dwarf shrubs. The Karoo is associated with karoo bushes, grass and a climate that has great seasonal and daily temperature differences. The Acacia Savanna is distinguished by the great range of endemic species to Namibia, including grass species and acacia species such as camelthorn and blackthorn. The broad leafed Savanna is characterized by high species difference particularly the deciduous tree species such as the Mopane and a rainfall ranging between 450-700mm per annum. Lastly the Wetland and Coastal/Marine vegetations such as the Northern Namibian floodplains along streams and rivers, lakes and ponds. The country is a semi-arid, dry country where extreme temperatures are experiences in some parts (Peters, 2010). It is globally known for being a biodiversity hotspot with areas that have exceedingly high species wealth and endemism of global importance (Namibian Ministry of Environment and Tourism, 2010). These extreme climatic conditions may force the plants to develop and adapt various mechanism of survival, which may be of significance to plant medicinal nature. Consequently it is also important to note that the southern regions of Hardap and //Karas, are situated near to the Sperrgebiet area, which is one of the most important

conservation areas in the world. The Succulent Karoo Ecosystem has the highest diversity of succulent flora globally. Thus over 10,000 plant species have been identified in this ecosystem including 1,954 endemic plant species (Namibian Ministry of Environment and Tourism, 2010).

2.6 Plant use by the Nama people especially in the southern Namibia

In the rural area of Namibia, where medical facilities and services are scarce patients, walk or use a donkey drawn cart to reach the nearest, medical center. Thus indigenous people in this areas use medicinal plants especially in the rural areas of the Southern Namibia. Consequently the use of medicinal plants as primary health care in the Regions of Hardap and the //Karas by the Nama people plays an important role as it, attends to treating various ailments and diseases, at low cost. Since the local Nama community member cannot afford the expensive modern pharmaceuticals and health care.

The traditionally used medicinal plants are readily available near to the homestead and freely accessible for use hence affordable and available. These traditionally used medicinal plants are also culturally acceptable in many African communities (Maroyi, 2013). The plants that are investigated in the study namely the *Acanthosicyos naudinianus , Fockea angustifolia , cf Salvadora persia* and *Nymania capensis* , are used by the various Nama communities to treat fever, headaches, diarrhea, abdominal pain , cough, skin lesions inflammation and sicknesses that are associated with HIV. Different Nama communities in the two regions each have their own specific approach to health in treating various ailments and diseases. In this respect there are however

different ways of collecting, preparing and administering these herbal concoction for the
different treatment

2.7 Mechanism of action of anticancer medicine of both natural and synthetic compounds, and protein regulation pathways

Anticancer agents from various sources such as natural and synthetics, compounds have been reported to show various peptide cationic interactions with cancer cells through various modes of action (Y. B. Huang, He, Jiang, & Chen, 2012). These compounds effects the cytoplasmic embrane disruption via micellization or pore formation, and induction of apoptosis (Y.-B. Huang, Wang, Wang, Liu, & Chen, 2011). To understand the anticancer mechanism of action of compounds and extracts, there are assay in place to understanding the pathways. Protein regulation that's involved in the anticancer activity that have been reported Apoptosis assay using Annexin V FITC/ propidium iodide Cell death by apoptosis is an intrinsic process which results when membrane phospholipids usually found on inner section of cell plasma membrane, phosphatidylserine and phosphatidylethanolamine are exposed on outer membrane section. These are then targeted for phagocytosis. This assay uses extracts on cancerous cell lines, a fluorescent tag, Annexin V fluoroisothiocynate which binds to phosphatidylserine and phosphatidylethanolamine as an indication of cells undergoing apoptosis (Preethy *et al.,* 2012).

Several studies such as (Navarro et al., 2009; X. Zhu, Chen, & Subramanian, 2014) have showed the sensitive and selective of various methods/techniques which plays a great

role in the identifying various proteins regulations and predicting the probable pathways involved , in understanding the mode of action involved. Thus through the identifying the metabolite acquisition hit rate and the quality of spectra of the techniques such as liquid chromatography−mass spectrometry (LC−MS), the MS/MS-mass spectrum acquired through a information-dependent acquisition (IDA), SWATH (sequential window acquisition of all theoretical fragment-ion spectra), and finally the MS All or the MSE techniques.

Hence the study by (X. Zhu et al., 2014) demonstrated that the technique employed by the current study on the investigation of mechanism of action *Acanthosicyos naudinianus* root *Fockea angustifolia* whole plant and the tuber, *cf Salvadora persia* root and *Nymania capensis* root , the SWATH – ms exhibited the greatest outcome in comparison to the MS-MS. Due to the better quality of MS2 spectra with an identical MS2 acquisition hit rate. The apoptotic cell death which takes place in a series of cascade event is a form of cell death that is favorable for anticancer drug discovery. There are various ways to determine whether a certain plant extract or compound induces apoptosis in cancer cell lines. These procedures assist in understanding the mechanism of action, as which biochemical pathways are activated and how they go about causing programmed cell death. For instance the mitochondria dependent pathway for apoptosis is directed by Bcl-2-family proteins. The Bax/Bcl-2 regulates caspase-9 and caspase-3, which eventually leads to apoptosis (Accardi et al., 2015; Q. Li et al., 2011). An apoptotic response is cause by a cellular stress due to a transcriptional up-regulation or posttranslational reactions which activates pro-apoptotic regulators such as

BH3-only factors. The BH3 is the only group of proteins that interact with the multi-domain Bcl-2 proteins, Bax or Bak (Godard & Camus, 2012). The p53 is another protein that undergoes activation through the up-regulation of various proteins at levels as well as by regulatory modulation. Hence p53 operates as a transcription factor inducing the expression of genes mediating growth arrest, DNA repair and apoptosis (C. Behl and C. Ziegler, 2014).

Cell death can either be through apoptosis and necrosis. Necrotic cell death is due to an unpleasant cell damage which causes death of groups of cells within a tissue. Secondly apoptosis is a regulated form of cell death that may be induced or is pre programmed into the cell, which is characterized by specific DNA changes. Apoptosis It can be prompted if there is inaccuracy identified in DNA replication. The loss of this defensive mechanism would permit mutant cells to carry on to dividing and growing, thereby preserving mutations in subsequent cell divisions. Hence cytotoxic anticancer drugs operate by inducing mutations in cancer cells which are not sufficient to cause cell death, but which can be recognized by the cell, triggering apoptosis.

Numerous Cytoxan CTX drugs for cancer treatment are compounds in nature that are extracted from plants such as the phytochemical, antioxidants, although there are also synthetic compounds. The mechanism and site of act of this compounds are the determinants of whether the compounds are anti-metabolites, genotoxic agents and mitotic spindle inhibitors (Chorawala, Oza, & Shah, 2012). The anti-metabolites, anticancer agents, such as Folate antagonists, Methotrexate act on the cancer cell

through the Inhibition of the dihydrofolate reductase and thus affect nucleoside metabolism. Pyrimidine antagonists such as 5-Flourouracil, Cytarabine, Gemcitabine, Capecitabine, obstruct pyrimidine nucleotide formation through incorporation of itself into newly synthesized DNA within the cells (Ding & Zhou, 2012). Purine antagonists act through the inhibition of the synthetic steps during S-phase seen in figure 1 of replication (Payne & Miles, n.d.).

The alkylation of cancer cells happens by launching alkyl agents groups such as Cisplatin, Cyclophosphamide, Melphalan, Temazolomide, Carmustine, Ifosfamide, Streptozotocin into DNA to construct cross linking between two DNA strands by that restrain protein production (Chorawala et al., 2012). The second class of cytoxan drugs are the genotoxic agents this compounds bind to DNA and directly or indirectly affect the replication which in turn induces the apoptosis (Alawode, 2013; Swift & Golsteyn, 2014; R. Y. L. Tsai & Pederson, 2014). Meanwhile intercalating agents drugs such as the epirubicin, doxorubicin, dactinomycin attaches to DNA through intercalation between specific bases pair which than obstruct the DNA synthesis which is the main aim towards achieving cell death. Furthermore the enzyme inhibitors such as etoposide, topotecan, irinotecan. Etoposide: Inhibits topoisomerase II thus prevent resealing of DNA which leads to cell death. While the topotecan/Irinotecan plays an important role in Inhibiting the topoisomerase I which allows single strands break in DNA but this does not affect resealing (Chorawala et al., 2012).

The third groups of anticancer agents CTX drugs are the mitotic spindle inhibitors (Luqmani, 2005). These agents include the Vinca alkaloids, vincristine and vinblastine,

which arrest the cell division in metaphase by binding to tubulin that well discussed in detail in figure 1 (Ding & Zhou, 2012; Mukhtar, Adhami, & Mukhtar, 2015; A. Tsai et al., 2014). Taxanes derivatives of paclitaxel and the docetaxel agents, Stabilize polymerization of tubulins and inhibit the disassembly of microtubules within the cancer cells (Chorawala et al., 2012; Ding & Zhou, 2012). Imatinib are a protein tyrosine Kinase inhibitors which is aimed at the inhibition this enzyme, through the inhibition of proliferation of myeloid cell.

While gefitinib and erlotinib are Epidermal Growth factor Receptor (egfr) Inhibitors, which activates the EGFR induced dimerisation and intracellular activation of protein tyrosine kinase (Ding & Zhou, 2012; Saha, Adhikary, Bhattacharyya, Das, & Sa, 2012). There are new anticancer agents that used in the conventional medicine and these are agents such re the Proteosome Inhibitors that work by preventing degradation of intracellular protein leading to activation of signaling cascade, of the cell cycle arrest and apoptosis, some of the agents such as Bortezomib are in this group and others such as the aromatase Inhibitors for instance anastrozole letrozole, exemestane aromatase, responsible for conversion of testosterone to estradiol (Accardi et al., 2015; Chorawala et al., 2012).

As part of the control mechanisms involved during normal cell cycle progression, p21 or cyclin-dependent kinase inhibitor binds and inhibits the activity of cycli-CDK2 or CDK1 complexes, therefore serving to cause cell cycle arrest at G1. There are various studies that detect the activation and expression of different protein such as p21 in cancerous cell lines to understand the mechanism of action involved adopted from

Boukes & Van de Venter (2011). Furthermore, expression of other proteins such as caspase, NF-kB/p65, COX-2 and p53 are also investigated in various studies to determine the mechanism by which plant extracts cause apoptotic cellular death (Zhang *et al.,* 2011).

Furthermore various compounds being it plant compounds or synthetic may cause the activation of numerous genes in the cancerous cells which further leads to the up and down regulation of some of the protein. The regulation of these proteins induces cancer cell death through different cellular pathways, which are favourable for cancer therapeutic. Some compounds stimulate apoptosis in cancer cells through the hindering of the Bcl2. Hence this results in the up-regulation expression of pro-apoptotic Bax (Chorawala et al., 2012). Apart from that, these compounds at time also cause the liberation of Smac/DIABLO from mitochondria, which antagonize the inhibitors of apoptosis proteins released from mitochondria (Chaabane, Mohamed, Jaksik, Ripk, & Vhl, 2013). The Beclin-1 when introduces into colon cancer cells that lost beclin-1 or expressed it at low levels represses the development of the cancer cells, indicating that beclin-1 expression can be used as a therapeutic strategy. The steady nature of p53 accumulation in the nucleus and binding thereof to specific DNA sequences causes to trans activation of a number of pro- apoptotic genes, such as those encoding members of the Bcl2 family, the BH3 only proteins of Bax, Noxa and Puma (Chorawala et al., 2012) , which results in cancer cell death by apoptosis. The other is also that in apoptotic cells, p53 co- immunoprecipitates with Bcl2, Bcl-XL and Bak (Chaabane et al., 2013).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Research design

The study investigated the *in vitro* anti-cancer properties of *Acanthosicyos naudinianus* ,*Fockea angustifolia , cf Salvadora persia* and *Nymania capensis* found in Hardap and //Karas regions generated both quantitative and qualitative data. The qualitative design was for the screening of the presence or absence of phytochemicals using thin layer chromatography (TLC). The quantitative design involved quantification of selected cell viability assays, as well as the cytotoxicity assays. The SWATH-MS assay produced both qualitative and quantitative data. The qualitative design for SWATH-MS involved data from the presence and absence of proteins in the biological samples. While qualitative data was produced from the number of peptides that were identified in the treated cancer cell sample.

3.2 The plant population

Plants material that was used during this study was collected from the Hardap and the //Karas regions based on ethnobotanical knowledge that exists within the local community supported by ethno-pharmacological uses of the ethno-medicines. The strategy was focused on plants use to treat ailments similar or related to the symptoms of cancers. This was achieved through a snow ball, un-stratified sampling interview.

3. 3 Sampling

3.3.1 Selection and collection of plant material

The selection of plants was based on the use of these plants as anticancer treatment agents in traditional settings, based on ethno botanical knowledge. Different parts of the plants were collected and voucher specimens' were prepared using a plant press and sent to the National Herbarium at the National Botanical Research Institute for scientific identification

3.4 Research workflow

The following procedures in the flow chart shows the activities that were carried out during the study, however each of these will be discussed in detail in the paragraphs that will follow thereafter.

3.4.1 Flow chart for procedures

Collection of plant materials → Drying of plant material → Preparation of Aqueous and organic plant extracts → Phytochemical screening

Cytotoxicity assay ← Anticancer assay ← Cell culture and maintenance ← Antioxidant activity

Cytotoxicity assay → SWATH Mass Spectometry
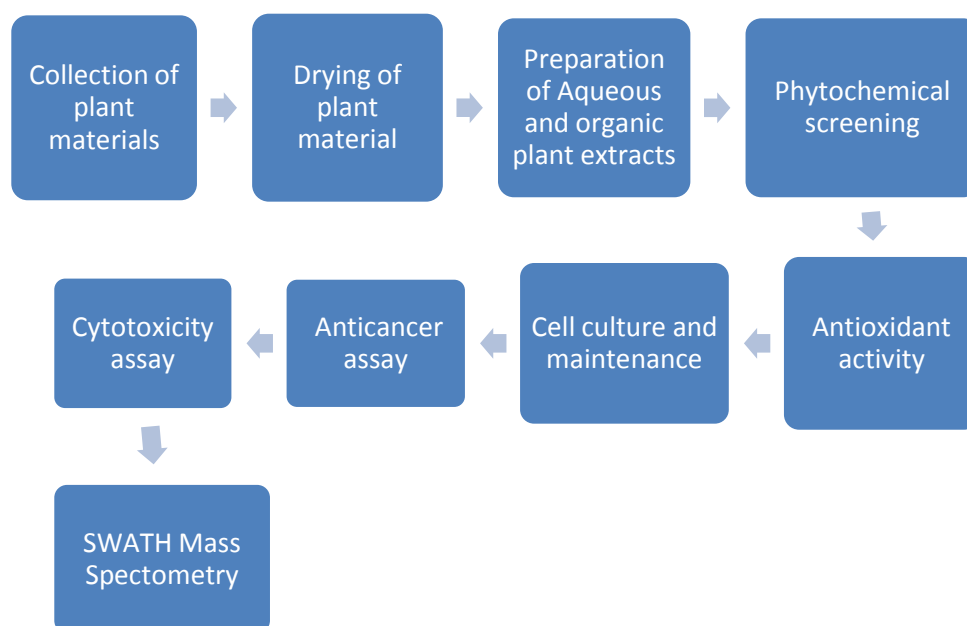
3.5 Collection of plant materials

Medicinal plants collected in *!Goxas* village Hardap, and *Itzawisi* village //Karas regions , in southern Namibia were selected on the basis of their local use. A research permit was applied for from the Ministry of Environment and Tourism for collecting plant samples. For the purpose of identification of the plants, voucher specimens were prepared using leaves, flowering parts and fruits from the collected plants where possible. The collected plant parts were then pressed in the plant press as seen in figure 8 containing newspapers, a flimsy and blot paper by flattening out the specimens within minutes of collection to preserve the integrity of the plant parts as they dry. The plant press was then tightened securely attaching the straps.

The upper and bottom frames

The Board found at the Top and bottom

Blotting paper

The frame straps
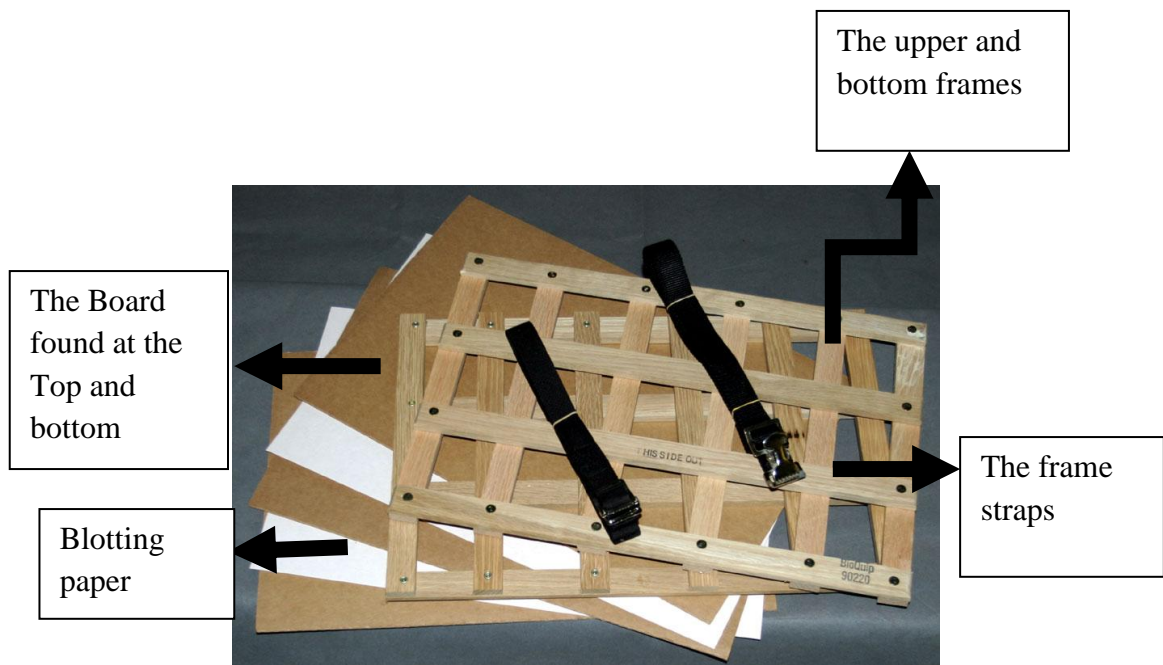
Figure 8: Plant press and its component

The plants were tagged using a unique collection code/ number and a form was filed in describing the specific habitat/ecosystem of the plant collected. The aspects of habitat/ecosystem that were outlined in the form were information on the records of the latitude and longitude of the site using a GPS unit for scientific identification of the plants at the herbarium, National Botanical Research Institute (NBRI). Plant parts based on the ethno-medicinal uses of *Acanthosicyos naudinianus, Fockea angustifolia , cf Salvadora persia* and *Nymania capensis* were collected in the //Karas and the Hardap regions for the investigative purpose of the study.

3.6 Preparation of the plant part samples

Once the collected plants reached the laboratory plant parts of *Acanthosicyos naudinianus* root *Fockea angustifolia* whole plant and the tuber, *cf Salvadora persia* root and *Nymania capensis* root were rinsed using running tap water to remove soil and debris then air dried at room temperature indoors for 4 weeks. The tuber and root were sliced into smaller pieces to aid in drying further for an additional week. The dried plant material were then ground individually using a blender to into powder. The blender was cleaned using hot water and 10% bleach, between grinding different plant parts to prevent contamination of samples. The powdered form of each plant sample was weighed and then stored at 4°C fridge prior to using preparation of plant extracts.

3.7 Preparation of plant extracts

3.7.1 Aqueous extract

Ten grams of plant powder from each of the following samples, from the *Acanthosicyos naudinianus* root, *Fockea angustifolia* tuber and whole plant material*; cf Salvadora persia*rootand *Nymania capensis* root, were weighed out and placed in 100 ml volumetric/conical flask separately one flask per plant part. A volume of 100 ml of distilled water was added to each sample and allowed to stand for 48 hours with regular mixing. After 48 hours, the extracts were each filtered using Whatman filter papers, into clean volumetric/ conical flasks using separating funnels to separate solid plant material from the aqueous extract. The filtrates were transferred into round bottom flasks and dried on a rotary evaporator. This was followed by lyophilization of the dried aqueous extracts using a freeze dryer for four to six hours. The plant extracts at the bottom of the round flask were then scrapped off and weighed before being stored at -20°C until required. The aqueous percentage yield was calculated by dividing the mass of dry plant extract with the original mass of plant material used for extraction purposes multiplied by a 100.

3.7.2 Organic extracts

Organic ethanolic plant extraction was done using a similar approach to that of the aqueous distilled water. Ten grams of powdered *Acanthosicyos naudinianus* root, *Fockea angustifolia* tuber and whole plant material*, cf Salvadora persia*rootand *Nymania capensis* root, were separately weighed out and placed in 100ml

volumetric/conical flask separately. A volume of 100 ml of absolute ethanol was added to each of the powdered plant samples and left to stand for 48h with occasional gentle mixing. After a period of two days, the five extracts were separately filtered using Whattman filter paper, into clean volumetric/ conical flask using separating funnels. The clean organic extracts were transferred into round bottom flasks and the solvent was evaporated using a rotary evaporator. This was followed by further drying of plant material, using a freeze dryer for four to six hours. The dried organic plant extracts in the round bottom flasks were then scrapped off weighed and stored at -20°C until they were required. The organic percentage yield was calculated by dividing the mass of dry plant extract with the original mass of plant material used for extraction purposes multiplied by a 100.

3.8 Phytochemical screening

The phytochemical profiling of the five plant parts namely the *Acanthosicyos naudinianus*, *cf Salvadora persia, Nymania capensis* roots and *Fockea angustifolia* tuber and whole plant was carried out for all the 10 extracts, five aqueous(distilled water) and five organic (ethanol). The presence and absence of alkaloids, flavonoids, steroids, saponins, anthraquinones, and coumarines was determined using thin layer chromatography (TLC). The assay was carried out as described by Pascaline, Charles, Lukhoba, & George, 2011with some modifications.

The TLC silica gel 60 F_{254} aluminum plates (Merck KGaA 1.05554.0001) were developed as follows. A line was drawn in pencil one centimeter from the bottom of the plate, the stock solutions of the various plant extracts were prepared at a concentration of 600μg/ml. Aqueous and ethanol extracts as well as a positive control for each compound class were dissolved in distilled water and ethanol respectively. This was then spotted on to the chromatography plate. Each of the plant extract spots were placed one centimeter apart from each other including a spot for a control for each phytochemical class using a thin capillary tube. The spots were air dried before application of the next spot to control the spot size on the chromatogram thus avoiding cross contamination of the different plant extracts on the TLC plate.

Consequently different solvent tanks were prepared to test for the presence and absence of various phytochemical classes as seen in Table 1 below. The solvent front was allowed to move up a 5/6 of chromatogram plate and it was indicated by drawing a line through it. All TLC plates were dried for 10 minutes before presence or absence of compounds was detected after spraying or exposure to UV light as described in Table 1 were followed for the phytochemical analysis of the crudes plant extract. The thin-layer chromatograms were analyze, by using the positive controls as seen in Table 1 as standards for comparison of the spot color and the rf values. The different rates at which the various phytochemical class of compounds moved across the chromatograms.

Table 1: The Thin Layer Chromatography procedure for identification of phytochemical compounds

Compounds	Mobile phase	Spraying reagent
Alkaloids standard: Quinine	Chloroform: methanol: 25% ammonia (8:2:0.5)	Dragendorff's reagent
Flavonoids standard: Quercetin	chloroform and methanol (19:1)	5g of aluminium chloride in 500g of ethanol.
Saponins standard: Triterpenoids	chloroform, glacial acetic acid, methanol and water (64:34:12:8)	Iodine vapors.
Steroids standard: βSitosterol	chloroform, glacial acetic acid, methanol, and water (64:34:12:8)	p-anisaldehyde was sprayed onto the plates in 50ml glacial acetic acid and 97% sulphuric acid and heated at 105^0C
Coumarines	Chloroform	10% potassium hydroxide in methanol

3.9 Maintenance of cell cultures

The growth inhibitory effects of the different plant extracts were tested using a 3-cell line panel consisting of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells by the Sulforhodamine B (SRB) assay. according to Voigt, 2005) with some modifications. Cell lines were routinely maintained as a monolayer cell culture at 37ºC, 5% CO_2, 95% air and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50μg/ml gentamicin (complete media).The WI-38 cell line - normal Human Fetal Lung Fibroblast from ECACC was routinely maintained as a monolayer cell culture for the cytotoxicity assay, at 37ºC, 5% CO_2, 95% air and 100% relative humidity in EMEM containing 10% fetal bovine serum, 2 mM L-glutamine and 50μg/ml gentamicin.

3.10 Anticancer assay

Cell lines from confluent flasks of TK10, UACC62 and MCF7 were detached by adding 3 ml of 1x trypsin to each T25 flask for 5 minutes. Fresh complete media was added to inactivate the trypsin. The detached cells were then transferred to a 15 ml centrifuge tube and centrifuged at 200 rpm for 2 minutes. The supernatant was removed and the pellet was re-suspended in fresh media and the number of cells was determined using a haemocytometer. The Haemocytometer was used to calculate the cell density, through dye exclusion staining differentiating viable cells from dead cell. The final cell density was adjusted to the density of 7-10 000 cells/well platesfor each cell line.

The cell lines were seeded into 96 well microtiter platesand incubated for 24h. Stock solution for the plant extracts were prepared by dissolving one mg of the plants extract of *Acanthosicyos naudinianus* root *Fockea angustifolia* whole plant and the tuber, *cf Salvadora persia* root and *Nymania capensis* root in 1ml DMSO with 1000 times dilution, using complete RPMI media ,followed by serial dilution using complete media to produce five different concentrations of (100, 50, 25, 12.5, 6.25) µg/ml. The three cell lines were treated by incubating the cells in 100 µl plants extracts diluted in complete media, while complete media with the diluted DMSO was used as a negative control while the etoposide was used as a positive control. The plates were incubated at 37ºC, 5% CO_2, 95% air and 100% relative humidityfor 48 hours after addition of the plant extract.

Viable cells were subsequently fixed to the bottom of each well with cold 50% trichloroacetic acid, and placed in the refrigerator for 2 hours. Following this, the trichloroacetic acid was washed off with 1% acetic acid, and the plates were blotted on clean dry tissue paper and the plates were further dried at room temperature. The sulfurhodamine dye was prepared by dissolving 8 grams of the dye into two litres of 1% acetic acid which was prepared by adding 1980 ml of distilled water into 20 ml of acetic acid. Then the 100µl of 0.4% Sulfurhodamine was pipetted into each of the wells in the 96 wells plates and the plates were incubated at 37ºC, 5% CO_2, 95% air and 100% relative humidity for 30 minutes. Subsequently the plates were washed off to remove unbound dye with 1% acetic acid, then blotted using clean dry tissue paper and left to dry at room temperature for 24 hours. Lastly 10 mM Tris base was use to extract protein

bound sulfurodamine dye, by shaking the 96 well plates to dissolve the contents. Optical density was then determined at 540 nm using a multi-well spectrophotometer and the data analysis was performed using Graph Pad Prism software. The 50% cell growth inhibition (IC_{50}) was determined by non-linear regression.

3.11 Cytotoxicity assay

The cytotoxic effects of the compounds were tested by Sulforhodamine B (SRB) assay using a similar procedure to that of the anticancer assay using a fibroblast cell line,(WI-38). This procedure was also adapted from (Voigt, 2005).The cell lines from confluent flasks WI-38 were detached using 1X trypsin with 3ml of trypsin being added to each T25 flask for 5 minutes. Fresh complete media was added to inactivate the trypsin. The cells were then transferred to a flask and centrifuged at 200 rpm for 2 minutes. The supernatant was removed and the pellet was re-suspended in fresh media and the number of cells was determined. The Hemocytometer was use to calculate cell density. This was then used to inoculate a volume of 100µl of cells in 96-well microtiter plates at plating densities of 7-10 000 cells/well the plates were incubated for 24 hours. In addition the stock solution for the plant extracts were prepared by dissolving 1mg of the plants extract in 1ml DMSO with 1000X times dilution, using complete RPMI media, followed by serial dilution using same complete media to produce five (5) different concentrations of (100, 50, 25, 12.5, 6.25) µg/ml. Subsequent to 24 hours incubation, the cells were treated with the plant extracts (experiment drug) of 100µl which was pipette to into the correct wells. Untreated cells were used as negative controls, wells containing

medium and no cells were used as the blanks.   The standard anticancer drug that was used for the experimentas a positive control was etoposide. The 96 well plates were incubated at 37ºC, 5% CO_2, 95% air and 100% relative humidity for 48 h after addition of the plant extract.

Trichloroacetic acid was prepared by dissolving 250 g in 500 ml of distilled water, cold 50% trichloroacetic acid was used to fix viable cells to the bottom of each well in the plates and then they were placed in the refrigerator for 2 hours. After the two hours, the trichloroacetic acid was washed off with 1% acetic acid, after which the plates were blotted on clean dry tissue paper and the plates were further dried at room temperature. The 0.4% Sulfurhodamine was pipette into each of the wells in the 96 wells plates for the anticancer screen, data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC50) was determined by non-linear regression.

3.12 Antioxidant assay

The antioxidant activity of the plant extracts of *Acanthosicyos naudinianus ,Fockea angustifolia , cf Salvadora persia* and *Nymania capensis* were determined by the DPPH assay (2,2-diphenyl-2- picrylhydrazyl hydrate) according to the procedure used by (Rishi & Sneha, 2012) with some modifications. The radical scavenging ability of plant extracts the plants at 5 five different concentrations of (6.25, 12.5, 25. 50, 100) μg/ ml. A concentration of ($6x10^{-5}M$) of DPPH was prepared by weighing out 88.7 mg of DPPH and dissolved with (1000 x10) ml of methanol. Ascorbic acid (vitamin C) was used as a

standard in this experiment. Ninety six (96) well plates were prepared with plants extracts of *Acanthosicyos naudinianus ,Fockea angustifolia , cf Salvadora persia* and *Nymania capensis* at 5 five concentration of (6.25, 12.5, 25. 50, 100) μg/ ml in triplicates for each of the plant extract samples, including the ascorbic acid. The ($6x10^{-5}$M) DPPH (2,2-diphenyl-2- picrylhydrazyl hydrate) was added to the plant extract of *Acanthosicyos naudinianus ,Fockea angustifolia , cf Salvadora persia* and *Nymania capensis* and the standard (ascorbic acid) in the 96 well plate, thesewere kept in the dark for 30 minutes at room temperature. Subsequently, the optical densityfor the cells was measured at 540 nm on a multi-well spectrophotometer. The data on absorbance, optical density was used to obtain the percentage inhibition of the plant extracts of *Acanthosicyos naudinianus ,Fockea angustifolia , cf Salvadora persia* and *Nymania capensis* including that of ascorbic acid on the DPPH.

3.13 Mechanism of action analysis using Sequential Window Acquisition of all Theoretical Spectra (SWATH) Mass Spectra

3.13.1 Cell culture treatment

Nine T75 flasks Human cell line of renal cancer (TK10) were incubated for 24 hours as described in Section 3, cells were treated with the *Acanthosicyos naudinianus* extract at concentration of 5 μg/ml and 10 μg/ml in triplicate with three flasks containing untreated cells served as a control. The flasks were incubated for 48 hours after treatment. Trypsin was added to each T75 flask to detach the cells which were then centrifuged at200 rpm for 2 minutes. The resulting pellets were washed in Phosphate

Buffer Saline three times and re-suspended in lysis buffer (6 M Urea, 10 mM NaPPi, 20 mM Tris pH 8) before lysis by freeze-thawing followed by sonication. The supernatants, containing the extracted proteins, were collected and protein concentration was determined using Bradford assay as seen in appendix two, 10 mg of each extract was analyzed using SDS-PAGE, 500 mg aliquots were stored in a -80° C freezer

3.13.2 Clean-up and Digestion

Three aliquots each for the control, 5 μg/ml and 1 μg/ml *Acanthosicyos naudinianus* treated cells were thawed once. The concentration of the protein in the cell sample was checked using the Bradford assay. The cell samples were than cleaned up using Acetone precipitation and pellets re-suspended in 80 ml, 4M Urea, 20 mM Tris pH 8. The protein concentration was checked again using the Bradford assay and 10 mg of each extract was analyzed using SDS-PAGE. Protein of 300 mg per sample was reduced ad DTT and alkylated using IAA. In-solution digestion was performed using Lys C for3 hours at 30 °C, followed by trypsinization for 21 hours at 37 °C on all the samples. SDS-PAGE was carried out on 10 mg of each extract. Each sample was split in 3 x 100 mg aliquots which were vacuum dried to completion and stored at -80 °C for Liquid Chromatography-Tandem mass spectrometry (LC-MS/MS) analysis. Three aliquots each for the control, 5 μg/ml and 10 μg/ml *Acanthosicyos naudinianus* treated cell proteins were analyzed. The samples were re-suspended in 20 ml, 2% ACN / 0.2% FA and spiked with 0.5 ml HRM RT peptide standards (Biognosys), 10 ml of each sample was used for LC-SWATH analysis (Protein Quantitation runs) on an AB Sciex 6600 Triple TOF MS coupled to a Dionex 3500 RLSC system.

3.14 Data analysis

Table 2: Analysis of the experimental data

Experimental Assay	Data analysis
Anticancer, and Cytotoxicity	GraphPad Prism software, to calculate 50% of cell growth inhibition (IC_{50}) by on-linear regression using a sigmoidarc, with Z' factor > 0.5 to determine whether response in the study is large enough to warrant further attention.
Phytochemical screen	Qualitative data, analyzing the presence and absence of compounds on the chromatogram with reference to compound rf values.
Antioxidant assay	Quantitative data was generated from the antioxidant DPPH scavenging activity by percentage inhibition.
SWATH – MS	Both qualitative and quantitative data. The analysis of protein was on an AB Sciex 6600 Triple TOF MS coupled to a Dionex 3500 RLSC system.

3.15 Research Ethics

The research ethics that were observed during this study of the *in vitro* anti-cancer properties of *Acanthosicyos naudinianus, Fockea angustifolia, cf Salvadora persia* and *Nymania capensis* in the Hardap and the //Karas regions, are as follows: Plant collection permits were obtained from the Ministry of Environment and tourism for collecting plant samples in a non-destructive manner from the Hardap and //Karas regions.

CHAPTER FOUR: RESULTS

The table below show the plants that were collected namely, the *Acanthosicyos naudinianus*, *Fockea angustifolia*, *cf Salvadora persia*and *Nymania capensis* plants from the southern part of Namibia in the Hardap and //Karas regions near the *!Goxas* and *Itzawisis* villages respectively the location coordinates of each of the plants is given in the table.

Table 3: Plants collected for the study

Voucher number	Plant Local Name	Scientific Names	Plant part	Region (village)	Location coordinates	
HS001	*Ka-!nomab*	*Acanthosicyos naudinianus*	Root	Hardap, *!Goxas*	26.239060	18.163391
HS002	*≠Habas*	*Fockea angustifolia*	Tuber	Hardap, *!Goxas*	26.234415	18.161969
HS002	*≠Habas*	*Fockea angustifolia*	Whole plant	Hardap *!Goxas*	26.234415	18.161969
HS004	*≠Nu-≠khoeb*	*Cf Salvadora persia*	Root	//Karas, *Itzawisis*	26.222388	18.159428
HS006	*!Kheape*	*Nymania capensis*	Root	//Karas *Itzawisis*	26.234415	18.161969

The plants that were collected *Acanthosicyos naudinianus* ,*Fockea angustifolia* , *cf Salvadora Persia* and *Nymania capensis* are shown in figure below are plants found in Hardap and the //Karas regions.

(A)	(B)	(C)
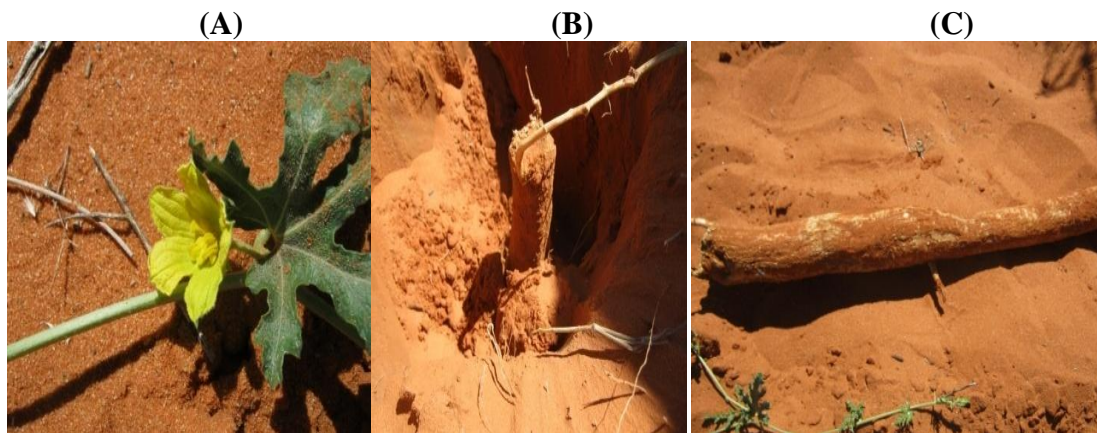
Figure 9: *Acanthosicyos naudinianus* plant (A) flower and leaves, and the ((B) (C)) root

(A)	(B)
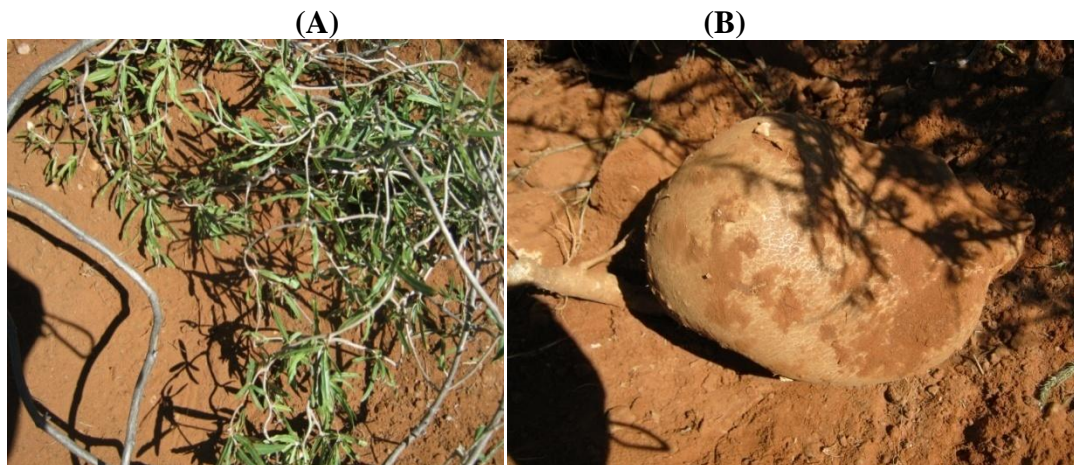
Figure 10:*Fockea angustifolia* (A) stems, leafs and (B) the tuber
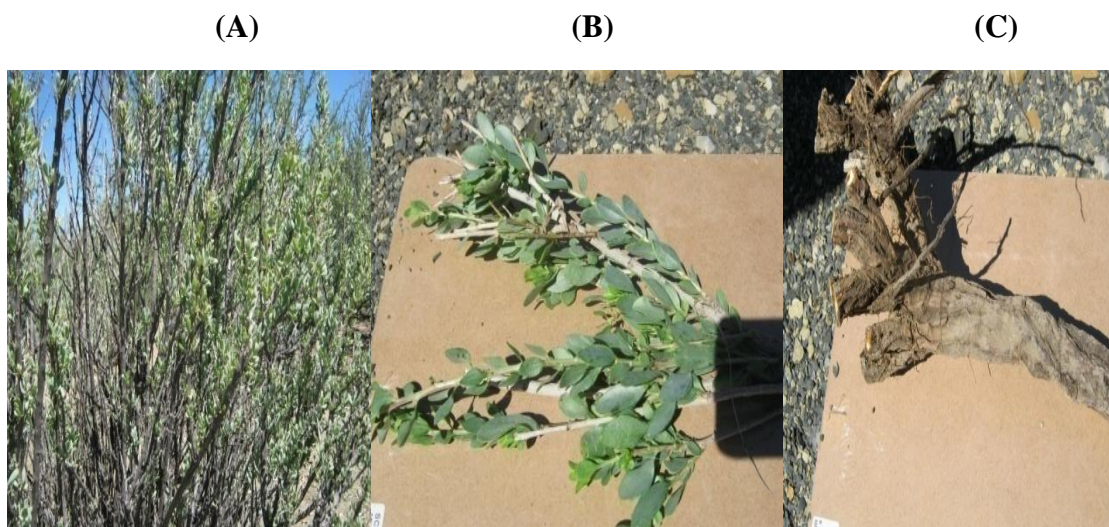
Figure 11: *cf Salvadora persia* plant, (A) (B) leafs attached to stem and (C) the root

Figure 12:*Nymania capensis* plant

4.1 Percentage Yield with reference to the effect of the Aqueous and Ethanol extractions system.

Plant extracts prepared using aqueous and organic compounds showed the yield as can be seen in figure 13, and appendix one. The aqueous *Fockea angustifolia* plant extract

showed the highest yield of about 13.6 % followed by the *Acanthosicyos naudinianus* plant extract of about 9.4%. While *Nymania capensis* plant extract yielded the least about 4.8 % for the aqueous extracts. The ethanol extract of *Fockea angustifolia* plant yielded the highest percentage of about 18.6% as compared to all the other ethanol plant extracts followed by *Nymania capensis* plant extracts with about 13.8% yield while all the other four plant extracts had percentage yield below 10% as can seen in figure 13 below
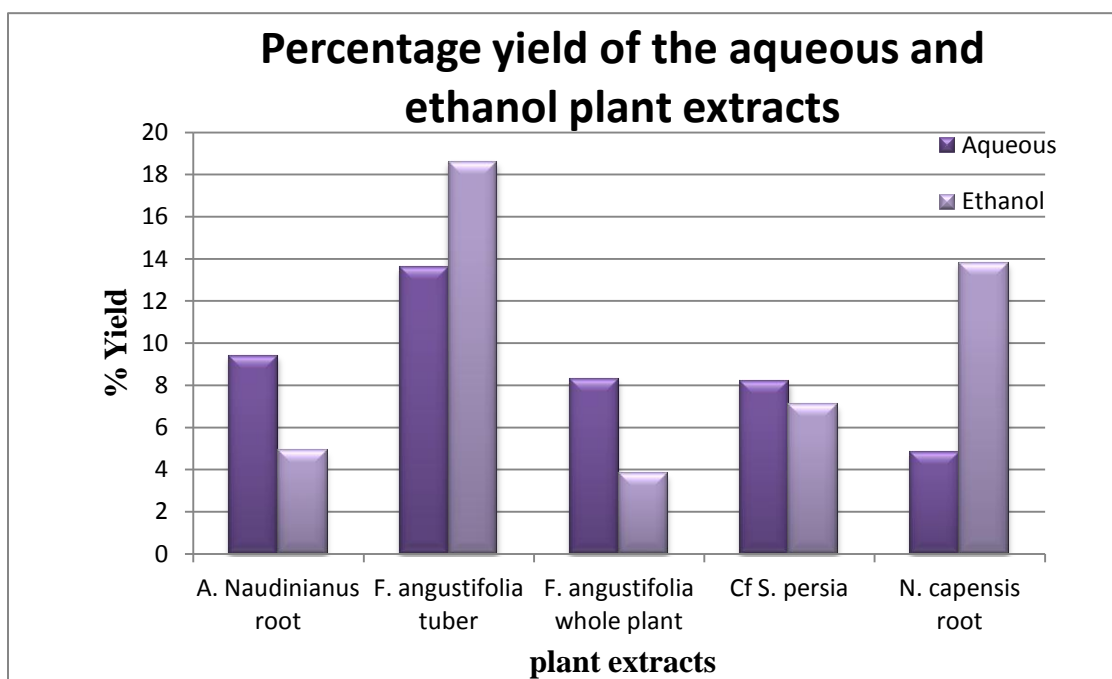
Figure 13: The Percentage yield of both aqueous and ethanol plant extracts

4.2 Phytochemical analysis

The phytochemical screen of five different classes of compounds revealed the presence and or absence of these compound in the ten plant extracts (both aqueous and ethanol). The tables 4 and 5 below show the results for the specific classes of compounds that were screened for in the extracts. Furthermore the tables highlights that there is a trend

between the aqueous and the organic plant extracts where the ethanol extracts have more of the screened compounds present, as compared to the aqueous plant extract.

Table 4 illustrates the presence and absence of phytochemical class compounds in the crude plant extracts. The plant extracts which was found to contain all phytochemicals classes of compounds screened for, namely alkaloid, flavanoid, coumarine, anthraquinone and saponins were the *Fockea angustifolia* tuber, and the *Nymania capensis* root plant extracts Alkaloid, flavonoids, coumarines and anthraquinones were detected in the *cf Salvadora persia* root. The whole plant extract of *Fockea angustifolia* only detected the presence of coumarines, anthraquinones, and saponins. And lastly the *Nymania capensis* root crude plant extract detected the presence of saponins.

Table 4: Profiling of phytochemical class in aqueous plant extract

Plant Aqueous extracts	Alkaloids	Flavonoids	Coumarines	Anthraquinones	Saponins
Acanthosicyos naudinianus root	-	-	-	-	+
Fockea angustifolia tuber	+	+	+	+	++
Fockea angustifolia *WP	-	-	+	++	++
cf Salvadora persia root	+	+	+	+	-
Nymania capensis root	++	+	+	++	++

Key: ++++ Very high presence, +++ high presence, ++ moderate presence, + present, - absent

*WP: Whole Plants

The ethanol crude plant extracts of four of the screen five plants extracts detected the presence of the all five phytochemical class of compound see in table 5. The *Fockea angustifolia* tuber extract exhibited the presence of alkaloid, flavonoids, coumarines, anthraquinones and saponins. Whereas the ethanol whole plant extract of the same plant also showed alkaloids, flavonoids, coumarines, anthraquinones and saponins to be present. Hence alkaloids, flavonoids, coumarines, anthraquinones and saponins were also detected in the ethanol plant extracts of the *cf Salvadora persia* root. The *Nymania capensis* root extract also exhibited the presence of alkaloids, flavonoids, coumarines, anthraquinones and saponins. Lastly the ethanol extract of *Acanthosicyos naudinianus* showed the presence of only flavonoids, coumarines and saponins on the thin layer chromatography.

Table 5: Profiling of phytochemical class in the ethanol plant extract

Plant organic extracts	Alkaloids	Flavonoids	Coumarines	Anthraquinones	Saponins
Acanthosicyos naudinianus root	-	++	+++	-	+
Fockea angustifolia tuber	++	+++	++++	++++	+
Fockea angustifolia *WP	+	+++	++	++++++	++
cf Salvadora persia root	+	+++	+++++	++++++	++
*Nymania capensis*root	+	+++	+++	++++	+

Key: ++++ Very high presence, +++ high presence, ++ moderate presence, + present, - absent *WP: Whole Plants

66

4.3 Anticancer activity screen

The anticancer screen which used the Sulforhodamine B (SRB) assay to test the growth inhibitory effects of the different aqueous and ethanol plant extractsof *Acanthosicyos naudinianus* ,*Fockea angustifolia* , *cf Salvadora persia*and *Nymania capensis* and the standard drug of Etoposide were tested in the 3-cell line panel consisting of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells. The SRB assay measured the drug-induced cytotoxicity and cell proliferation. Hence the graph below illustrates the inhibitory effect of the standard drug of etoposide which was achieved through the use of a simple statistical analysis of IC_{50} values, which is the concentration at which 50% or half of the cell are inhibited.
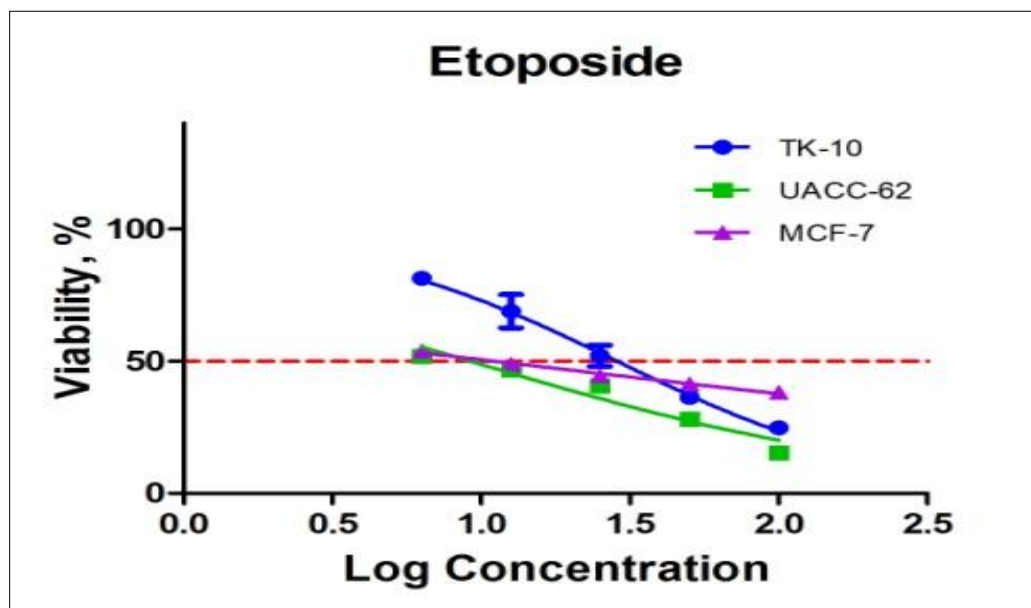
Figure 14: The percentage cell viability of Tk-10, UACC-62, and MCF-7 cancer cell line

The Etoposide standard drug exhibited inhibitory activity against all three cell lines of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells. The IC_{50} values were calculated as follows, TK10 was 28.64 µg/ml, UACC-62 9.19 µg/ml, and lastly MCF-7 11.07 µg/ml as shown in figure 14.

To distinguish and compare the inhibitory activity of the *Acanthosicyos naudinianus* both the aqueous and the ethanol extracts of the experimental plant extract drug the graph (figure 15 A) below was develop based on the IC_{50} values of the *Acanthosicyos naudinianus* experimental plant extracts drug against the three cancer cell lines of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells. The aqueous plant extract of *Acanthosicyos naudinianus* inhibited growth of all three cells lines. The *Acanthosicyos naudinianus* aqueous plant extract showed an IC_{50} of <6.25 µg/ml against the TK-10 cancer cell lines, 16 µg/ml against the UACC-62 cancer cell lines, and 48.85 µg/ml against MCF-7 cancer cell lines.

Figure 15 (B) illustrates the inhibitory activity of the *Acanthosicyos naudinianus* ethanol plant extract, which showed that the TK-10 cancer cell lines were inhibited at IC_{50} value of 56.52 µg/ml, UACC-62 cancer cell line at of 76.77µg/ml, whereas the MCF-7 cell lines was inhibited at an IC_{50} value of 95.71µg/ml.

(A)   **(B)**

Figure 15: The percentage cell viability of Tk-10, UACC-62, and MCF-7 cancer cell line after treatment with (A) aqueous *Acanthosicyos naudinianus* plant extracts (B) ethanol *Acanthosicyos naudinianus* plant extracts

The inhibitory activity of the aqueous *cf Salvadora persia* root seen in Figure 16 (A) shows IC_{50} values that were all greater than 100µg/ml for all three cancer cell lines of Tk-10, UACC-62 and MCF-7. Whereas the ethanol extracts of the same plant of *cf Salvadora persia* root in figure (B) exhibited IC_{50} values of 31.28 µg/ml against the TK-10 cancer cell lines, 33.43 µg/ml against the UACC-62 cancer cell lines and 41.67 µg/ml against the MCF-7 cell line.

(A)   **(B)**

Figure 16: The percentage cell viability of Tk-10, UACC-62, and MCF-7 cancer cell line after treatment with (A) aqueous root of *cf Salvadora persia* plant extracts and (B) ethanol root of *cf Salvadora persia* plant extracts

The aqueous *Nymania capensis* root plant extract showed inhibition activity at an IC$_{50}$ of 100 µg/ml, for the TK-10 cancer cell lines , and f the UACC-62 cancer cell lines The MFC-7 was inhibited at an IC$_{50}$ of 92.93 µg/ml as demonstrated in figure 17 (A).Figure 17 (B) shows the ethanol HS006 *Nymania capensis* root plant extract had an IC$_{50}$ of 76.32 µg/ml against the TK-10 cancer cell line, 91.78 µg/ml for theUACC-62 cancer cell line and it was greater than 100 µg/ml for the MCF-7 cell lines.

(A)   **(B)**
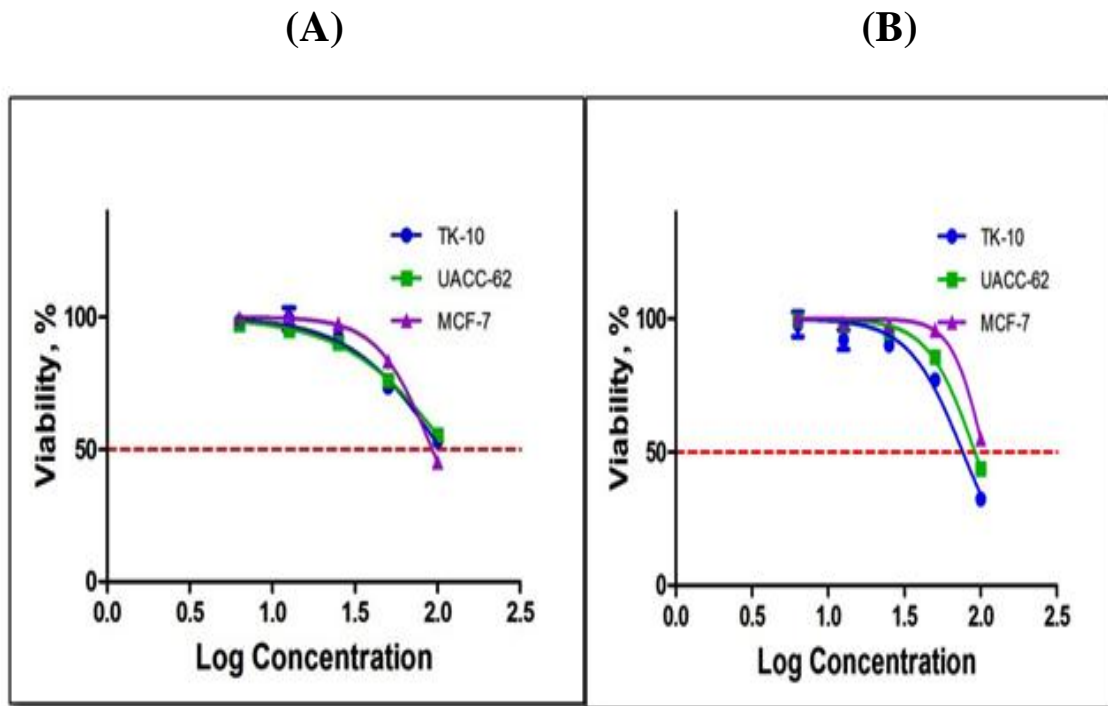
Figure 17 : The percentage cell viability of Tk-10, UACC-62, and MCF-7 cancer cell line after treatment with (A) aqueous root of *Nymania capensis* plant extracts and (B) ethanol root of Nymania *capensis* plant extracts

The inhibitory activity based on the IC_{50} values of whole plant extracts of *Fockea angustifolia* are shown in figure 18 (A) and (B), for the aqueous Whole plant extracts was greater than 100µg/ml against all three cancer cell lines. The ethanol extract had IC_{50} values of 100µg/ml for the TK-10 cancer cell lines and the UACC-62 cancer cell lines, whilst it was83.81µg/ml for MCF-7 cancer cell lines.

(A) **(B)**
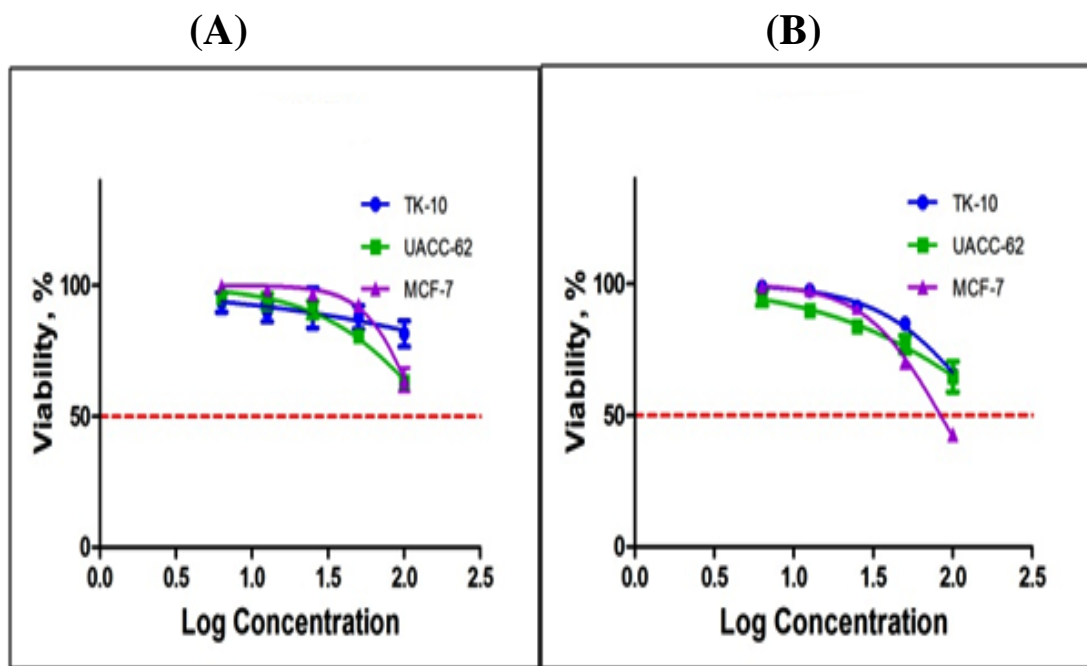
Figure 18: The percentage cell viability of Tk-10, UACC-62, and MCF-7 cancer cell line after treatment with (A) aqueous HS002 Whole plant *Fockea angustifolia* plant extracts and (B) ethanol HS002 Whole plant *Fockea angustifolia* plant extracts

Similarly to the activity of the aqueous whole plant extracts of *Fockea angustifolia* shown in Figure18(A). The tuber extract of *Fockea angustifolia* also showedIC$_{50}$valuesthat were all greater than100μg/ml for all three cell lines of TK-10, UACC-62 and MCF-7.The ethanol plant extracts (figure 19 B) also showed IC$_{50}$values that were greater than 100μg/ml.

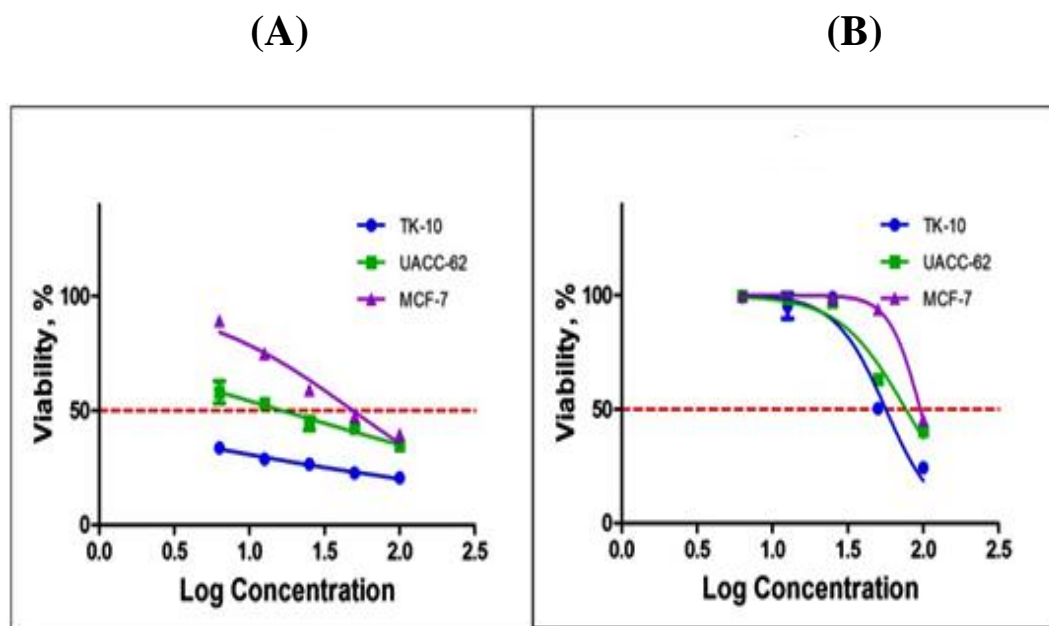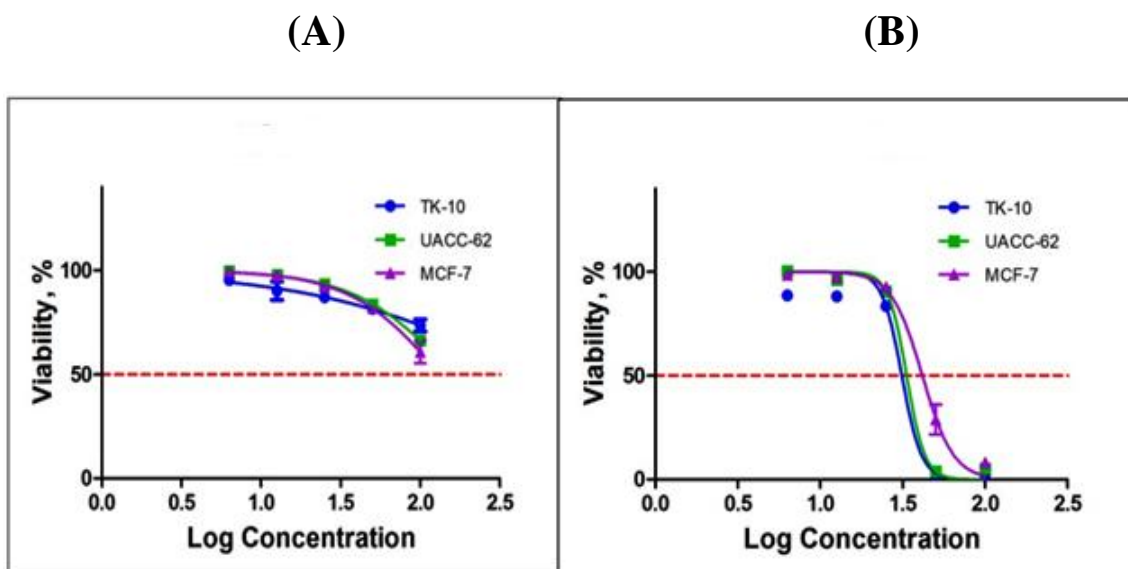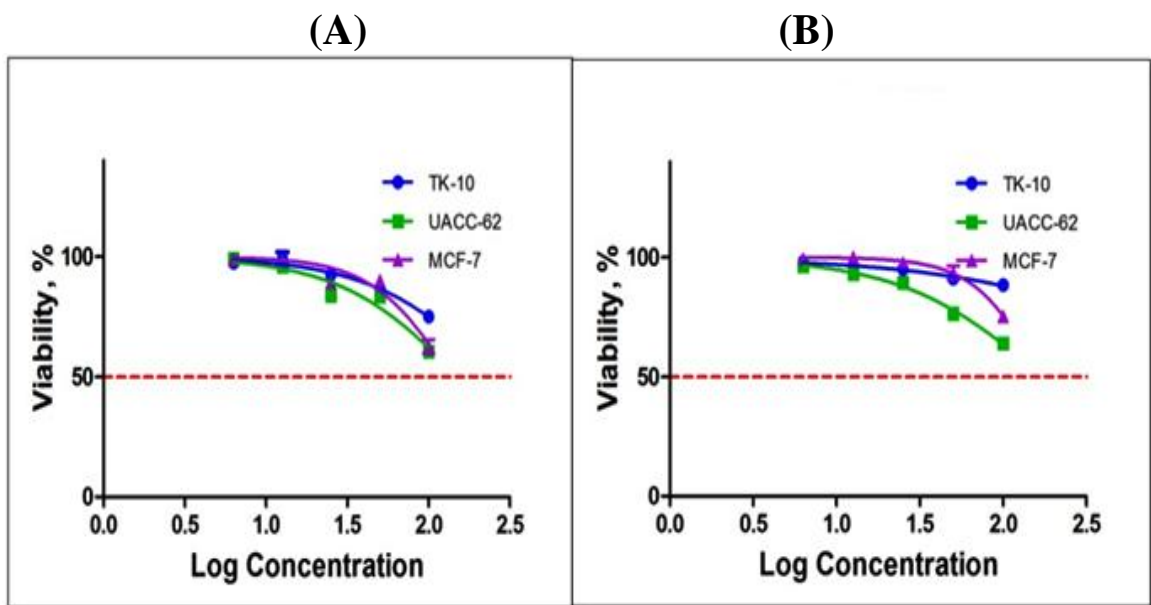Figure 19: The percentage cell viability of Tk-10, UACC-62, and MCF-7 cancer cell line after treatment with (A) aqueous tuber *Fockea angustifolia* plant extracts (B) ethanol tuber *Fockea angustifolia* plant extracts

The IC_{50} values of the various plant extracts ranged from between 6.25µg/ml and 100µg/ml (Table 6). The table also compares the IC_{50} values of various plant extracts, against the standard drug of Etoposide, with aqueous plant extract of *Acanthosicyos naudinianus* exhibiting most potent anticancer activity against the TK-10 renal cancer cell lines.

Table 6: IC_{50} Anticancer activity of Aqueous and Ethanol plant extracts.

Crude plant extract	IC_{50} Value ± SD (µg/mL)		
	TK-10, µg/ml	UACC-62,µg/ml	MCF-7,µg/ml
Aqueous plant extracts			
Acanthosicyos naudinianus root	<6.25±2.21	16.41± 1.84	48.85± 0.32
Fockea angustifolia Whole plant	>100± 4.94	>100± 2.36	>100± 4.46
Fockea angustifolia tuber	>100± 2.14	>100± 0.81	>100± 2.86
cf Salvadora persia root	>100± 2.92	>100± 0.38	>100± 5.41
Nymania capensis root	>100± 1.05	>100± 2.37	92.93± 0.34
Ethanol plant extracts			
Acanthosicyos naudinianus root	56.52± 0.36	76.77± 1.58	95.71± 0.81
Fockea angustifolia Whole plant	>100±>1.33	>100± 5.84	83.81± 1.61
Fockea angustifolia tuber	>100±>0.90	>100± 0.36	>100± 1.82
cf Salvadora persia root	31.28±>0.96	33.43±0.58	41.67± 0.09
Nymania capensis root	76.32± 1.70	91.78± 1.30	>100±0.30
Etoposide	**28.64±4.02**	**9.19±0.82**	**11.07±0.94**

4.4 Cytotoxicity Assay

The cytotoxicity effects of the plant extracts of *Acanthosicyos naudinianus,Fockea angustifolia , cf Salvadora persia*and *Nymania capensis* and the standard drug of Etoposide were tested with Sulforhodamine B (SRB) assay. Similar to the anticancer screen assay, however this screen employed the use of primary cell line of WI-38 cell line - normal Human Fetal Lung Fibroblast.Hence the SRB assay considered the drug-induced cytotoxicity. Therefore the graph representation demonstrates the viability of theprimary cell line of WI-38 cell line - normal Human Fetal Lung Fibroblast after the addition of experimental drugs the plant extracts of *Acanthosicyos naudinianus ,Fockea angustifolia , cf Salvadora persia*and *Nymania capensis* and the standard drug of Etoposide. The statistical analysis of the cytotoxicity screen is based on the IC_{50} values. Figure 21shows the cytotoxicity of Etoposide the positive control of this study had an IC_{50} of 5.1µg/ml.
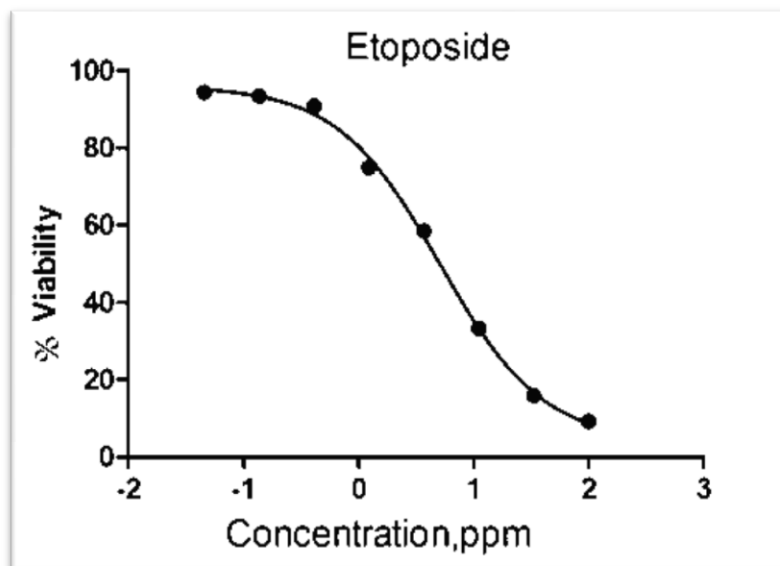
Figure 20: The viability of WI-38 cell line after treatment with Etoposide

The cytotoxcity effects of the *Acanthosicyos naudinianus* ,*Fockea angustifolia* , *cf Salvadora persia* and *Nymania capensis* extracts, and the standard drug of Etoposide, are summarized in table 7 based on the IC_{50} values which ranged from between <6.25 and 100> (µg/ml). The table also compares the IC_{50} values of various plant extracts, against the standard drug of Etoposide. The aqueous *Acanthosicyos naudinianus* plant extractwas estimated to be moderately hazardous, with an IC_{50}value of (12 µg/ml), while samples the ethanol *Acanthosicyos naudinianus* and *cf Salvadora persia* plant extracts were weakly Hazardous. The rest of the extracts were classified under as Low Hazard effect details of dose curve graph for each of the plant extracts can be seen in appendix four.

Table 7 : Cytotoxicity of aqueous and ethanol plant extracts against the human fibroblast cell lines

Crude plant extract	$IC_{50} \pm SD$ (µg/mL)of WI-38, µg/ml	
	Aqueous plant extract	**Ethanol plant extract**
Acanthosicyos naudinianus root	12± 0.28	83.34± 0.93
Fockea angustifolia whole Plant	>100± 2.09	>100± 4.88
Fockea angustifolia tuber	>100± 1.07	>100± 1.52
cf Salvadora persia root	>100± 0.37	37.88± 2.49
Nymania capensis root	>100± 0.42	>100± 1.90
Etoposide	5.1± 3.04	

4.5 Antioxidant activity

4.5.1Antioxidant activity of the aqueous plant extracts

In the current study the radical scavenging activity of the *Acanthosicyos naudinianus* *,Fockea angustifolia , cf Salvadora persia*and *Nymania capensis* aqueous plant extracts were determined and compared to the of the standard ascorbic acid, a known antioxidant agent. The overall antioxidant activity of the aqueous plant extract of *Acanthosicyos naudinianu,Fockea angustifolia , cf Salvadora persia*and *Nymania capensis* is demonstrated in Figure 21. The graph shows the percentage inhibition of the plant extracts at five different concentrations of(6.25, 12.5, 25. 50, 100) µg mL⁻¹, at a concentration of (6x10^{-5}M) DPPH(2,2-diphenyl-2- picrylhydrazyl hydrate).

The free radical scavenging activities of the aqueous plant extracts alongside the standard, exhibits that the Ascorbic acid showed activity of (30.34%) at a concentration of 50µg/ml, and 81.61% at 100µg/ml. Although this is the highest percentage inhibition, it is also the only inhibition above the 50%. Free radical scavenging activity of *Acanthosicyo naudinianus* shows 36.11% inhibition lower than 50% at a concentration of 50µg/ml, *Fockea angustifolia* extracts showed (21.71%) scavenging activity at the concentration of 50µg/ml was lower than the *Acanthosicyos naudinian.* The *cf Salvadora persia* and *Nymania capensis* plant extracts both also exhibited the lowest percentage inhibition of 15.4% and 15.81% respectively both at the concentration 50µg/ml.
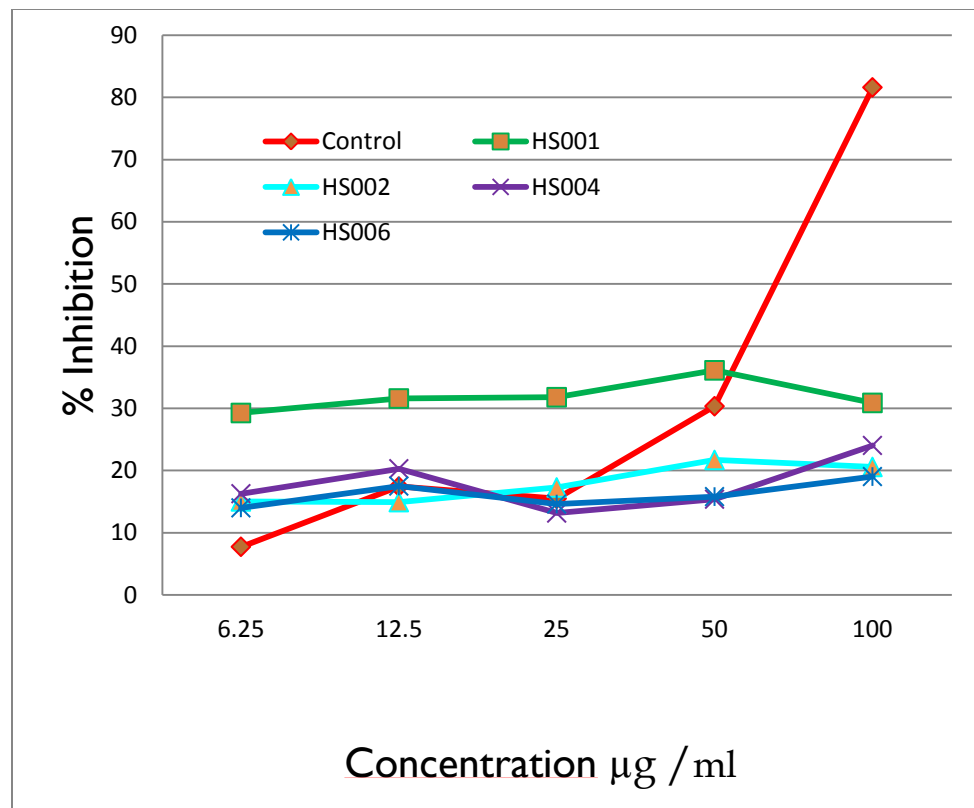
Figure 21: Percentage inhibition of the aqueous plant extracts on DPPH

4.5.2 Antioxidant activity of the ethanol plant extracts

The radical scavenging activity of the ethanol plant extracts of the *Acanthosicyos naudinianus, Fockea angustifolia, cf Salvadora persia* and *Nymania capensis* are shown in figure 22, at. Five different concentrations (6.25, 12.5, 25. 50, 100μg /ml), at a concentration of (6x10^{-5}M) DPPH (2,2-diphenyl-2- picrylhydrazyl hydrate)

The free radical scavenging activities of Ascorbic acid showed a high activity (32.03%) from a concentration of 50 μg/ml. Free radical scavenging activity of *Acanthosicyos naudinianus* 22.55% at a concentration of 50μg/ml. *Fockea angustifolia* extracts 16.48 %) scavenging activity at the concentration of at 50 μg/ml which was lower than the

Acanthosicyos naudinianus. Finally the *cf Salvadora Persia* and *Nymania capensis* plant extracts both also exhibited percentage inhibition below 50 %, both at the concentration 50 µg/ml. See appendix five for further details.
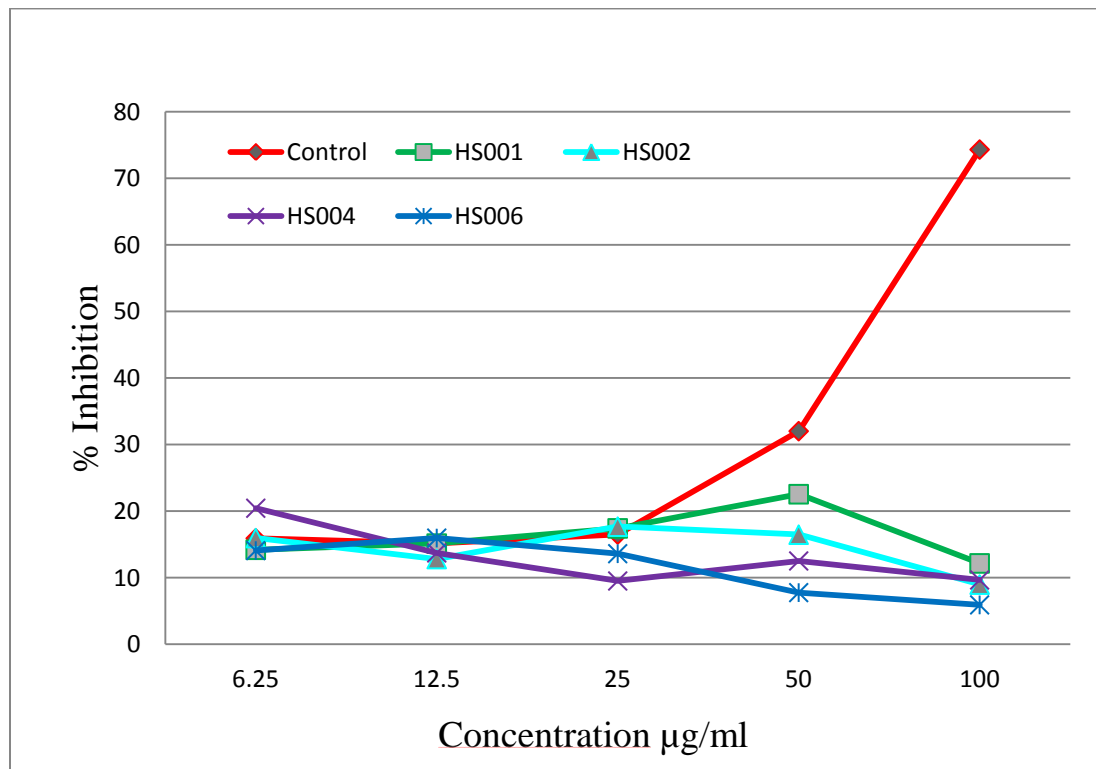
Figure 22: Percentage inhibition of the ethanol plant extracts on DPPH

The statistical analysis, showed outcome of two sample t-test, the P values from the two-tailed test, of the *Acanthosicyos naudinianus* plant extract in comparison to ascorbic acid, revealed that the $p < 0.05$, therefore there is a statistically significant difference between the *Acanthosicyos naudinianus* plant extract and the ascorbic acid ($p < 6.01519^{-16}$). *Fockea angustifolia* there is a statistical significant difference of *Fockea angustifolia* ($p < 0.000834$) between the *Fockea angustifolia* plant and ascorbic acid.

Fockea angustifolia The *cf Salvadora persia* plant extract as seen in table 8 shows the likelihood of uncertainty in samples in comparison to ascorbic acid, with $p < 0.002788$), therefore there is a statistically significant difference between the two groups. The *Nymania capensis* plant extract in comparison to ascorbic acid, revealed $p < 0.05$, therefore there is a statistically significant difference *Nymania capensis* since ($p < 0.000103$), details of this can be seen in appendix five. Overall there was no statistical significant difference between the different plant extracts of both aqueous and ethanol. Based on percentage inhibition the p value is 0.37 which is greater than 0.05, Hence the probability that he observe results are due to random chance is high.

Table 8: Statistical evaluation of the P values ethanol plant extracts against DPPH

Ethanol plant extracts			
Plant extracts	Plant mean values	Control mean values	P/value
Acanthosicyos naudinianus	0.165	30.53	6.01519^{-16}
Fockea angustifolia	17.91231	30.53	0.000834
cf Salvadora persia	17.8403	30.53	0.001394
Nymania capensis	16.20048	30.53	0.000103

Acanthosicyos naudinianus ,*Fockea angustifolia Nymania capensis* .Table 9 shows the statistical outcome, using the two sample t-test for the ethanol plant extracts of *Acanthosicyos naudinianus, Fockea angustifolia, cf Salvadora persia* and *Nymania capensis*. *Acanthosicyos naudinianus.* There is a statistically significant difference between the *Acanthosicyos naudinianus* and the ascorbic acid since ($p < 0.001233733$). *Acanthosicyos naudinianus* While *Fockea angustifolia* plant extract in comparison to ascorbic acid, also showed statistically significant difference amongst the two

groups, since *Fockea angustifolia* (p < 0.000477). *Fockea angustifolia* the *cf Salvadora persia* plant extract as seen in the table 9 illustrates the p < 0.05, therefore statistically significant difference between the *cf Salvadora persia* and the standard ascorbic acid was observed at (p < 0.000903848). Lastly there was a statistically significant difference between the *Nymania capensis* plant extract and the ascorbic acid standard at *Nymania capensis* (p < 0.000584).*Nymania capensis*

Table 9: Statistical evaluation of the P values aqueous plant extracts against DPPH

Aqueous plant extract			
Plant extracts	**Plant mean values**	**Control mean values**	**P/value**
Acanthosicyos naudinianus	16.29934721	30.53	0.001233733
Fockea angustifolia	14.38722	30.53	0.000477
cf Salvadora persia	13.18180016	30.53	0.000903848
Nymania capensis	11.47722	30.53	0.000584

4.6 SWATH-MS results and analysis

The *Acanthosicyos naudinianus* treated cell protein sample at concentration of 5μg/ml and 10μg/ml showed the output identification of about 50550 of 108763 fragment ions. By the uses Generated by a LC-MS/MS Protein Identification runs from a Spectral Library on an AB Sciex 6600 Triple TOF MS coupled to a Dionex 3500 RLSC system as can be seen in the table 10.

Table 10: Output for protein identification runs for the spectral Library.

Proteomics	
Group Classification	**Totals groups present in the sample**
Precursors	8701 of 8782
Modified Peptide	8203 of 8281
Peptides	8185 of 8263
Proteotypic peptides	7332
Protein groups	1251(1257)[*]
Proteins	1326(1473)[*]
Single Hits	226 (205)[*]
Fragments	50550 of 108763

Sum of specific peptide match - without brackets,

[*]Sum of significant specific peptide matching accordance with the search engine - in brackets

The Figure 24 shows final spectral library data which consisted of 8701 precursors (blue sphere), 8185 peptides (green sphere) and 1251 unique proteins (yellow sphere) in the *Acanthosicyos naudinianus* treated cell protein sample. Through the Max Quant with a cut-off of 1% FDR at spectral, peptide and protein levels (using Biognosys Spectronaut software).
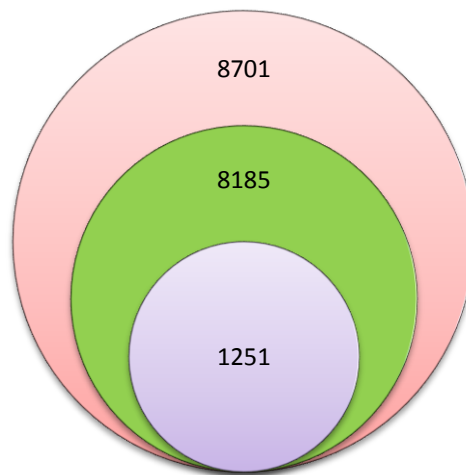
Figure 23: Protein spectral library data for *Acanthosicyos naudinianus* treated cell sample

The Bradford assay was used to measure the concentration of total proteins in the *Acanthosicyos naudinianus* treated TK-10 cell sample see Appendix 2 . The proteins were visualized on a gel by SDS-PAGE (figure 24), which confirmed the band correspondence of the proteins after extraction of the sample.

Figure 24 shows the SDS-PAGE profiles of the protein from *Acanthosicyos naudinianus* treated TK-10 cells after sample extraction at concentrations of 5µg /ml and 10µg /ml alongside the control sample. The first three lanes contain three control samples of cntrl 1, 2, and 3, followed by the three replicates of the 5µg /ml *Acanthosicyos naudinianus* treated TK-10 cell protein sample. The three 10µg /ml

Acanthosicyos naudinianus treated TK-10 cell protein sample are in the last three lanes of the gel. The SDS- PAGE performed before and after acetone precipitation ensured the comparison of successful denaturation and purification of the protein in the biological sample. The Bradford assay seen in Appendix 2 performed before the SDS-page gel measured the quantity and concentration of total proteins in the *Acanthosicyos naudinianus* treated TK-10 cell sample.
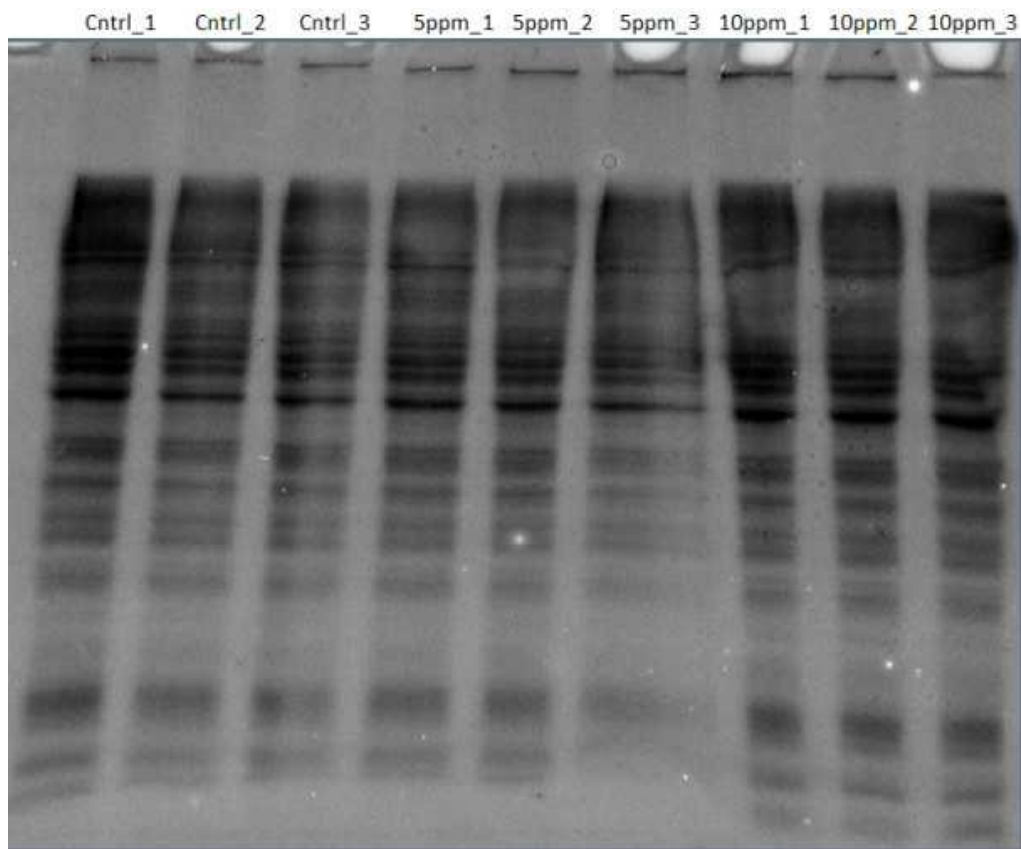
Figure 24: SDS-PAGE of 10µg per sample post extraction

The SDS-PAGE profiles of the *Acanthosicyos naudinianus* treated TK-10 cell protein after acetone precipitation at concentration of 5µg /ml and 10µg /ml alongside the control sample can be seen in figure 26 .The first lane contains the ladder, followed by the three control samples of cntrl 1, 2, and 3 then starting from the fifth lane three replicates of the 5 µg /ml *Acanthosicyos naudinianus* treated TK-10 cell protein sample. Lastly the three 10 µg /ml *Acanthosicyos naudinianus* treated TK-10 cell protein sample are in the last three lanes of the gel. The gel verified the successful denaturation, and purification of the proteins from undesirable substances in the biological sample. As Protein purification aids in establishing a clear Identification of the protein through SWATH- MS, and the possible biological mode of action of the specific proteins in the sample.
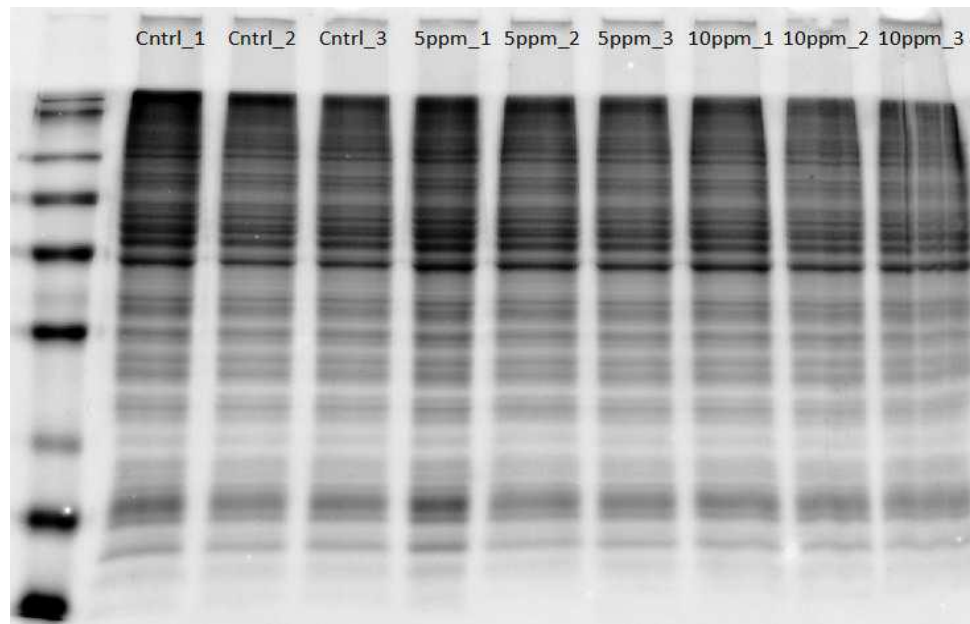
Figure 25 : SDS-PAGE of 10ug per sample post acetone precipitation

 Protein identification peaks from the *Acanthosicyos   naudinianus* treated TK-10 cells. The two peak spectra of the *Acanthosicyos naudinianus* treated TK-10 cell protein sample tested the reproducibility of the sample. Both diagrams A and B generated identical findings of the spectral peak. The SWATH-mass spectrometer achieved good reproducibility as can be seen in the figure above the schematic representation of the peak shapes of the peptide ions, demonstrating how the peaks of individual ions may overlap, due to their close proximity to each other, through intensity of the peak against retention time, in both figure 26 (A) and the figure 26 (B)

(A)
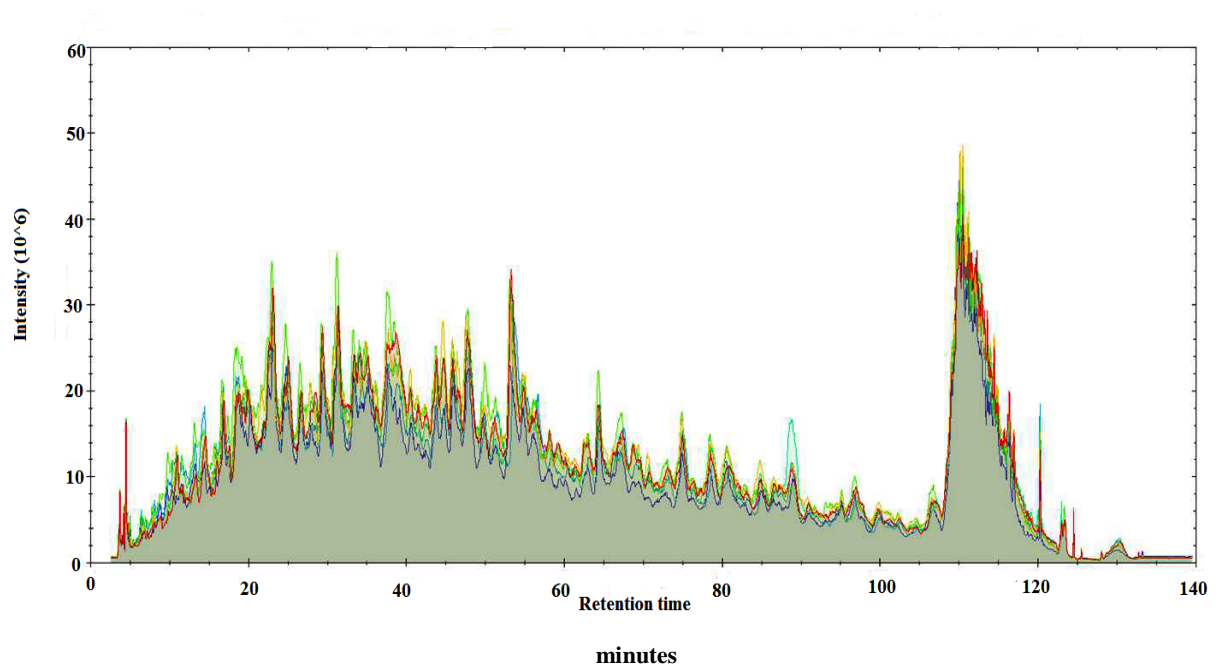
Key: 5µg/ml_1 ------------5µg/ml_2 ------------5µg/ml_3 ------------

Control_1 ------------Control_2 ------------Control_3 ------------

(B)

Key: 10µg/ml_1 ------------10µg/ml_2------------10µg/ml_3 ------------

Control_1 ------------Control_2------------Control_3 ------------
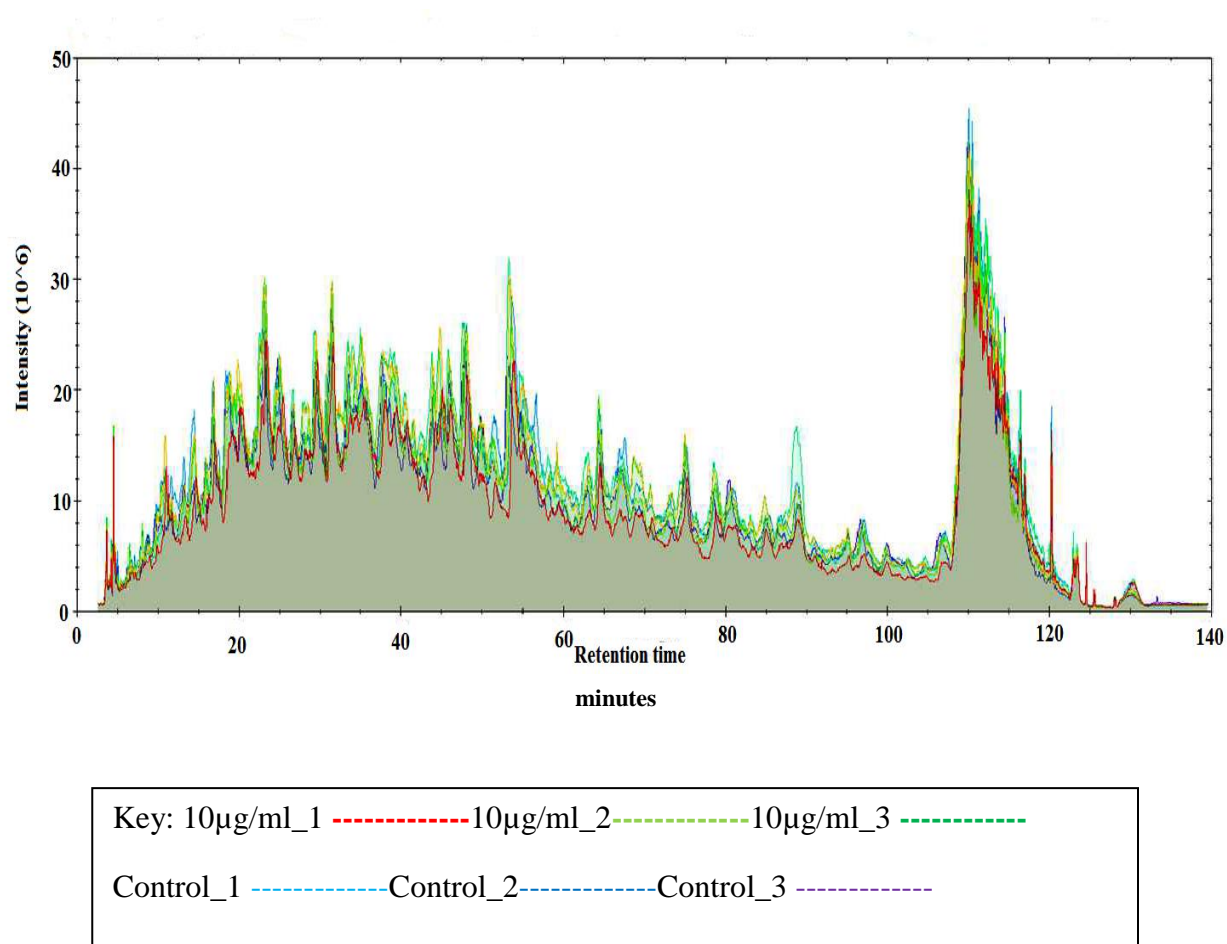
Figure 26: Illustrates individual ions peak intensity of the peak against retention time for the *Acanthosicyos naudinianus* treated TK-10 cells extracts

The SWATH-MS runs were processed using Spectronaut software where peak areas were extracted from each SWATH run and matched to the spectral library so that peptides and proteins can be annotated hence Table 11 below shows the identification coverage matching in the Spectral library and the SWATH

Table 11: Identification coverage matching the Spectral library and SWATH

Identification coverage matching the Spectral library and SWATH			
Sample ID	Control	HS001 5µg/ml	HS001 10µg/ml
Proteins	93%	95 %	93 %
Peptides	80 %	83 %	78 %
Transition	80 %	83%	76 %

The sum quantity of P09493 protein at a concentration of 5µg/ml was about 23.7 x 10^3. While 10 µg/ml was approximately 27.0 x10^3 however the sum quantity of the control was less than both test concentration at the 11.8x10^3. This demonstrate the high presence of the P09493 protein based on their sum quantity compared to the control hence the P09493 protein was unregulated in the *Acanthosicyos naudinianus* treated TK-10 cell sample. The post normalization with reference to the area intensity of all condition, including the replicates can be seen in appendix six for all protein groups to be discussed below.
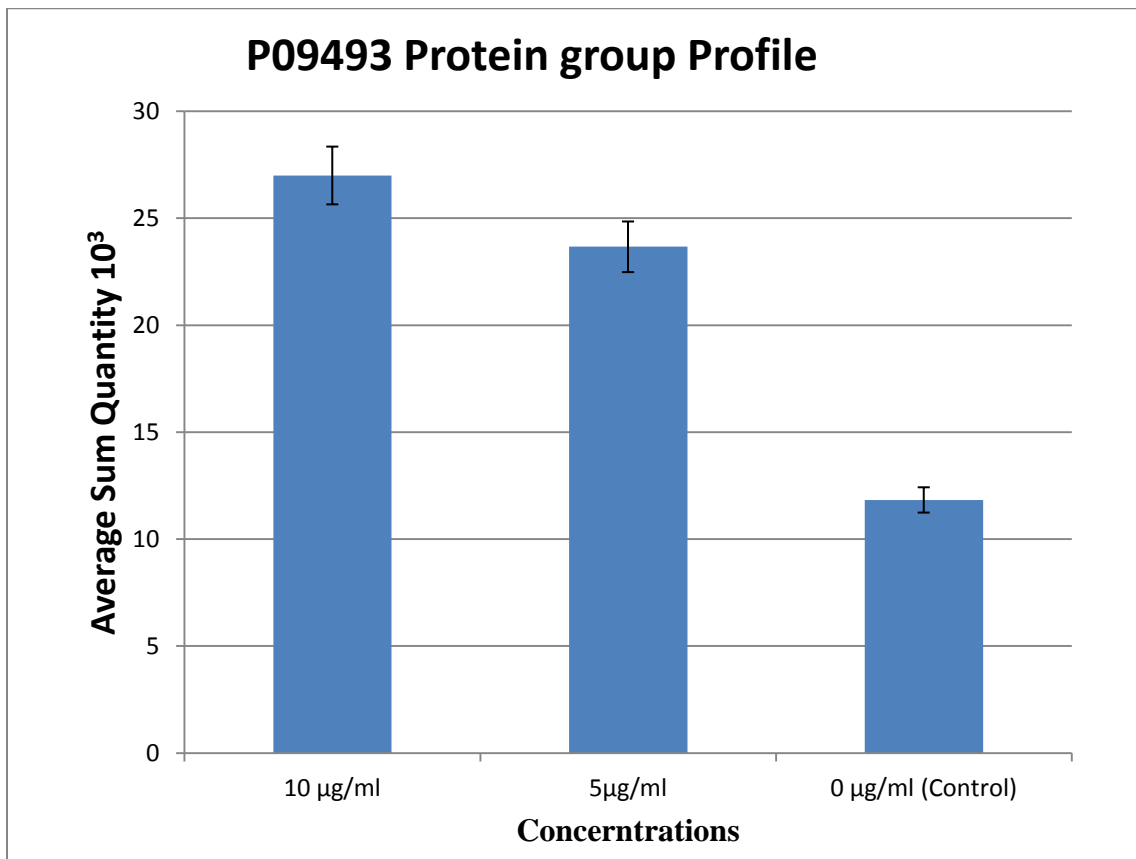
Figure 27: Sum quantities for the P09493 protein profile

The Q562R1 protein had sum quantities of 26.7 $x10^2$ and 28.0 $x10^2$ at concentrations of 5µg/ml and 10µg/ml, respectively of *Acanthosicyos naudinianus* treated TK-10 renal cancer cell sample. Overall both test concentrations were higher than that of the control which was about15.8x10^2. Therefore there was an up-regulation of the Q562R1 protein.
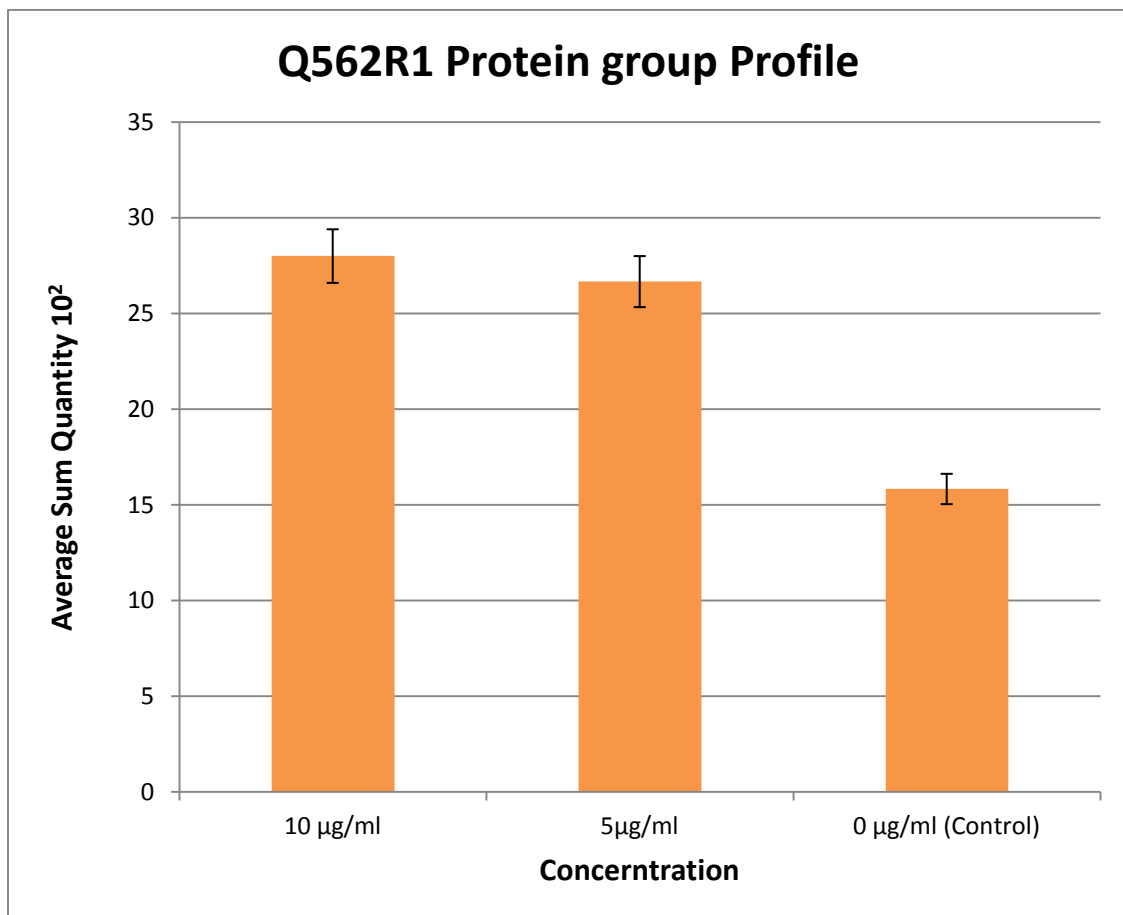
Figure 28: Sum quantities for the Q56R1 protein profile

The *Acanthosicyos naudinianus* treated TK-10 cell sample also demonstrated the up-regulation of P84550 protein with sum quantity of about 28 $x10^3$ and 34 $x10^3$at a concentration of 5µg/ml and 10µg/ml respectively, while the control's sum quantity was approximately 20 $x10^3$.
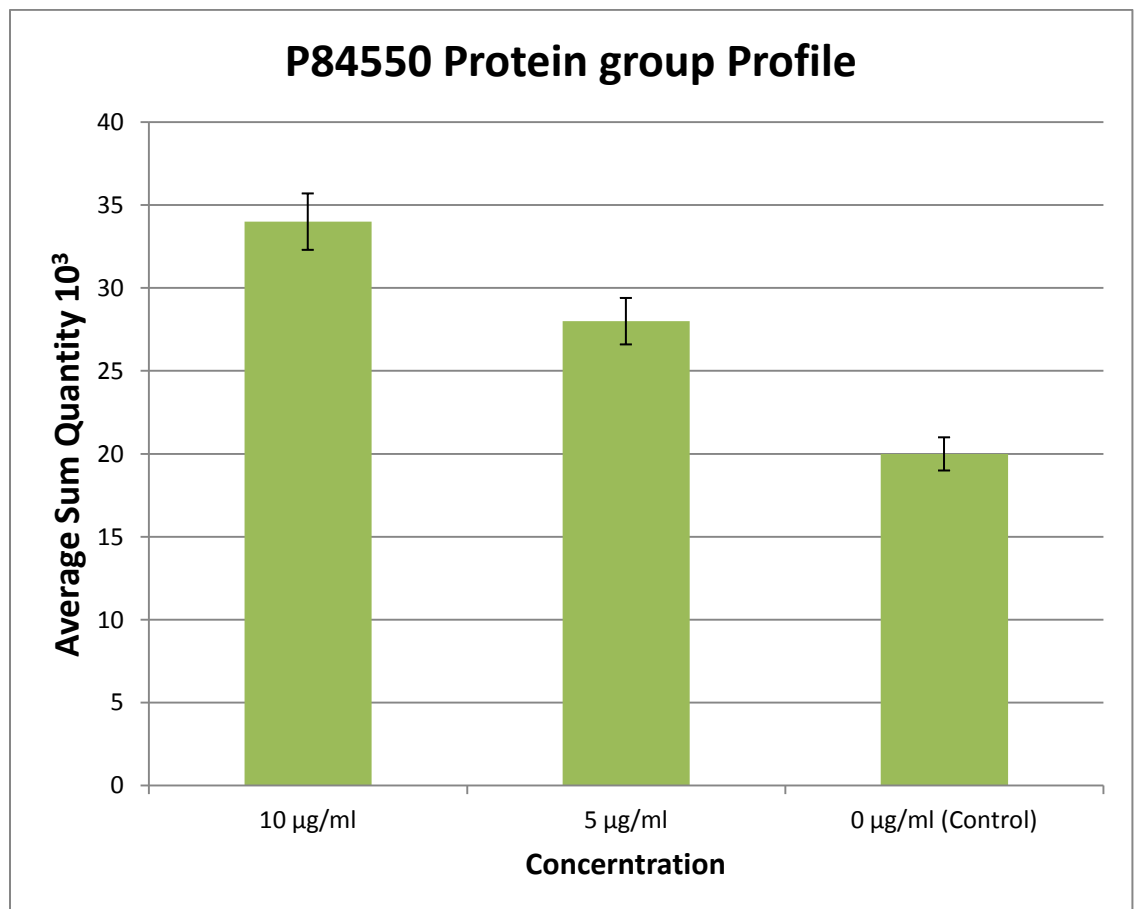
Figure 29: Sum quantities for the P09493 protein profile

The Q5TZA2 protein was the only down regulated protein observed in the *Acanthosicyos naudinianus* treated TK-10 cell sample, since both test concentration of 5µg/ml and 10µg/ml, had sum quantity less than the control about 19 $x10^2$ and 32.7 $x10^2$ respectively. While the sum quantity, of the control was 56 $x10^2$. Hence demonstrating the down regulation of Q5TZA2 protein based on their sum quantities.
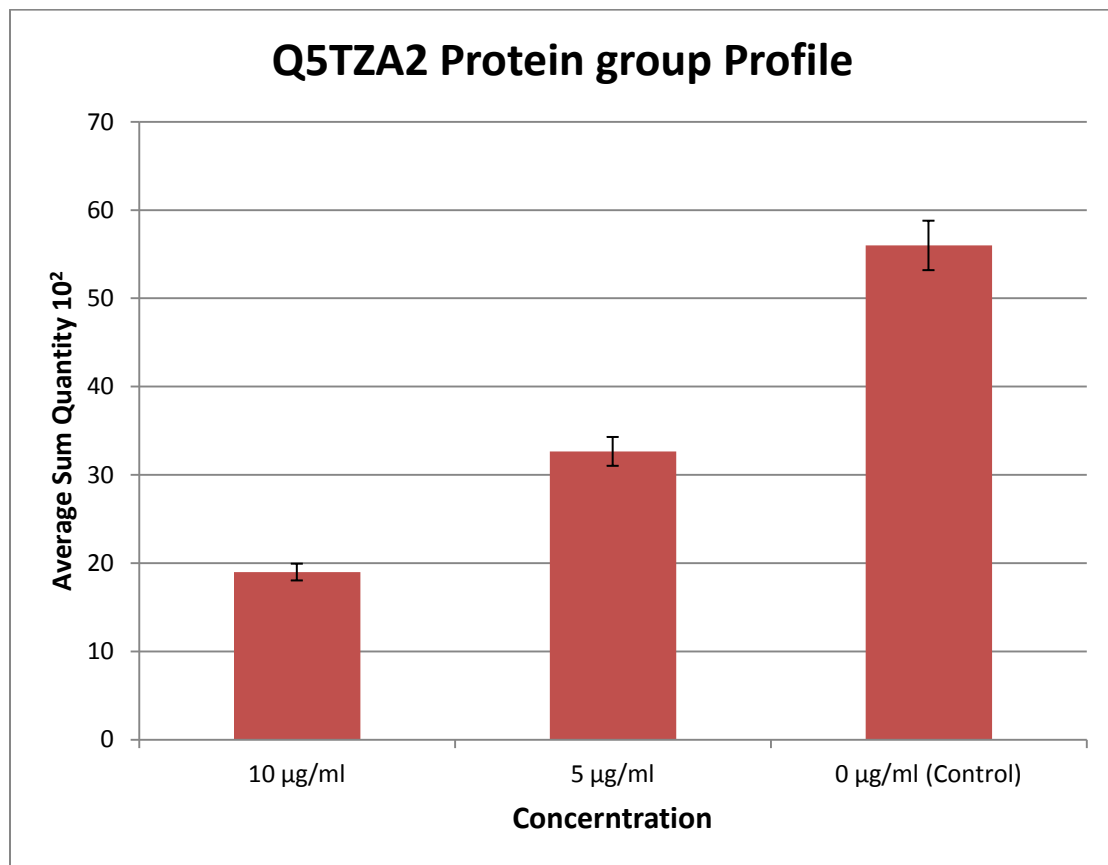
Figure 30: Sum quantities for the P09493 protein profile

Proteins that were up and down regulated as a result of treatment with the *Acanthosicyos naudinianus* plant extracts at two concentrations of 5 µg /ml and 10µg /ml in replicates of three, are Summarized and illustrated in table 17. One out of the identified three proteins showed down-regulation of the *Acanthosicyos naudinianus* plant extracts treated TK-10 cell sample at a concentration of 5µg /ml. The protein is identified as Q5TZA2 with a P/value of 0.00041, hence a significant statistical difference was observed, because the P/value < 0.05. The other two proteins were up regulated namely P09493 and Q562R1.

The P09493 protein was up regulated at 5µg /ml and 10µg /ml concentration of the *Acanthosicyos naudinianus* treated TK-10 cell samples. This particular protein at 5µg /ml had a P/value of 7.1E10, which demonstrated no statistical significant difference, since P/value > 0.05. At 10µg /ml *Acanthosicyos naudinianus* plant extracts treated TK-10 cell sample also showed an up regulation of P09493 protein with a P/value < 0.05 which is 0.0000000001. The second protein which is up regulated is the Q562R1 at a concentration of 5µg /ml it had a P/value of 0.00097 while the 10µg /ml *Acanthosicyos naudinianus* plant extracts treated TK-10 cell sample had a P/value of 0.0015.

Table 12: Significantly Up / Down regulated proteins

Condition Numerator	Condition Denominator (µg/ml)	Protein ID	Average Log Ratio	Average Log Ratio	Standard Deviation (%)	Pvalue	Qvalue
Cntrl	5	P09493	-0.88	0.88	30	7.1E10	3.1E-0.7
Cntrl	5	Q562R1	-0.82	0.82	29	9.7E-0.4	4.3E-02
Cntrl	5	Q5TZA2	0.74	0.74	22	4.1E-04	2.3E-02
Cntrl	10	P09493	-1.25	1.25	38	1.0E-10	2.2E-08
Cntrl	10	Q562R1	-0.82	0.82	32	1.5E-03	4.7E02
Cntrl	10ppm	P84550	-0.7	0.7	0.3	1.4E-03	4.0E-02

Key: Down   Regulated in treated samples

Up   Regulated in treated samples

The graph in figure 31 depicts the posttranslational modification peptide peaks, using the peptide score range distribution. The changes in protein expression in each of two biological conditions before, and after translation induced by treatment, are significant as it assist in differentiating the peptides in terms of their functionalities in the cells. As seen in figure 34 protein match hit scores increases with decrease in the peptide peak rank which indicates a general trend that scoring functions   reviewed quality corresponding possible peptide interpretations in the test samples   at 5µg/ml and 10µg/ml alongside the control from the query spectra data base.
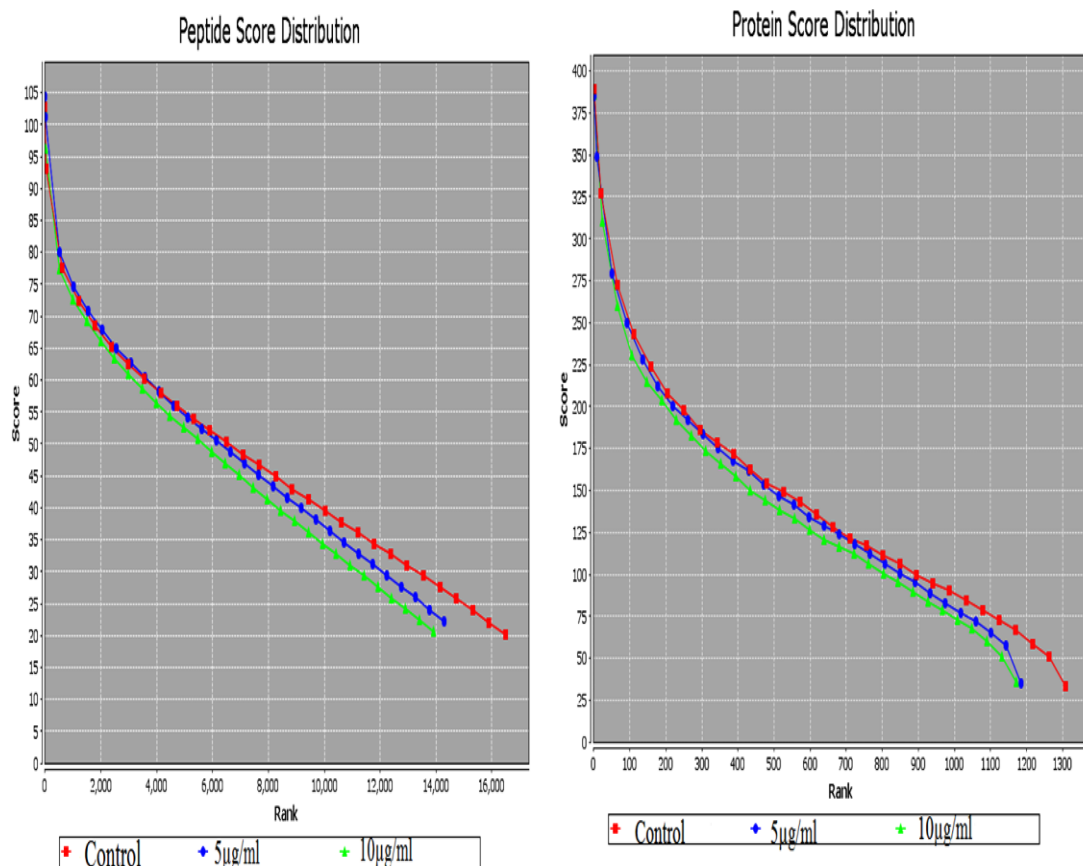
Figure 31: The simultaneous comparison of the expression of proteins between two different biological states through the posttranslational modifications peak ranks.

Overlap of human proteins, a subset annotated with the peptide numbers in the biological sample of *Acanthosicyos naudinianus* treated TK-10 cells extracts. About 9569 peptide were found to have all three peaks of posttranslational modifications of 6, 17 and 23. The individual posttranslational modification coverage of the peptide numbers were less than that of the common combine set with evidence provided in the figure 32. The individual had about 3869 peptide while the individual 5 µg/ml had 2034, and last the 10 µg/ml, had 1864 peptides identified. The peptides that were found to be present in both the control and 10 µg/ml totalled 1418. The commonly identified proteins between the control and 5 µg/ml were 1636. Finally about 1046 peptides were found to be common in the 5 µg/ml and 10 µg/ml.
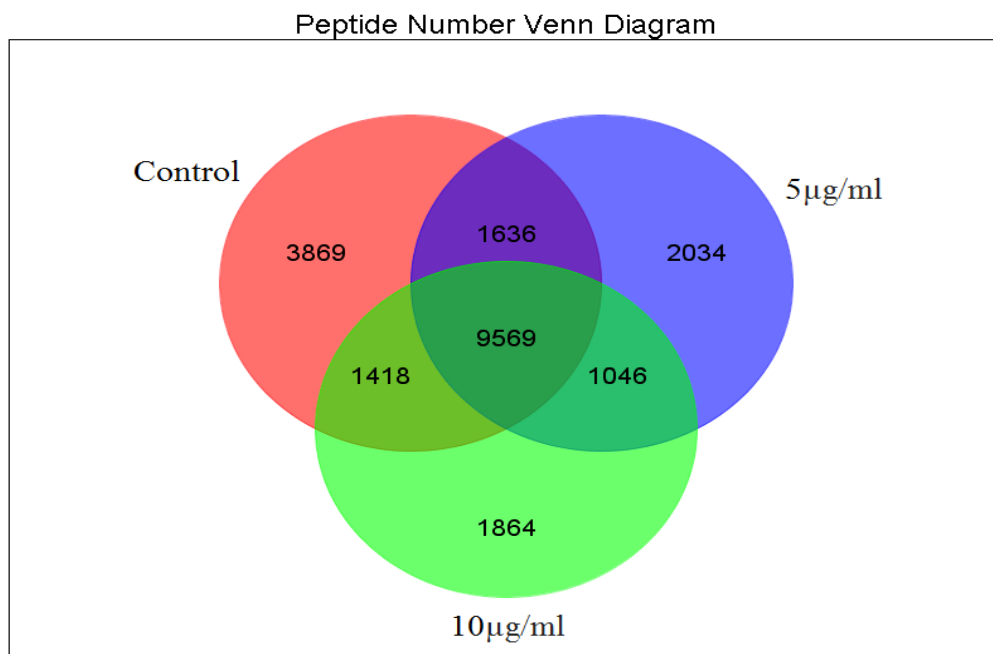
Figure 32: Venn diagram showing the Before treatment of the TK- 10 cancer cell lines with aqueous *Acanthosicyos naudinianus* plant extract with comparison between peptide numbers in control, 5µg/ml and 10µg/ml

The number of peptide that were identified by the individual peak PTM groups were 143 peptides in the control. The 5 µg/ml only identified 41 peptides, while the 10µg/ml identified about two more peptide than the 5 µg/ml. The peptides that were identified to be common among the control and 10 µg/ml were 60 as seen in the diagram in figure 34. While the number of identified peptides commonly between the control and 5µg/ml was about 75. Commonly identified peptide between the 5µg/ml and 10µg/ml were 39. While the about 1030 peptides were identified to be common amongst all three set of control, 5µg/ml and lastly 10µg/ml.

Protein Number Venn Diagram
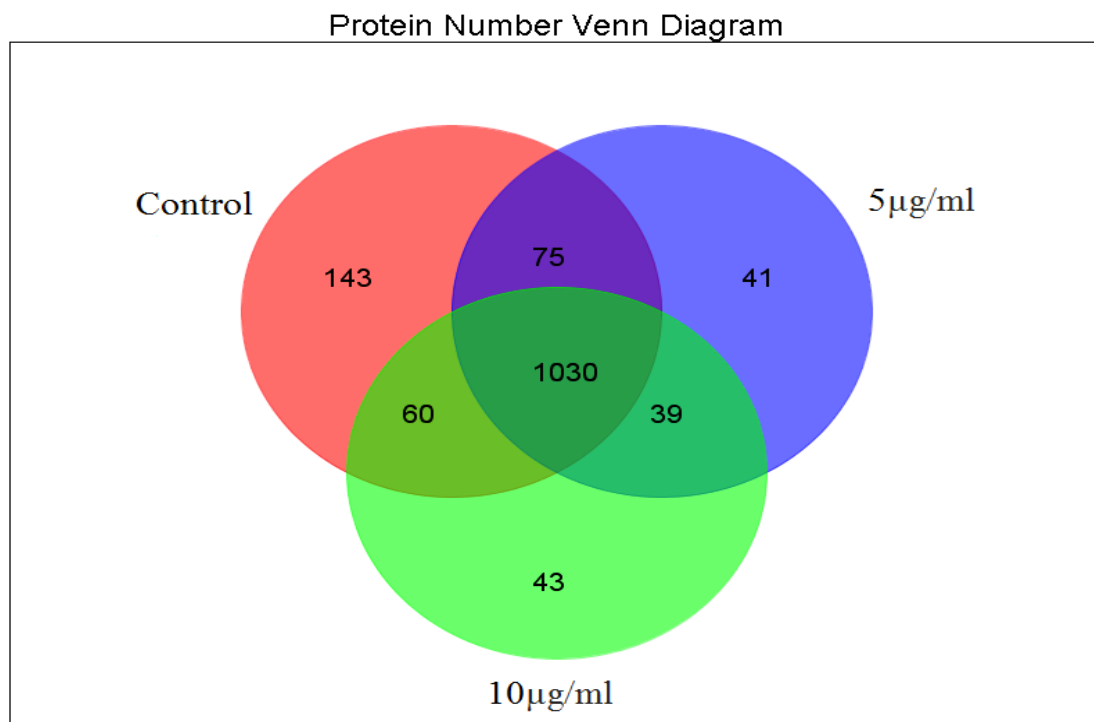
Figure 33: Venn diagram showing the After treatment of the TK- 10 cancer cell lines with aqueous *Acanthosicyos naudinianus* plant extract with comparison between peptide numbers in control, 5µg/ml and 10µg/ml

4.7 Gene ontology of the biological process involving different components ad their functions.

The findings of the gene ontology search detected important implications, in terms of the functionality of the component in the biological pathway. The result showed that the molecular functions of the components involved, seen in figures 34. There is activation of protein heterodimerization activity, through protein binding, with a p-value range of 10^{-3} to 10^{-5}. The transcription regulatory regions sequence –specific DNA binding is also active at a p-value of 10^{-3} to 10^{-5}.
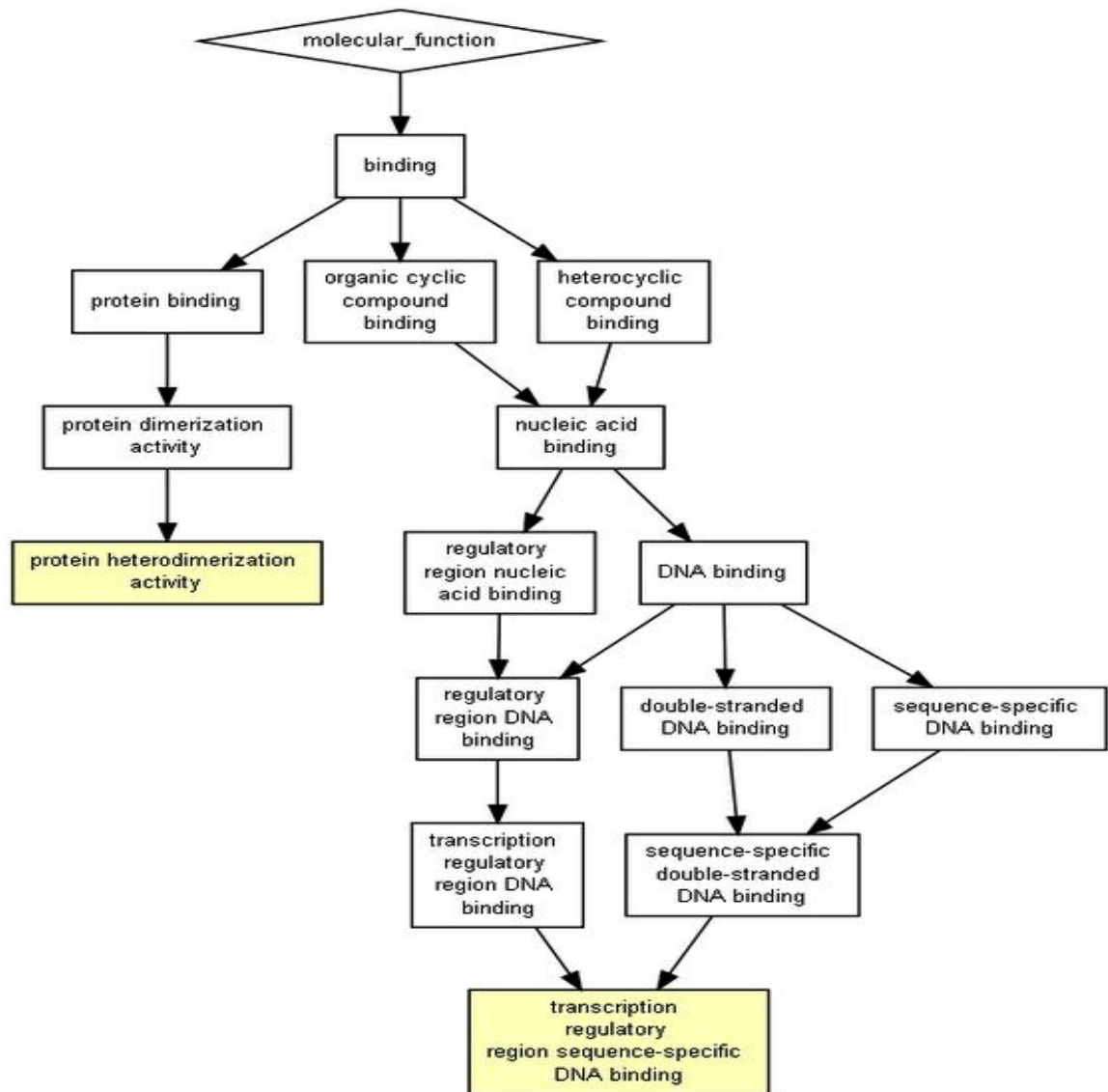
Figure 34: Functions of pathway components from gene oncology for possible mechanism of action.

Key: p-value color scale

$>10^{-3}$	10^{-3} to 10^{-5}	10^{-5} to 10^{-7}	10^{-7} to 10^{-9}	$< 10^{-9}$

4.8 Gene ontology components

This study observed evidence that there is a definite enrichment for nuclear nucleosome proteins in the aqueous *Acanthosicyos naudinianus*   treated TK-10 renal cancer cell samples as seen in figure 35. This enrichment is through the cellular component activating macromolecules complex, which could be either by protein DNA complexes or the protein complexes. The protein complex induces the activity of the DNA packing complex at a color p-value of 10^{-5} to 10^{-7}. This packing complex activates the nucleosome which than enriches the nuclear nucleosome. The results of this gene ontology indicated the possibility of enriching the nuclear nucleosome through the nuclear chromosome part, at a color scale p-value of about 10^{-3} to 10^{-5} further details on gene ontology can be seen in appendix three.

Figure 35: The pathway components of the gene oncology for possible mechanism of action.

Key: p-value color scale

$>10^{-3}$	10^{-3} to 10^{-5}	10^{-5} to 10^{-7}	10^{-7} to 10^{-9}	$< 10^{-9}$

CHAPTER FIVE: DISCUSSION

The use of medicinal plants and plant derived compounds and products, has been reported in several studies to have contributed to various field of pharmaceuticals either through alternative, complimentary or supplementary medicine worldwide (Adnan *et al*., 2014; Haider & Zhong, 2014; Ramasubramania Raja. R Sreenivasulu M, 2015).

5.1 Phytochemical classes' compounds associated with anticancer activity namely alkaloids, flavonoids, anthraquinones, coumarins, and saponins

Therapeutic properties of these medicinal plants may be attributed to the presence of phytochemical class compounds (Enyiukwu *et al*., 2014). In this study it was found that the plant extracts of both the aqueous and ethanol extracts of *Acanthosicyos naudinianus*, *Fockea angustifolia , cf Salvadora persia*a and *Nymania capensis*, under investigation in this study exhibited the presence of various classes of phytochemical constituents such as the saponins, coumarines, alkaloids, anthraquinones and flavonoids. As can be seen in table 4 and 5 the ethanol extracts of the all plants showed the presence of more phytochemical class of compounds than the aqueous plant extracts that are used in the traditional settings, due to the wide polar range of the solvent systems (Tiwari, Kumar, Mandeep, Kaur, & Kaur, 2011). Prior studies also have noted that the organic solvent systems extract phytochemical compounds much more effectively than the aqueous extraction system this is may be due to the difference in polarities of the extraction solvents (Dushimemaria & Mumbengegwi, 2015). Furthermore flavanoids, saponins and coumarine phytochemical class of compounds were found to be more readily present in the ethanol than in the aqueous plant extracts of

Acanthosicyos naudinianus root, *Fockea angustifolia* tuber and whole plant*, cf Salvadora persia*a root and *Nymania capensis* root as compared to the other compound classes such as anthraquinones, alkaloids as can be seen in Tables 4 and 5.

The saponins class of compounds that were present in the plant extracts are argued to have health benefits in human through physiochemical reactions (Aberoumand, 2012). Most importantly they are known to induce apoptosis in some human cancer hence presenting anticancer properties (Ntsoelinyan, Manduna, & Mashele, 2014). Alongside their ability to fight infections, through cytolysis by complexing with cell membrane bilayers creating pore for penetrations (Enyiukwu *et al*., 2014; Nikhil, Shikha, Anil, & Prakash, 2012), saponins are also known to be immunomodulators, and antioxidants according to previous studies such as that of (Fuchs & Weng, 2011).

While the coumarins class of compounds was detected in most of the plant extracts namely the ethanol and aqueous extracts of the plants of *Acanthosicyos naudinianus, Fockea angustifolia, cf Salvadora persia* and *Nymania capensis ,*they are reported to have pharmacological properties of anti-tumor activity by induced apoptosis (Bronikowska, Szliszka, Jaworska, Czuba, & Krol, 2012)in various cancer cell lines namely leukemia, lung, renal, and breast cancers (Musa *et al*., 2011), with reports on clinical trials against prostate, melanoma, and renal cancers(Musa *et al*., 2011).

The coumarins class of compounds that were found to be present in the plant extrcts have antioxidant properties resulting from their radical scavenging ability, which plays an important part in reducing oxidative stress in terminal illness such as cancer (Dai &

Mumper, 2010).The presence of other class of compounds such as the flavonoids, are known to have shielding properties against UV radiation protecting the plants, through preventing oxidative stress, this may have contributed to the observed anticancer potential of the plants used in this study which as used as traditional medicinal plants in Nama communities (Ntsoelinyan *et al*., 2014).

This study also demonstrated the presence of the alkaloids class of compounds and the anthraquinones known for their anti-inflammatory properties (Dave & Ledwani, 2012) in the ethanol extracts of *Fockea angustifolia, cf Salvadora persia* and *Nymania capensis* .The alkaloids class of compounds and their derivatives are physiologically very active agents therapeutically (Enyiukwu *et al*., 2014; Ntsoelinyan *et al*., 2014).Alkaloids isolated from the *Catharanthus roseus* plants known as the vinca alkaloidsnamely the vinblastine and vincristine have been found to have anticancer properties(Mu *et al*., 2012) therefore the presence of this class of compounds, potentially means possible anticancer activity of the plant extracts against the three human cancer cell lines of (MCF-7, UACC-62, and TK-10).The rich phytochemical bioactive compounds produce by these plants from the hot dry Hardap and //Karas region of Namibia, are also influenced by the environmental stress on the plants such as the diversity in climate (Akula & Ravishankar, 2011).

5.2 Antioxidant activity associated with anticancer activity for compounds such as alkaloids, flavonoids, anthraquinones, coumarins, and saponins

Past research has shown the degradation that is caused by free radicals in degenerative diseases and ailments such as tumor promotion, and carcinogenesis due to the oxidative

damage of living cells (Alsarhan, 2014), can be reduce by the antioxidants compound (Nisha & Deshwal, 2011). These compounds play an important part in reducing oxidative stresses, which deteriorate the normal function of the cells in the human body particularly in case of disease such cancer (Dai & Mumper, 2010). This makes alkaloids, flavanoids, coumarine and saponins that were found to be present in the plant extracts in this study suitable agents for chemoprevention as they suppress, and chelate oxidation catalyzed by irons (Dai & Mumper, 2010). Thus antioxidants are useful potential of protecting biological systems such as the three cancerous cell lines investigated in this particular study against oxidative stress.

 This study revealed a general low antioxidant activity by aqueous and ethanol plants extracts despite the presence of various class of phytochemical such has the phenolic compounds of   flavonoids and coumarines with reported antioxidant activity (Ntsoelinyan *et al*., 2014) and frequently associated with high free radical scavenging ability, amongst plant compounds (Graidist *et al*., 2015). The phenolic class of compounds in some plants has been found to be even more potent antioxidants than Vitamin C and E and carotenoids (Dai & Mumper, 2010).The current study found general trend of low antioxidant activity of the plant extract samples, exhibiting free radical scavenging activity lower than 50% inhibition at a concentration of 50μg/ml. Hence the observed low antioxidant radical scavenging of the plant extracts may be due to low phenol content of the extracts (Iqbal, Abu, & Lim, 2015).

5.3 Anti-cancer activities of plants extracts on MCF-7, UACC-62, TK-10 cancer cell lines and the cytotoxicity of these extracts on non-cancerous cell lines

The aqueous *Acanthosicyos naudinianus* plant extract was more potent than etoposide, a standard cancer drug, particularly against the TK-10 human cancer cell line. The low sensitivity, of etoposide in these *in vitro* cancer models may be due to possible factors such as poor cellular absorption or the cellular kinetics of the etoposide drug towards renal, melanoma and breast cancer (Rezonja, Knez, Cufer, & Mrhar, 2013). The screen for the experimental extracts revealed that, the aqueous root plant extract of *Acanthosicyos naudinianus* exhibited the greatest inhibition in the cells line of TK10 (renal), compared to the UACC62 (melanoma) and MCF7 (breast) cancer cell lines to a certain extent, with an IC_{50} value of <6.25 µg/ml against the TK-10 cancer cell lines.

This was potent in vitro anticancer activity. This is due to the low dose concentration of the plants extract required to inhibit the growth of renal cell lines. Natural products and their derivative have been identified as potent anti-cancer agents in the past from various studies (Prakash *et al*., 2013) and the findings of this study seem to be contributing to this field. This is based on the guidelines that defines the recommended percentage inhibition and the (IC_{50}) of proliferation in terms of anticancer activity and cytotoxic (Vijayarathna & Sasidharan, 2012).

Contrary to expectations, this study indicated weak activity of the ethanol *Acanthosicyos naudinianus* extracts compared to the aqueous plant extract of *Acanthosicyos*

*naudinianus,*a concoction similarly prepared by the traditional Nama communities in the southern regions of Namibia treating symptoms of cancer. The observation may be due to the ability of the water to dissolve, solubilize and extract potent bioactive compounds of similar polar range and the advantage that it targets a wider polar range (Tiwari *et al*., 2011).

While organic solvents are known to extracts bioactive compounds that are generally more effective than the aqueous (Prakash *et al*., 2013; Solowey *et al*., 2014) thus presenting increased chances of potential anticancer activity. Also the observed potent anticancer activity of this aqueous extract may be attributed to the presence of water soluble classes of compounds such as the flavonoids as seen in table 4, 5 and 6 known for their antioxidant properties (Zainuddin & Sul'ain, 2015) which plays an important role in anti-proliferative properties (Prakash *et al*., 2013). Thus a dose-dependent inhibition was observed in the screen of aqueous and ethanol *Acanthosicyos naudinianus* extracts against the three cancer cell lines. Therefore the possible anticancer activity observed during this screen, also shows the wealth in medicinal indigenous plants in Namibia (Cheikhyoussef, Shapi, Matengu, & Ashekele, 2011; Maroyi & Cheikhyoussef, 2015).

Plants have bio-active compounds with health benefits such as the phenols (Gul, Ahmad, Kondapi, Qureshi, & Ghazi, 2013) but some of the bio-active compounds may also contain probable deleterious compounds (Ifeoma & Oluwakanyinsol, 2013). Therefore

cytotoxicity of these various plants extracts and derivatives were tested to evaluate safety and efficacy (Zainuddin & Sul'ain, 2015)for human consumption. The crude plant extracts in this study exhibited a general trend of cytotoxicity ranging between low to moderately hazardous extracts. With the most potent aqueous *Acanthosicyos naudinianus* plant extract estimated as moderate hazardous while the ethanol *Acanthosicyos naudinianus* and *cf Salvadora persia* plants extracts of the study were estimated to be weakly Hazardous as can be seen in table 6.

All other plant extracts of the study were classified as low hazardous against the normal primary cell lines of human fetal lung fibroblast WI-38 see appendix four on Cytotoxicity of plant extracts. The toxicity test revealed that the IC_{50} values for the standard etoposide drug was more toxic based on the standard guidelines and also compared to the crude plant extracts used in this study (Vijayarathna & Sasidharan, 2012). The safety and toxicity of particularly medicinal plant derived products such as herbal supplements or complimentary alternative medicine, has mainly become a focal point in recent years since there is no clarity in distinguishing between active and toxic compounds of certain plants when used as treatment in many traditional settings (Ifeoma & Oluwakanyinsol, 2013; Rajalakshmi, Jayachitra, Gopal, & Krithiga, 2014).

5.4 The mechanisms of action involved in the anticancer activity of the plant extracts.

The SWATH- ms technique most importantly changes all the peptides ionized in the biological sample into a reusable digital map of proteomics (Liu *et al*., 2014). This study has demonstrated the significant contribution of the technique in understanding the change in protein expression patterns after exposure to the experimental drug, which in this case is the *Acanthosicyos naudinianus* plant extract. An added advantage of the SWATH-MS which was also observed in this study was the high technical and good quantification reproducibility, between biological replicates for concentrations 5µg/ml and 10µg/ml, a scenario also seen in reports such as (Liu *et al*., 2013, 2014; Zhang *et al.*, 2015).

Protein expression profiles were found to be similar between the different SWATH- run and the libraries. The difference in protein expression profiles may be due to the difference in fold change which can be attributed to the difference in the number of peptides present within the library (Rosenberger *et al*., 2014). Overall the expression profiles of differentially expressed proteins, of the treated TK-10 cells identified four proteins which were up and down regulated due to the treatment of TK-10 cancer cell lines with the aqueous *Acanthosicyos naudinianus* plant extract at two concentrations of 5µg/ml and 10 µg/ml alongside the control. Three of the proteins were up-regulated while one was down regulated at concentrations 5µg/ml and 10µg/ml. The first and most important protein which was up-regulated was the P84550 protein SKI from the family of transcriptional corepressor 1 , this protein has the ability to act as an transcriptional corepressor of LBX1, which inhibits the Bone Morphogenetic Protein (BMP) signalling pathway (Mannervik, Nibu, Zhang, & Levine, 1999; von Kitzing, Jonas, & Sakmann,

2006).The human BMP pathway has constituent proteins which plays an critical role in the regulations of proliferation, differentiation and apoptosis of cancerous cells (R. N. Wang *et al*., 2014).Since the BMP signal pathway is reported to promote cancer growth and metastasis (Alarmo & Kallioniemi, 2010; Melotti, Ruiz, & Lorente-trigos, 2010) the inhibition of the BMP signal pathway by P84550 protein may suggest that the observed anticancer activity and the mode of activation of aqueous *Acanthosicyos naudinianus* plant extract against the TK-10 renal cancer cell lines may through apoptosis induced by inhibition of the BMP signalling pathway.

The second protein which was up-regulated was P09493 (Tropomyosin α1), this protein is also known as the actin filament binding protein in muscle and non-muscle cells (Rao, Rivera-Santiago, Li, Lehman, & Dominguez, 2012). Most importantly the Tropomyosin α1protein regulates the endothelial cell response to oxidative stress following its phosphorylation, the protein also suppresses breast cancer, (MCF-7 cell lines) hence a tumor suppressor protein (Zerradi, Houle, & Huot, 2015).The protein together with the troponin multiplex plays a critical role of motility in the vertebrate striated muscle contractions generally the P09493 protein regulates the smooth muscle contraction by interaction with caldesmon, while in non-muscle cells it's implicated in stabilizing cytoskeleton actin filaments (Geisler *et al*., 2015; Rao *et al*., 2012; Zerradi *et al*., 2015).

The third up-regulated protein is the Q562R1 (β Actin-like Protein2) (X. Li et al., 2012)the actin group of proteins are greatly preserved with contribution to cell motility and are widely expressed in all eukaryotic cells(Baykal *et al*., 2013). Furthermore the

Q562R1 (β Actin-like Protein2) proteins' contribution towards understanding role it plays in cancer is steady increase, however, the number of studies is still limited. The Q5TZA2 (Rootletin) protein a main structural constituent of the ciliary rootlet, a cytoskeletal-like structure found in ciliated cells of the basal body at the proximal end of a cilium and extends proximally toward the cell nucleus. Rootletin was the only down regulated protein identified. This protein plays a vital part in the initiation of cellular mitosis (G. Wang *et al*., 2014) an important stage in the cell cycle. The protein is a regulator of the centrosome cohesion and disjunction (G. Wang *et al*., 2014) therefore the down regulation of this particular protein may than distort the progression of the cancer cell cycle. This may contribute to the observed anticancer properties of the aqueous *Acanthosicyos   naudinianus* plant extract.

5.5 The post-translational modifications (PTM) and the gene ontology

To further understand the anticancer mechanism of action of the aqueous *Acanthosicyos naudinianus* plant extracts against the TK-10 renal cancer cell , the post-translational modifications (PTM) and the gene ontology was investigated. The post-translational modifications on proteins affects the potential functions of the proteins through covalent bonds at small chemical moieties to selected amino acid residues (Duan & Walther, 2015). The PTM also affects the cellular functionalities, such as metabolism, signal transduction, and protein stability through processes such as phosphorylation, glycosylation, methylation, and acetylation (Minguez *et al*., 2015). The number of peptides detected and identified in the biological sample after the treatment the *Acanthosicyos naudinianus* plant extract showed a limited number of peptides in general

throughout the two concentration conditions of the 5µg/ml and 10µg/ml. However the highest number of peptides that were detected in the samples was in the 10µg/ml treatment. According to the data from the gene ontology screen, we can infer that the functions of the component present in the biological pathway of a possible mechanism of action, involves the activation of various processes and pathways such as the nucleosome assemble activation and the Endoplasmic Reticulum (ER) to Golgi vesicle mediated activities as seen in Appendix 3.

The molecular functionality of these components in the above mentioned process contributes to the activation of other molecules such as the Histone group of proteins. These proteins activate different pathways as a result seen in the diagram in Appendix 3. The initial bind of the molecules to other components such as proteins, organic cyclic compounds, and heterocyclic compound consequently stimulates nucleic acid binding which further attach to DNA and regulatory regions nucleic acid, followed by sequence specific double stranded DNA , which significantly activates the transcription regulatory regions sequence –specific DNA . The anticancer mode of action of the aqueous *Acanthosicyos naudinianus* may be based on the enrichment of the nuclear nucleosome in the chromotin as seen in figures 36.The enrichment of the nuclear nucleosome by the Histone protein, clarifies the reason as to why only few up/ down regulated species were detected than what was expected in the SWATH runs seen in table 17 in the results section. The reason for this is that the Histone proteins are difficult to analyze via MS firstly because the protein group are part of the chromatin (Banerjee *et al*., 2014)and thus tend to be lost during sample preparation specifically during the spinning out the

DNA (Rodriguez-Collazo, Leuba, & Zlatanova, 2009) and secondly they contain a very high percentage of Lysine residues which results in many small peptides post trypsin digestion which are not detected via MS (Arnaudo & Garcia, 2013; Rodriguez-Collazo *et al.*, 2009).

However gene Ontology analysis revealed the identification of Histone protein abundance or over-representation at 5µg/ml and 10µg/ml of *Acanthosicyos naudinianus* plant extract. This histone code protein's activity is affected by various enzymes through alterations of methylate, acetylate, phosphorylate and ubiquitylate or the reverse thereof such as the deacetylation (Chervona & Costa, 2012; Minguez *et al.*, 2015)or through the amino-acid deposit in the core histone during DNA replication, and transcription and repair (Jensen, 2006). The ability of aqueous *Acanthosicyos naudinianus* plant extract to interfere with the histone code function by inhibiting histone deacetylases enzyme function, obstructs gene activation and copying in altered cells, preventing further cancer progression, and inducing apoptosis (Kim & Bae, 2011). Thus based on the results from the gene ontology it is the apoptosis pathway through which the anticancer mechanism of the aqueous *Acanthosicyos naudinianus* plant extract acts to kill the TK-10 renal cancer cells. Lastly the knowledge that the mechanism of action aqueous *Acanthosicyos naudinianus* plant extract is the vital to further studying the plant extracts for potential plant derived anticancer agents.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

The present study was designed to determine the *in vitro* anti-cancer properties of *Acanthosicyos naudinianus, Fockea angustifolia, cf Salvadora persia* and *Nymania capensis* found in Hardap and the //Karas regions and the possible mechanisms involved in the anticancer activity of the plant extracts. Phytochemical class compounds of Anthraquinones, Alkaloids, Saponins, Flavonoids and Coumarines were found to be present in the plants extracts of *Acanthosicyos naudinianus, Fockea angustifolia, cf Salvadora persia* and *Nymania capensis ,* these secondary metabolic compounds have health benefits, particularly they have anti-tumor activity and chemotherapeutic properties.

 Most significantly the research has also shown that the extracts of the *Acanthosicyos naudinianus , Fockea angustifolia, cf Salvadora persia*and *Nymania capensis* plant extracts had anticancer activity. Aqueous *Acanthosicyos naudinianus* plant extract had the most potent anticancer activity against the renal cancer cell lines with an IC_{50} value of less than 6.25µg/ml compared to all the other plant extracts and cancer cell lines in the study. In addition, the study showed low cytotoxicity emanating from the plant extracts except for aqueous *Acanthosicyos naudinianus*, which were moderately cytotoxic, hence this may be further investigated by doing purification and isolation to separate activity from the moderate toxicity. One of the more significant findings to emerge from this study was that mechanism of action of the aqueous *Acanthosicyos naudinianus* plant extracts against the renal cancer cell lines, which was through the

Apoptotic pathway of the nuclear nucleosome enrichment with the up-regulation of the Histone proteins in the chromatin. Therefore enhanced specificity of proteins such a Histones against cancer cells may present effective probable anticancer therapeutics, in drug discovery and development research (Y. B. Huang et al., 2012).The use of traditional medicinal plant by the Nama communities for treatment, prevention and management of cancer and symptoms similar to that of cancer has been valorized by this particular study. Consequently this study has also confirmed the underlying, rationality for the use of these plants in the Nama communities through the scientific link associated with the use of medicinal plants by the Nama communities in the Hardap and //Karas regions of Namibia as primary health care. And as a result the research has shown the probable anticancer activity, through induced apoptosis of some of these medicinal plants.

Recommendation for future studies

- There is still need for fractionation and purification of some of the plant extracts such as the aqueous *Acanthosicyos naudinianus* plant extracts, to separate the potent activity observed from the moderate toxicity,

- Further in *vitro* and in *vivo* testing studies are recommended.

- Last Analytical chemistry, for the plant phytochemistry

References

Abdul, B., & Hassan, R. (2012). Main Critical Side Effects Associated with Chemotherapy Used in Cancer Treatment. *Pharmaceutica Analytica Acta*, *3*(5), 4172.

Aberoumand, A. (2012). Screening of Phytochemical Compounds and Toxic Proteinaceous Protease Inhibitor in Some Lesser-known Food Based Plants and Their Effects and Potential Applications in Food. *International Journal of Food Science and Nutrition Engineering*, *2*(3), 16–20.

Abraham, R. T., & Abraham, R. T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases, *Cold Spring Harbor Laboratory Press*. 2177–2196.

Accardi, F., Toscani, D., Bolzoni, M., Palma, B. D., Aversa, F., & Giuliani, N. (2015). Mechanism of Action of Bortezomib and the New Proteasome Inhibitors on Myeloma Cells and the Bone Microenvironment : Impact on Myeloma-Induced Alterations of Bone Remodeling, *Hindawi Publishing Corporation BioMed Research International 2015,*1-13.

Adnan, M., Bibi, R., Mussarat, S., Tariq, A., & Shinwari, Z. K. (2014). Ethnomedicinal and phytochemical review of Pakistani medicinal plants used as antibacterial agents against Escherichia coli. al. *Annals of Clinical Microbiology and Antimicrobials 13*(40), 1–18.

Agnieszka Jamio1kowska. (2014). The role of some secondary metabolites in the health status of sweet pepper (*capsicum annuum* l .) grown in the field. *Acta Sci. Pol., Hortorum Cultus 13*(2) 15-30.

Ahmed, K. (2013). Early Prevention and Detection of Skin Cancer Risk using Data Mining. *International J*ournal of Computer *Applications 62*(4), 0975 – 8887.

Akula, R., & Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, *6*(11), 1720–1731.

Alam, S., & Khan, F. (2014). QSAR and docking studies on xanthone derivatives for anticancer activity targeting DNA topoisomerase II. *Drug Design, Development and Therapy*, *8*, 183–195.

Alamgir, A. N. M., Rahman, A., & Rahman, M. (2014). Secondary Metabolites and Antioxidant Activity of the Crude Leaf Extract of Bacopa monniera (L .) Pennel . and Coccinia grandis (L .) J . Voigt, *3*(1), 226–230.

Alarmo, E.-L., & Kallioniemi, A. (2010). Bone morphogenetic proteins in breast cancer: dual role in tumourigenesis. *Endocrine-Related Cancer*, *17*(2), R123–R139.

Alawode, T. T. (2013). An overview of the anti-cancer properties of some plants used in traditional medicine in Nigeria. *International Research Journal of Biochemistry and Bioinformatics 3*(1), 7–14.

Alieldin, N. H., Abo-Elazm, O. M., Bilal, D., Salem, S. E., Gouda, E., Elmongy, M., & Ibrahim, A. S. (2014). Age at diagnosis in women with non-metastatic breast cancer: Is it related to prognosis *Journal of the Egyptian National Cancer Institute*, *26*(1), 23–30.

Alison, M. R., & College, I. (2001). Introductory article on Cancer. *Encyclopedia of life sciences Nature Publishing Group*

Alitalo, a, & Detmar, M. (2012). Interaction of tumor cells and lymphatic vessels in cancer progression. *Oncogene*, *31*(42), 4499–4508.

Allen, D. E., & Hatfield, G. (2004). Medicinal Plants in Folk Tradition an ethnobotany of Britain and Irland. *An ethnobotany of britain & ireland,* 11-143.

Alsarhan, A. (2014). Review on Some Malaysian Traditional Medicinal Plants with Therapeutic Properties, *Journal of Basic & Applied Sciences,* (August 2015), 148–159.

American cancer society. (2012). Breast Cancer Facts & Figures.

American cancer society. (2014). Breast Cancer What is breast cancer

American Cancer Society. (2014). Kidney Cancer (Adult) - Renal Cell Carcinoma Overview What is cancer .

American cancer society. (2015). Melanoma Skin Cancer What is cancer.

Arnaudo, A. M., & Garcia, B. a. (2013). Proteomic characterization of novel histone post-translational modifications. *Epigenetics & Chromatin*, *6*(1), 24.

Banerjee, A., Sanyal, S., Kulkarni, K. K., Jana, K., Roy, S., Das, C., & Dasgupta, D. (2014). Anticancer drug mithramycin interacts with core histones: An additional mode of action of the DNA groove binder. *FEBS Open Bio*, *4*, 987–995.

Baykal, A. T., Baykal, B., Serhatli, M., Adigüzel, Z., Tunçer, M. A., Kaçar, Ö., … Açilan Ayhan, C. (2013). Proteomic evidence for the plasticity of cultured vascular smooth muscle cells. *Turkish Journal of Biology*, *37*, 414–425.

Ben-shlomo, R. (2014). Chronodisruption , cell cycle checkpoints and DNA repair. *Indian Journal of Experimental Biology, 52*(May), 399–403.

Bertoli, C., Skotheim, J. M., & Bruin, R. A. M. De. (2013). Control of cell cycle transcription during G1 and S phases. *Nature Publishing Group*, *14*(8), 518–528.

Bertrand, K. A., Tamimi, R. M., Scott, C. G., Jensen, M. R., Pankratz, V. S., Visscher, D., Vachon, C. M. (2013). Mammographic density and risk of breast cancer by age and tumor characteristics. *Breast Cancer Research, 15*(R104),1-13.

Bhat, R. (2014). Medicinal plants and traditional practices of Xhosa people in the Transkei region of Eastern Cape , South Africa, *Indian Journal of Traditional Knowledge, 13*(April), 292–298.

Bichakjian, C. K., Halpern, A. C., Johnson, T. M., Foote Hood, A., Grichnik, J. M., Swetter, S. M., … Smith Begolka, W. (2011). Guidelines of care for the management of primary cutaneous melanoma. *Journal of the American Academy of Dermatology*, *65*(5), 1032–1047.

Boyle, P. (2012). Triple-negative breast cancer: Epidemiological considerations and recommendations. *Annals of Oncology*, *23*(SUPPL. 6), 8–13.

Bristow, S. L., Leman, A. R., Kovacs, L. A. S., Deckard, A., Harer, J., & Haase, S. B. (2014). Checkpoints couple transcription network oscillator dynamics to cell-cycle progression. *Genome Biology, 15*(446), 1–10.

Bronikowska, J., Szliszka, E., Jaworska, D., Czuba, Z. P., & Krol, W. (2012). The coumarin psoralidin enhances anticancer effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *Molecules (Basel, Switzerland)*, *17*(6), 6449–6464.

Bruni L, Barrionuevo-Rosas L, Albero G, Aldea M, Serrano B, Valencia S, Brotons M, Mena M, Cosano R, Muñoz J, Bosch FX, de Sanjosé S, Castellsagué X. Human Papillomavirus and Related Diseases in Namibia. Summary Report. *ICO Information Centre on HPV and Cancer (HPV Information Centre). 2015*, 03-20.

C. Behl and C. Ziegler. (2014). Cell Cycle : The Life Cycle of a Cell. *Molecular Mechanisms and Implications for Disease*, 9-19.

Chaabane, W., Mohamed, D. U., Jaksik, E. R., Ripk, Á., & Vhl, (2013). Autophagy , Apoptosis , Mitoptosis and Necrosis : Interdependence Between Those Pathways and Effects on Cancer, *Arch. Immunol. Ther. Exp. 2013 (*61*), 43–58.

Cheikhyoussef, A., Shapi, M., Matengu, K., & Ashekele, H. M. (2011). Ethnobotanical study of indigenous knowledge on medicinal plant use by traditional healers in Oshikoto region , Namibia. *Journal of Ethnobiology and Ethnomedicine*, *7*(1), 1-10.

Chen, Y., Williams, V., Filippova, M., Filippov, V., & Duerksen-hughes, P. (2014). Viral Carcinogenesis: Factors Inducing DNA Damage and Virus Integration, *Cancer journal 2014*(6) 2155–2186.

Chervona, Y., & Costa, M. (2012). Histone modifications and cancer: biomarkers of prognosis *American Journal of Cancer Research*, *2*(5), 589–97.

Chinsembu, K. C., & 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*, 25. http://doi.org/10.1186/1746-4269-6-25

Chorawala, M. R., Oza, P. M., & Shah, G. B. (2012). Review Article Mechanisms of Anticancer Drugs Resistance : An Overview, *4*(1), 1–9.

Chow, W., Dong, L., & Devesa, S. (2010). Epidemiology and risk factors for kidney cancer. *Nature Reviews Urology*, *7*(5), 245–257.

Christgen, M., & Derksen, P. W. (2015). Lobular breast cancer: molecular basis, mouse and cellular models. *Breast Cancer Research*, *17*(1), 1–9.

Coady, Y., & Boylan, F. (2014). Ethnopharmacology in Ireland: An overview. *Brazilian Journal of Pharmacognosy*, *24*(2), 197–205.

Collins, K., Jacks, T., & Pavletich, N. P. (1997). The cell cycle and cancer, *Proc. Natl. Acad. Sci. USA 94*(April), 2776–2778.

Dai, J., & Mumper, R. J. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules*, *15*(10), 7313–7352.

Dave, H., & Ledwani, L. (2012). A review on anthraquinones isolated from Cassia species and their applications. *Indian Journal of Natural Products and Resources*, *3*(3), 291–319.

Dennert, G., & Horneber, M. (2006). Selenium for alleviating the side effects of chemotherapy, radiotherapy and surgery in cancer patients. *The Cochrane Database of Systematic Reviews*, (3), CD005037.

Ding, Z., & Zhou, G. (2012). Cytotoxic Chemotherapy and CD4 + Effector T Cells : An Emerging Alliance for Durable Antitumor Effects, *2012*.

Dolle, J. M., Daling, J. R., White, E., Brinton, L. A., Doody, D. R., Porter, P. L., & Malone, K. E. (2009). Risk Factors for Triple-Negative Breast Cancer in Women Under the Age of 45 Years, *Cancer Epidemiol Biomarkers Prev 18*(4), 1157–1167.

Dorothy A Shead, Laura J Hanisch , Lacey Marlow, Maria Ho, Nicole Mcmillian , Susan Kidney, R. C. (2014). Guidelines for Patients ®. *Network, national comprehensive cancer*.1-106.

Dossus, L., & Benusiglio, P. R. (2015). Lobular breast cancer: incidence and genetic and non-genetic risk factors. *Breast Cancer Research*, *17*(1), 1–8.

Duan, G., & Walther, D. (2015). The Roles of Post-translational Modifications in the Context of Protein Interaction Networks. *PLoS Computational Biology*, *11*(2), 1–23. http://doi.org/10.1371/journal.pcbi.1004049

Dummer, R., Green, A., Pittelkow, M. R., Iwatsuki, K., Elwan, N. M., & Zipser, M. C. (2011). Skin cancer - A world-wide perspective. *Epidemiology of Malignant Melanoma*, 1–398.

Dummer, R., Hauschild, A., Guggenheim, M., Keilholz, U., & Pentheroudakis, G. (2012). clinical practice guidelines Cutaneous melanoma : ESMO Clinical Practice clinical practice guidelines. *Annals of Oncology 23*(August 2008).

Dushimemaria, F., & Mumbengegwi, D. R. (2015). Palliative treatment of cancer in resource poor settings : Traditional medicine perspective. *Journal of Pharmacognosy and Phytotherapy,* Full *7*(May), 73–79.

Enyiukwu, D. N., Awurum, A. N., Ononuju, C. C., & Nwaneri, J. A. (2014). Significance of characterization of secondary metabolites from extracts of higher plants in plant disease management, Internation journal of advace agricultural research 2014*(2)*, 8–28.

Escudier, B., Eisen, T., Porta, C., Patard, J. J., Khoo, V., Algaba, F., … Kataja, V. (2012). clinical practice guidelines Renal cell carcinoma : ESMO Clinical Practice Guidelines for diagnosis , treatment and follow-up † clinical practice guidelines. *Annals of Oncolog, 23*(September 2008).

Evan, B., Chaney-Graves, K., DeSantis, C., Ferlay;, J., Forman, D., Nathan, G., Sitas, F. (2011). Cancer in Africa. *American Cancer Society*.

Fabbrocini, G., Triassi, M., Mauriello, M. C., Torre, G., Annunziata, M. C., de Vita, V., Monfrecola, G. (2010). Epidemiology of skin cancer: Role of some environmental factors. *Cancers*, *2*(4), 1980–1989.

Feature. (2008). Glossary of Common Forms of Cancer. *International Journal of Pharmaceutical Compounding*, *12*(1), 2008.

Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., … Bray, F. (2013). Fact Sheets by Cancer. *GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet].Lyon, France: International Agency for Research on Cancer; 2013.*

Fuchs, H., & Weng, A. (2011). Chemistry and pharmacology of saponins : special focus on cytotoxic properties. *Botanics: Targets and Therapy*, (JANUARY), 19–29.

Gavhane, Y. N., Shete, a S., Bhagat, a K., Shinde, V. R., Bhong, K. K., Khairnar, G. a, & Yadav, a V. (2011). Solid Tumors : Facts , Challenges and Solutions. *International Journal of Pharma Sciences and Research*, *2*(1), 1–12.

Gawkrodger, D. J. (2004). Occupational skin cancers. *Occupational Medicine*, *54*(7), 458–463.

Geisler, C., Gaisa, N. T., Pfister, D., Fuessel, S., Kristiansen, G., Braunschweig, T., … Henkel, C. (2015). Identification and validation of potential new biomarkers for prostate cancer diagnosis and prognosis using 2D-DIGE and MS. *Biomed Res Int*, *2015*, 454256.

Godar, D. E. (2011). Worldwide Increasing Incidences of Cutaneous Malignant Melanoma, *2011*.

Godard, J., & Camus, A. (2012). Molecular Mechanisms That Differentiate Apoptosis from Programmed Necrosis, 1–8.

Graidist, P., Martla, M., & Sukpondma, Y. (2015). Cytotoxic Activity of Piper cubeba Extract in Breast Cancer Cell Lines. *Nutrients*, *7*(4), 2707–2718.

Gul, M. Z., Ahmad, F., Kondapi, A. K., Qureshi, I. a, & Ghazi, I. a. (2013). Antioxidant and antiproliferative activities of Abrus precatorius leaf extracts--an in vitro study. *BMC Complementary and Alternative Medicine*, *13*(1), 53.

Hackermüller, J., Reiche, K., Otto, C., Hösler, N., Blumert, C., Brocke-heidrich, K., … Ahnert, P. (2014). Cell cycle , oncogenic and tumor suppressor pathways regulate numerous long and macro non-protein-coding RNAs.

Haider, M., & Zhong, J. L. (2014). Ethno-medicinal Uses of Plants from District Bahawalpur , Pakistan, *6*(5), 183–190.

Halpern Allan, Perry Robins, M. S. (2011). Melanoma, *A Publication of The Skin Cancer Foundation 29*(1).

Hansen, C. C., Derrick, M., Warriach, I., Underwood, L. F., Cammack, J. T., & Riese, W. De. (2015). The Association between Autosomal Dominant Polycystic Kidney Disease and Renal Cell Carcinoma, *Open Journal of Urology*, *2015*(5), 84–90.

Hassan, A., Rahman, S., Deeba, F., & Mahmud, S. (2009). Antimicrobial activity of some plant extracts having hepatoprotective effects. *Journal of Medicinal Plants Research*, *3*(1), 20–23.

Hefti, M. M., Hu, R., Knoblauch, N. W., Collins, L. C., Haibe-Kains, B., Tamimi, R. M., & Beck, A. H. (2013). Estrogen receptor negative/progesterone receptor positive breast cancer is not a reproducible subtype. *Breast Cancer Research : BCR*, *15*(4), 68-71.

Henrion, M., Frampton, M., Scelo, G., Purdue, M., Ye, Y., Broderick, P., … Houlston, R. S. (2013). Common variation at 2q22.3 (ZEB2) influences the risk of renal cancer. *Human Molecular Genetics*, *22*(4), 825–831.

Heuvel, S. Van Den, General, M., & Cancer, H. (2005). Cell-cycle regulation, *WormBook content*,1–16.

Hiruma, W., Suruga, K., Kadokura, K., Tomita, T., Miyata, A., Sekino, Y., Yamaguchi, N. (2015). Antitumor Effects and Acute Oral Toxicity Studies of a Plant Extract Mixture Containing Rhus verniciflua and Some Other Herbs, *Open Journal of Immunology*, *2015*(5), 39–49.

Huang, Y. B., He, L. Y., Jiang, H. Y., & Chen, Y. X. (2012). Role of helicity on the anticancer mechanism of action of cationic-helical peptides. *International Journal of Molecular Sciences*, *13*(6), 6849–6862.

Huang, Y.-B., Wang, X.-F., Wang, H.-Y., Liu, Y., & Chen, Y. (2011). Studies on mechanism of action of anticancer peptides by modulation of hydrophobicity

within a defined structural framework. *Molecular Cancer Therapeutics*, *10*(3), 416–426.

Hyland Katherine. (2008). Tumor Suppressor Genes and Oncogenes : Genes that Prevent and Cause Cancer, *Biochemistry/Molecular Biology Lecture,*292–306.

Ifeoma, O., & Oluwakanyinsol, S. (2013). Screening of Herbal Medicines for Potential Toxicities. *New Insights into Toxicity and Drug Testing*, 244.

INwachukwu, C. N., Umeh, N. C., I., G. K., Slyvester, O., & Magnus, C. N. (2010). Identification And Traditional Uses Of Some Common Medicinal, *Report and opinion 2*(6), 1–8.

Iqbal, E., Abu, K., & Lim, L. B. L. (2015). Phytochemical screening , total phenolics and antioxidant activities of bark and leaf extracts of Goniothalamus velutinus (Airy Shaw) from Brunei Darussalam. *Journal of King Saud University - Science*, *27*(3), 224–232.

Jardines, L., Goyal, S., Fisher, P., Weitzel, J., Royce, M., & Goldfarb, S. B. (2015). Breast Cancer Overview : Risk Factors , Screening , Genetic Testing , and Prevention. *Home of the journal Oncology,* 1–20.

Jensen, O. N. (2006). Interpreting the protein language using proteomics. *Nature Reviews. Molecular Cell Biology*, *7*(6), 391–403.

Joanna, Q. (2015). Combination of Juvenile Papillomatosis , *Juvenile Fibroadenoma and Intraductal Carcinoma*, *5030*, 5027–5029.

Julsing, M. K., Koulman, A., Woerdenbag, H. J., Quax, W. J., & Kayser, O. (2006). Combinatorial biosynthesis of medicinal plant secondary metabolites. *Elsevier,23*, 265–279.

Khan, M. Y., Aliabbas, S., Kumar, V., & Rajkumar, S. (2009). Recent advances in medicinal plant biotechnology, *Indian journal of biotechnology 8*(January), 9–22.

Khare, C. P. (2007). Indian Medicinal Plants: An Illustrated Dictionary, *Springer,*001-900.

Kim, H.-J., & Bae, S.-C. (2011). Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. *American Journal of Translational Research*, *3*(2), 166–179.

Komen, S. G. (2014). What is Breast Cancer , 10–11.

Latif, N., Zaiden, R., Pham, D., & Rana, F. (2010). Clinical case study: Soft tissue sarcoma mimicking eosinophilic leukemia. *Clinical Advances in Hematology and Oncology*, *8*(12), 899–901.

Lee, K., Davies, T., & Mishima, M. (2012). Cytokinesis microtubule organisers at a glance. *Journal of cell sciences,125(15)* 3495-3500

Li, Q., Jiang, C., Zu, Y., Song, Z., Zhang, B., Meng, X., Zhang, L. (2011). SFE-CO2 extract from Typhonium giganteum Engl. tubers, induces apoptosis in human hepatoma SMMC-7721 cells involvement of a ROS-mediated mitochondrial pathway. *Molecules (Basel, Switzerland)*, *16*(10), 8228–42.

Li, X., Wang, Z., Liu, J., Tang, C., Duan, C., & Li, C. (2012). Proteomic analysis of differentially expressed proteins in normal human thyroid cells transfected with PPFP. *Endocrine-Related Cancer*, *19*(5), 681–694.

Liu, Y., Chen, J., Sethi, A., Li, Q. K., Chen, L., Collins, B., … Aebersold, R. (2014). Glycoproteomic Analysis of Prostate Cancer Tissues by SWATH Mass Spectrometry Discovers N-acylethanolamine Acid Amidase and Protein Tyrosine Kinase 7 as Signatures for Tumor Aggressiveness. *Molecular & Cellular Proteomics : MCP*, *13*(7), 1753–68.

Liu, Y., Hüttenhain, R., Surinova, S., Gillet, L. C. J., Mouritsen, J., Brunner, R., … Aebersold, R. (2013). Quantitative measurements of N-linked glycoproteins in human plasma by SWATH-MS. *Proteomics*, *13*(8), 1247–1256.

Lorente-trigos, A., Melotti, A., & Ruiz, A.(2010). Article BMP Signaling Promotes the Growth of Primary Human Colon Carcinomas in vivo. *Jornal of molecular cell biology,2*(6), 318–332.

Losonczy, G., Fazakas, F., Pfliegler, G., Komáromi, I., Balázs, E., Pénzes, K., & Berta, A. (2013). Three novel germ-line VHL mutations in Hungarian von Hippel-Lindau patients, including a nonsense mutation in a fifteen-year-old boy with renal cell carcinoma. *BMC Medical Genetics*, *14*(3), 1-8.

Lu, D., Hsiao, J. Y., Davey, N. E., Voorhis, V. A. Van, Foster, S. A., Tang, C., & Morgan, D. O. (2014). substrate degradation in mitosis, *The journal of cell biology,* *207*(1), 23–39.

Luqmani, Y. A. (2005). Mechanisms of Drug Resistance in, *14*(suppl 1), 35–48. http://doi.org/10.1159/000086183

Macmillan cancer support. (2012). Breast cancer. *Canerbackup,*1–12.

Mahoney, S., Arfuso, F., Millward, M., & Dharmarajan, A. (2014). The effects of phenoxodiol on the cell cycle of prostate cancer cell lines, *Cancer Cell International, 14*(110),1–12.

Makkar, H. P. S., Norvsambuu, T., Lkhagvatseren, S., & Becker, K. (2009). Plant Secondary Metabolites in some Medicinal Plants of Mongolia Used for Enhancing Animal Health and Production. T*ropicultura, 27*(3),159–167.

Malumbres, M., & Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm, *9*(mArCH). *Nature reviews cancer,9*(3)153-167.

Mannervik, M., Nibu, Y., Zhang, H., & Levine, M. (1999). Transcriptional coregulators in development. *Science Europepmc 284*(5414), 606-609.

Markevičius, M., & Jankevičius, F. (2015). Epidemiology and risk factors of renal cell carcinoma : literature review and correlation according to Lithuanian people epidemiological risk factors. *Lietuvos chirurgija 14* (3), 141–148.

Maroyi, A. (2013). Traditional use of medicinal plants in south-central Zimbabwe: review and perspectives. *Journal of Ethnobiology and Ethnomedicine*, *9*(31), 1-18

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

Mathew, S. S., Nieves, B., Sequeira, S., Sambandamoorthy, S., Pumiglia, K., Larsen, M., & Laflamme, S. E. (2014). Integrins promote cytokinesis through the RSK signaling axis. *Journal of cell science 2014*(127), 534–545.

Mcinerney, J. D., Micikas, L. B., Gardner, A. L., Scholar, V., Walsh, L. E., Clark, D. J., Andrews, K. (2014). Cell Biology and Cancer. *National Institutes of Health in collaboration with the National Cancer Institute,*1-198.

Mediani, A., Abas, F., Tan, C. P., & Khatib, A. (2014). Effects of Different Drying Methods and Storage Time on Free Radical Scavenging Activity and Total Phenolic Content of Cosmos caudatus. *Antioxidants, 2014*(3),358–370.

Minguez, P., Letunic, I., Parca, L., Garcia-Alonso, L., Dopazo, J., Huerta-Cepas, J., & Bork, P. (2015). PTMcode v2: A resource for functional associations of post-translational modifications within and between proteins. *Nucleic Acids Research*, *43*(1), 494–502.

Mohanty, M., & Pradhan, C. (2015). A Review on Phytochemistry , Bio-Efficacy , Medicinal and Ethno- Pharmaceutical Importance of Artocarpus altilis. *Human journal*, *3*(1), 219-231.

Mombach, J. C. M., Bugs, C. A., & Chaouiya, C. (2014). Modelling the onset of senescence at the G1 / S cell cycle checkpoint. *BMC Genomics*, *15*(7), 1-11.

Morebise, O. (2015). Medicinal plants of Dominica — Uses chemical constituents bioactivities and prospects. *Journal of medicinal plant science, 3*(5), 144–154.

Mu, F., Yang, L., Wang, W., Luo, M., Fu, Y., Guo, X., & Zu, Y. (2012). Negative-Pressure Cavitation Extraction of Four Main Vinca Alkaloids from Catharanthus roseus Leaves.*Molecule,2012*(17),8742–8752.

Mukhtar, E., Adhami, V. M., & Mukhtar, H. (2015).Targeting Microtubules by Natural Agents for Cancer Therapy. *National institute of health*,*13*(2), 275–284.

Murugesan, D., & Deviponnuswamy, R. (2014). Potential anti-inflammatory medicinal plants - a review. *International journal of pharmacy and pharmaceutical science,6*(4),43-49.

Musa, M. a, Badisa, V. L. D., Latinwo, L. M., Cooperwood, J., Sinclair, A., & Abdullah, A. (2011). Cytotoxic activity of new acetoxycoumarin derivatives in cancer cell lines. *Anticancer Research*, *31*(6), 2017–2022.

Naidu, V. L., Bahadur, A. N., & Kanungo, V. K. (2014). Medicinal Plants in Bhupdeopur Forest , Raigarh Chhattisgarh in Central India, *Int. J. Med. Arom. Plants 4*(1), 6–15.

Namibian Ministry of Environment and Tourism. (2010). Namibia ' s Fourth National Report to the United Nations Convention on Biological Diversity (UNCBD) August 2010 Compiled by the Namibian Ministry of Environment and Tourism, (August).

Narayanan, D. L., Saladi, R. N., & Fox, J. L. (2010). Ultraviolet radiation and skin cancer. *International Journal of Dermatology*, *49*(9), 978–986.

U.S. Department of health and human services Breast cancer.(2012). *National Institutes of Health National cancer institue.*

Navarro, P., Gillet, L., Carapito, C., Röst, H., Reiter, L., Rinner, O., Aebersold, R. (2009). SWATH-MS : a new data independent acquisition lc-ms methodology for quantitative complete proteome analysis, *Quantitative Proteomics* 49.

Nelson, H., Fu, R., Humphrey, L., Smith, M., Griffin, J., & Nygren, P. (2009). Comparative Effectiveness of Medications To Reduce Risk of Primary Breast Cancer in Women. *Comparative Effectiveness Review,* (17).

Nikhil, B., Shikha, B., Anil, P., & Prakash, N. (2012). Diverse pharmacological activities of 3-substituted coumarins: A review. *International Research Journal of Pharmacy*, *3*(7), 24–29.

Nisha, K., & Deshwal, R. K. (2011). Antioxidants and their protective action against DNA damage. *International Journal of Pharmacy and Pharmaceutical Sciences*,*3*(4), 28–32.

Ntsoelinyan, P. H., Manduna, S. S., & Mashele, I. T. (2014). The anticancer, antioxidant and phytochemical screening of Philenoptera violacea and Xanthocercis zambesiaca leaf, flower & twig extracts. *International Journal of Pharmacological Research*, *4*(2), 67–70.

Ode, O. J., Asuzu, I. U., & Ajayi, I. E. (2011). Bioassay-Guided Fractionation of the Crude Methanol Extract of Cassia singueana Leaves, *Journal of advanced scientific research,2*(4), 81–86.

Oliveira, A. M. F. ., Pinheiro, L. S., Pereira, Charlane Kelly Souto Matias, Wemerson Neves Gomes, R. A., Chaves, Otemberg Souza Souza, M. de F. V. de, Almeida, R. N. de, & Assis, T. S. de. (2012). Total Phenolic Content and Antioxidant Activity of Some Malvaceae Family Species. *Antioxidant,2012*(1), 33–43.

Oncology group. (2014). Cancer in Children & Adolescents. *Cancer facts and figure, 1*, 24-42.

Ozkan, G., Kamiloglu, S., Ozdal, T., & Boyacioglu, D. (2016). Potential Use of Turkish Medicinal Plants in the, 1–32.

Park, S. U., & National, C. (2012). Editorial : anticancer compounds from plants. *EXCLI Journal 2012*(11), 386–389.

Partridge, A. H., Rumble, R. B., Carey, L. A., Come, S. E., Davidson, N. E., Leo, A. Di, … Smith, I. E. (2014). Chemotherapy and Targeted Therapy for Women With Human Epidermal Growth Factor Receptor 2 – Negative (or unknown) Advanced Breast Cancer : American Society of Clinical Oncology Clinical Practice Guideline. *Journal of oncology*, *2014*(32), 1-16

Pascaline, J., Charles, M., Lukhoba, C., & George, O. (2011). Phytochemical constituents of some medicinal plants used by the Nandis of South Nandi district . *Journal of animal and plant sciences*. *9*(3), 1201–1210.

Payne, S., & Miles, D. (n.d.). Mechanisms of anticancer drugs, 34-46.

Perera, E., Gnaneswaran, N., Jennens, R., & Sinclair, R. (2013). Malignant Melanoma. Open access h*ealthcare*, *2*(1), 1–19.

Peters, J. (2010). Plant diversity patterns at different spatial scales in a semi-arid savanna ecosystem in central Namibia, *Diploma Thesis Landscape Ecology & Nature Conservation.*

Phosrithong, N., & Ungwitayatorn, Æ. J. (2010). Medicinal chemistry Molecular docking study on anticancer activity of plant-derived natural products, *Medicinal chemistry research, 2010*(19), 817–835.

Pinheiro, D., & Sunkel, C. (2014). Mechanisms of cell cycle control, *canalbq_n.º 9_fevereiro, 2012,* 4–17.

Poon, S. L., Mcpherson, J. R., Tan, P., Teh, B. T., & Rozen, S. G. (2014). Mutation signatures of carcinogen exposure : genome-wide detection and new opportunities for cancer prevention, *Genome medicine 6*(24), 1–14.

Prakash, O., Kumar, A., Kumar, P., & Ajeet, A. (2013). Anticancer Potential of Plants and Natural Products: A Review. *American Journal of Pharmacological Sciences*, *1*(6), 104–115.

Prasad S, T. A. (2015). Advanced Techniques in Biology & Medicine Traditional Medicine : The Goldmine for Modern Drugs, *Advance techniquesbiology & medicine 3*(1), 1–2.

Protzel, C., Maruschke, M., & Hakenberg, O. W. (2012). Epidemiology , Aetiology , and Pathogenesis of Renal Cell Carcinoma. *European Urology Supplements*, *11*(3), 52–59.

Qayyum, T., Oades, G., Horgan, P., Aitchison, M., & Edwards, J. (2012). The Epidemiology and Risk Factors for Renal Cancer. *Current Urology*, *6*(4), 169–174.

Radušienė, J., Karpavičienė, B., & Stanius, Ž. (2012). Effect of External and Internal Factors on Secondary Metabolites Accumulation in St. John's Worth. *Botanica Lithuanica*, *18*(2), 101–108.

Rajalakshmi, a, Jayachitra, a, Gopal, P., & Krithiga, N. (2014). Pharmacology & Toxicology Toxicity Analysis of different medicinal plant extracts in Swiss Albino Mice. *Pharmacology & Toxicology Research*, *1*(2), 1–6.

Rakha, E. A., Reis-filho, J. S., Baehner, F., Dabbs, D. J., Decker, T., Eusebi, V., … Ellis, I. O. (2010). Breast cancer prognostic classifi cation in the molecular era : the role of histological grade. *Breast Cancer Research, 12*(207), 1-12.

Ramakrishna, A., & Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, *6*(11), 1720–1731.

Ramasubramania Raja. R Sreenivasulu M. (2015). Medicinal plants secondary metabolites used in pharmaceutical importance-an overview. *World journal of pharmacy and pharmaceutical sciences* , *4*(04), 436–447.

Rao, J. N., Rivera-Santiago, R., Li, X. E., Lehman, W., & Dominguez, R. (2012). Structural analysis of smooth muscle tropomyosin and isoforms. *Journal of Biological Chemistry*, *287*(5), 3165–3174.

Rasool Hassan, B. A. (2012). Medicinal Plants (Importance and Uses). *Pharmaceutica Analytica Acta*, *03*(10), 4172.

Reed, A. E. M., Kutasovic, J. R., Lakhani, S. R., & Simpson, P. T. (2015). Invasive lobular carcinoma of the breast: morphology, biomarkers and 'omics. *Breast Cancer Research*, *17*(1), 12.

Remy, C., Marret, E., & Bonnet, F. (2005). Effects of acetaminophen on morphine side-effects and consumption after major surgery : meta-analysis of randomized controlled trials. *British Journal of Anaesthesia, 94* (4), 505–513.

Rezonja, R., Knez, L., Cufer, T., & Mrhar, A. (2013). Oral treatment with etoposide in small cell lung cancer – dilemmas and solutions. *Radiol Onco, 47*(1),1–13.

Rice, H., Bryant, S., Handley, C., & Hall, M. (2014). Oncogenes and Tumor Suppressor Genes : An Essential Building Block of Cancer. *Journal of the American Institute of Chemists*, *87*(2), 15–18.

Rishi, A., & Sneha, S. (2012). Antioxidant Activity of Pterocarpan (Phaseolus vulgaris) A Priliminary Assesment of Crude Extracts. *Research Journal of Pharmaceutical , Biological and Chemical Sciences*, *3*(3), 271–276.

Rodriguez-Collazo, P., Leuba, S. H., & Zlatanova, J. (2009). Robust methods for purification of histones from cultured mammalian cells with the preservation of their native modifications. *Nucleic Acids Research*, *37*(11), 1–15.

Rosenberger, G., Koh, C. C., Guo, T., Röst, H. L., Kouvonen, P., Collins, B. C., … Aebersold, R. (2014). A repository of assays to quantify 10,000 human proteins by SWATH-MS. *Scientific Data*, *1*, 140031.

Sagalowsky, A. I. (2011). Treatment Options For Patients With Stage I Nonseminomatous Testis Cancer : It ' s All Good. *European Urology*, *59*(4), 563–565.

Saha, S., Adhikary, A., Bhattacharyya, P., Das, T., & Sa, G. (2012). Death by Design : Where Curcumin Sensitizes Drug-resistant Tumours, *2584*, 2567–2584.

Samet, J. M., Avila-tang, E., Boffetta, P., Hannan, L. M., Olivo-marston, S., Thun, M. J., & Rudin, C. M. (2009). Lung Cancer in Never Smokers : Clinical Epidemiology. *American association for cancer research journal*, *15*(9), 5626–5646.

Savithramma, N., Rao, M. L., & Suhrulatha, D. (2011). Screening of Medicinal Plants for Secondary Metabolites. *Middle-east journa l of scientific research, 8*(3), 579–584.

Schairer, C., Li, Y., Frawley, P., Graubard, B. I., Wellman, R. D., Buist, D. S. M., … Miglioretti, D. L. (2013). Risk Factors for Inflammatory Breast Cancer and Other Invasive Breast Cancers.*Oxford University Press, 2013*. 1373–1384.

Sen, S., & Chakraborty, R. (2011). The Role of Antioxidants in Human Health. Oxidative Stress: Diagnostics, Prevention, and Therapy, *American Chemical Society 1083*, 1–37.

Shosan, L. O., Fawibe, O. O., Ajiboye, A. A., Abeegunrin, T. A., & Agboola, D. A. (2014). Ethnobotanical Survey of Medicinal Plants Used in Curing Some Diseases in Infants in Abeokuta South Local Government Area of Ogun State , Nigeria, *American Journal of Plant Sciences*, *2014*(5), 3258–3268.

Shuch, B., Amin, A., Armstrong, A. J., Eble, J. N., Ficarra, V., Lopez-Beltran, A., … Kutikov, A. (2014). Understanding Pathologic Variants of Renal Cell Carcinoma: Distilling Therapeutic Opportunities from Biologic Complexity. *European Urology*, *67*(1), 85–97.

Silva, N. C. C., & Fernandes Júnior, a. (2010). Biological properties of medicinal plants: a review of their antimicrobial activity. *The Journal of Venomous Animals and Toxins Including Tropical Diseases*, *16*(3), 402–413.

Siziopikou, K. P. (2013). Ductal carcinoma in situ of the breast: current concepts and future directions. *Archives of Pathology & Laboratory Medicine*, *137*(4), 462–6.

Sneyd, M. J., & Cox, B. (2013). A comparison of trends in melanoma mortality in New Zealand and Australia : the two countries with the highest melanoma incidence and mortality in the world. *BMC Cancer, 13*(372),1-9.

American Cancer Society.(2014). What are genes Gene mutations.1-123.

Solak, A., Yalaz, S., & Sivrikoz, O. N. (2013). A rare form of breast fibroadenoma : Intraductal fibroadenomatosis. *The Journal of Breast Health, 9*(1) 32–34.

Solowey, E., Lichtenstein, M., Sallon, S., Paavilainen, H., Solowey, E., & Lorberboum-galski, H. (2014). Evaluating Medicinal Plants for Anticancer Activity, *Scientific World Journal,2014,*1-12.

Swift, L. H., & Golsteyn, R. M. (2014). Genotoxic Anti-Cancer Agents and Their Relationship to DNA Damage , Mitosis , and Checkpoint Adaptation in Proliferating Cancer Cells. *International journal of molecular sciences, 2014*(15) 3403–3431.

Taylor, P., Igarashi, Y., Hanafusa, T., Gohda, F., Peterson, S., & Bills, G. (2014). Mycology : Species-level assessment of secondary metabolite diversity among Hamigera species and a taxonomic note on the genus. *An International Journal on Fungal Biology, 5*(3), 102-109.

Thomson, A. K., Heyworth, J. S., Girschik, J., Slevin, T., Saunders, C., & Fritschi, L. (2014). Beliefs and perceptions about the causes of breast cancer : a case-control study. *BMC Research Notes*, *7*(558), 1–8.

Tiwari, P., Kumar, B., Mandeep, K., Kaur, G., & Kaur, H. (2011). Phytochemical screening and Extraction: A Review. *Internationale Pharmaceutica Sciencia*, *1*(1), 98–106.

Tolentino, E. D. S., Centurion, B. S., Helena, L., Ferreira, C., De, A. P., Lq, L., Rzhyhu, U. (2011). Oral adverse effects of head and neck radiotherapy. *Journal of applied oral sciences 19*(5), 448–454.

Tsai, A., Wang, C., Liou, J., Pai, H., Hsiao, C., Chang, J., … Pan, S. (2014). Orally active microtubule-targeting agent , MPT0B271 , for the treatment of human non-small cell lung cancer alone and in combination with erlotinib. *Cell Death and Disease*, *5*(4), 1162–1173.

Tsai, R. Y. L., & Pederson, T. (2014). Connecting the nucleolus to the cell cycle and human disease.*The FASEB Journal, 28*(8), 1–7.

Urrego, D., Tomczak, A. P., Zahed, F., Stu, W., Pardo, L. A., & Zahed, F. (2014). Potassium channels in cell cycle and cell proliferation, *Royal Society,369,*1–9.

Veeresham, C., & Chitti, P. (2013). Therapeutic Agents from Tissue Cultures of Medicinal Plants. *Natural Products Chemistry & Research*, *1*(4), 1–5.

Vera-Badillo, F. E., Conde, E., & Duran, I. (2012). Chromophobe renal cell carcinoma: A review of an uncommon entity. *International Journal of Urology*, *19*(10), 894–900.

Verma, S., & Singh, S. P. (2008). Current and future status of herbal medicines. *Veterinary World*, *1*(11), 347–350.

Viale, G. (2012). The current state of breast cancer classification. *Annals of Oncology*, *23*(10), 1-4.

Vijayarathna, S., & Sasidharan, S. (2012). Cytotoxicity of methanol extracts of Elaeis guineensis on MCF-7 and Vero cell lines, *Asian Pacific Journal of Tropical Biomedicine 2*(10), 826–829.

Voigt, W. (2005). Sulforhodamine B assay and chemosensitivity. *Methods in Molecular Medicine*, *110*, 39–48.

Von Kitzing, E., Jonas, P., & Sakmann, B. (2006). The general transcription machinery and general cofactors. *Research in Veterinary Science*, *30*(3), 364-367.

Wang, G., Jiang, Q., & Zhang, C. (2014). The role of mitotic kinases in coupling the centrosome cycle with the assembly of the mitotic spindle, *Journal of cellsciences, 2014*(127), 1–12.

Wang, R. N., Green, J., Wang, Z., Deng, Y., Qiao, M., Peabody, M., Shi, L. L. (2014). Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes & Diseases*, *1*(1), 87–105.

Watchueng, J., & Gao, J. (2016). Efficient purification of paclitaxel from yews using high-performance displacement chromatography technique. *Journal of chromatography, 1218*(2011), 2929-2935.

World Health Organisation (WHO) (2004). Cancer the problem.1-2.

Williams, S. N., & Dienes, K. A. (2014). Sunscreen Sales , Socio-Economic Factors , and Melanoma Incidence in Northern Europe : A Population-Based Ecological Study. *SAGE journal,2014,*1-6

Wood, C. G., & Sandler, C. M. (2009). Papillary Renal Cell Carcinoma : Radio- relation and Spectrum, *Radiographics,* 2009(1), 741–755.

World Cancer Report. (2014). Press release Global battle against cancer won ' t be won with treatment alone Effective prevention measures urgently needed to prevent cancer crisis, (February), 3–4.

Wu, D., Zhang, S., Liu, Y., Wu, X., Zhu, G., Zhang, Y., Chen, A. (2015). Synthesis, Biological Activities, and Quantitative Structure–Activity Relationship (QSAR) Study of Novel Camptothecin Analogues. *Molecules,*2015(20),8634–8653.

Xiangming, H., Xiang, H., Zong, X., Yan, X., Yu, Y., Liu, G., & Zou, D. (2014). CDK2-AP1 inhibits growth of breast cancer cells by regulating cell cycle and increasing docetaxel sensitivity in vivo and in vitro. *Cancercell international 14*(130), 1–10.

Zainuddin, N. A. S. N., & Sul'ain, M. D. (2015). Phytochemical analysis , toxicity and cytotoxicity evaluation of dendropthoe pentandra leaves extracts, *International journal of applied biology and pharmaceutical technology,6*(1), 108–117.

Zerradi, M., Houle, F., & Huot, J. (2015). Regulation of Breast Cancer Progression by Phosphorylation of the Tumor Suppressor Tropomyosin-1 Alpha, (September), 783–792.

Zhang, Y., Bilbao, A., Bruderer, T., Luban, J., Strambio-De-Castillia, C., Lisacek, F., … Varesio, E. (2015). The use of variable Q1 isolation windows improves selectivity in LC-SWATH-MS acquisition. *Journal of Proteome Research*.

Zhu, K., Liu, Q., Zhou, Y., Tao, C., Zhao, Z., Sun, J., & Xu, H. (2015). Oncogenes and tumor suppressor genes : comparative genomics and network perspectives. *BMC Genomics*, *16*(7), 1-11.

Zhu, X., Chen, Y., & Subramanian, R. (2014). Comparison of information-dependent acquisition, SWATH, and MS(All) techniques in metabolite identification study employing ultrahigh-performance liquid chromatography-quadrupole time-of-flight mass spectrometry. *Analytical Chemistry*, *86*(2), 1202–9.

APPENDIX

Appendix ONE: Percentage Yield of the aqueous plant extracts

Table 13: The percentage yield of the aqueous plant extracts

Voucher number	Plant	Plant part	Initial mass (grams)	Final mass (grams)	% Yield
HS001	Ka-!Nomab	Root	10	0.94	9.4
HS002	≠Habas	Tuber	10	1.36	13.6
HS002	≠Habas	Whole plant	10	0.83	8.3
HS004	cf Salvadora persia	Root	10	0.82	8.2
HS006	!Kheape	Root	10	0.48	4.8

Table 14: The percentage yield of the ethanol plant extracts

Voucher number	Plant	Plant part	Initial mass (grams)	Final mass (grams)	% Yield
HS001	Ka-!Nomab	Root	10	0.49	4.9
HS002	≠Habas	Tuber	10	1.86	18.6
HS002	≠Habas	Whole plant	10	0.38	3.8
HS004	cf Salvadora persia	Root	10	0.71	7.1
HS006	!Kheape	Root	10	1.38	13.8

Appendix TWO: Bradford assay

The figure 36 below shows a linear regression plot for setting a standard to assume the overall relationship of at the 595 nm values (y-axis) versus their concentration in µg/ml (x-axis). Hence the unknown sample concentration was based on using the standard which shows concentration of proteins in the *Acanthosicyos naudinianus* treated TK-10 cell sample after extraction

	Diluted [ug/ul]	Undiluted [ug/ul]
CTRL 1	1.22	24.42
CTRL 2	1.33	26.54
CTRL 3	1.48	29.60
5ppm 1	1.29	25.82
5ppm 2	1.44	28.88
5ppm 3	1.57	31.30
10ppm 1	1.45	29.07
10ppm 2	1.30	26.03
10ppm 3	1.32	26.42

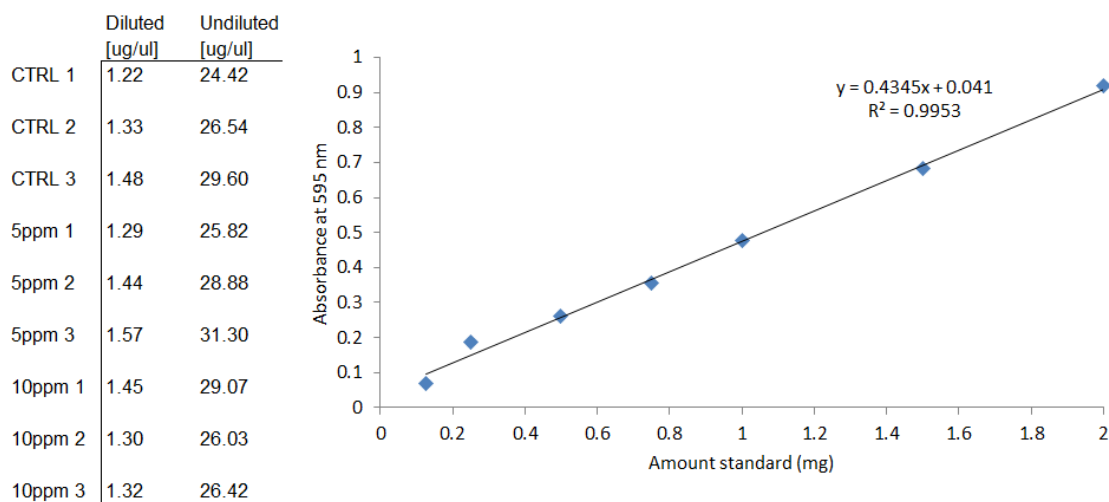$y = 0.4345x + 0.041$
$R^2 = 0.9953$

Figure 36:Bradford assay results samples post extraction

The figure 37 below shows a linear regression plot for setting a standard to assume the overall relationship of at the 595 nm values (y-axis) versus their concentration in µg/ml (x-axis). Hence the unknown sample concentration was based on using the standard which shows concentration of proteins in the *Acanthosicyos naudinianus* treated TK-10 cell sample after extraction, before acetone precipitation
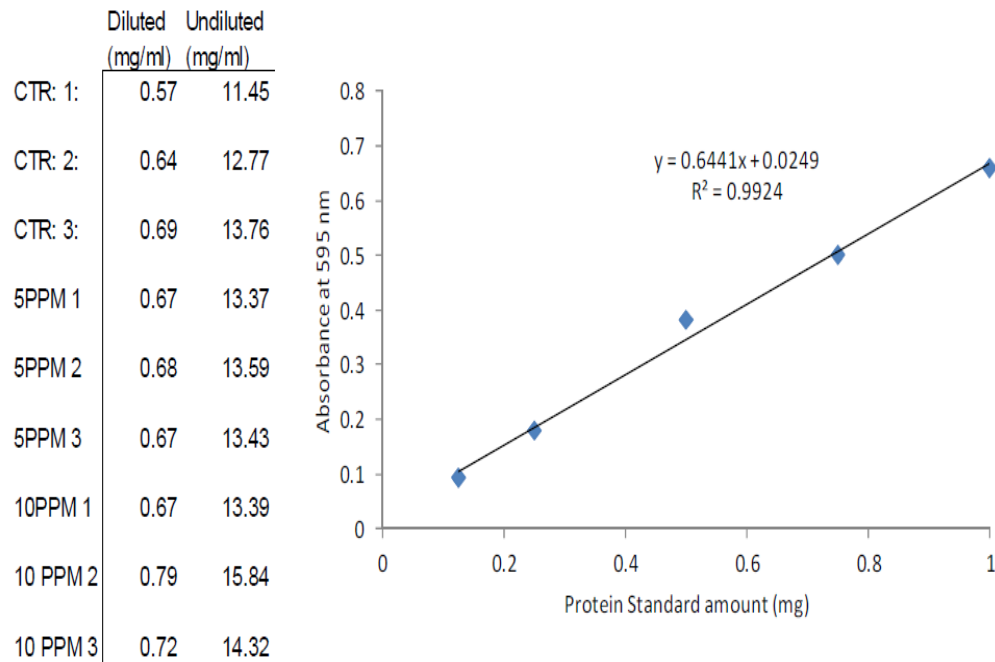
	Diluted (mg/ml)	Undiluted (mg/ml)
CTR: 1:	0.57	11.45
CTR: 2:	0.64	12.77
CTR: 3:	0.69	13.76
5PPM 1	0.67	13.37
5PPM 2	0.68	13.59
5PPM 3	0.67	13.43
10PPM 1	0.67	13.39
10 PPM 2	0.79	15.84
10 PPM 3	0.72	14.32

$y = 0.6441x + 0.0249$
$R^2 = 0.9924$

Figure 37: Bradford qualitative protein assay results of the samples post acetone precipitation

Bradford qualitative protein assay results are, based on the binding of Coomassie Brilliant Blue dye to a sample and comparing this binding to a standard generated by the reaction of known amounts of a standard protein, usually bovine serum albumin

Appendix THREE: Gene Ontology Analysis

Process involved in the potential anticancer mechanism of action of the aqueous *Acanthosicyos naudinianus* plant extract, on the TK-10 renal cancer cell line, as detected by the gene ontology analysis.
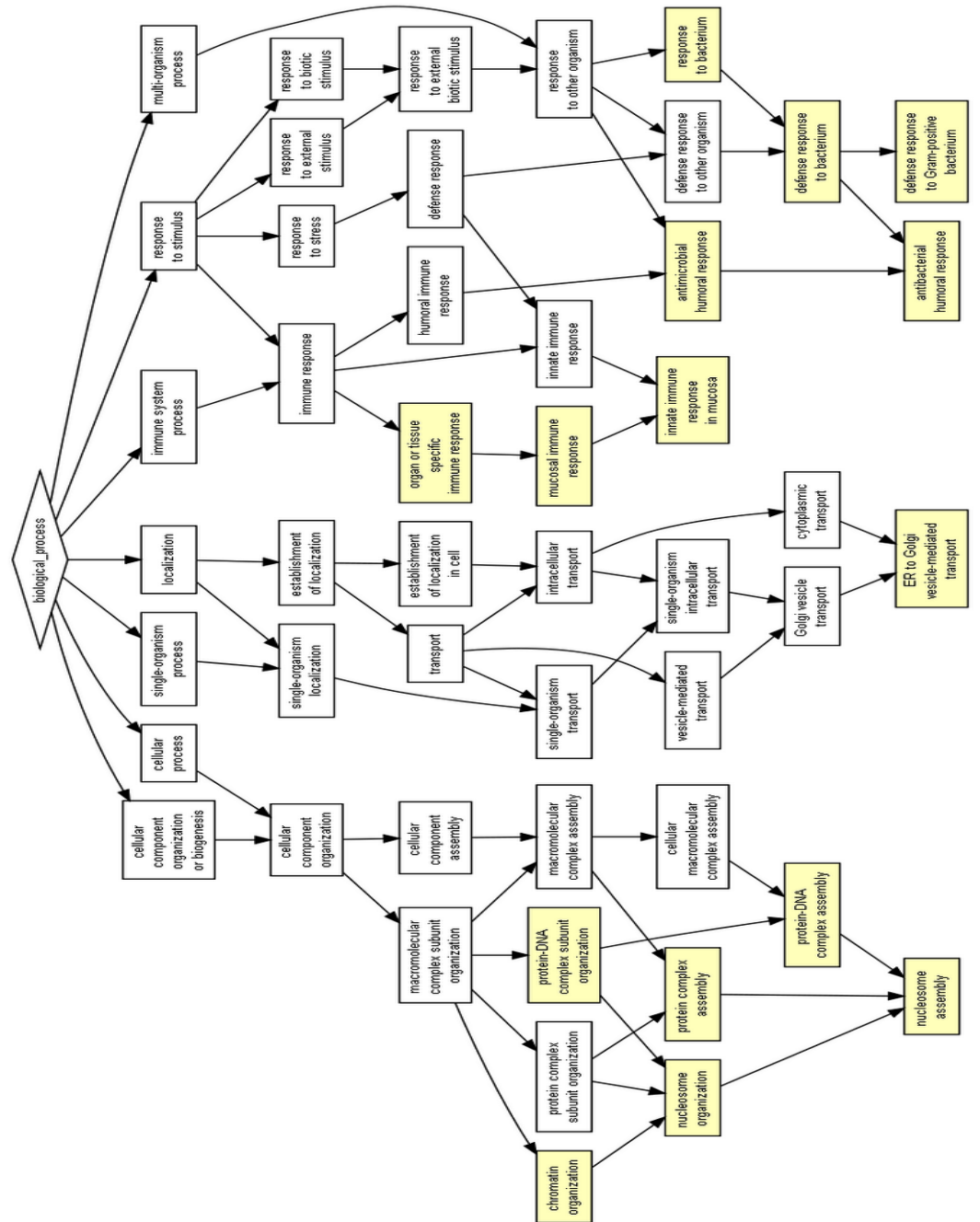
Appendix FOUR: Cytotoxicity Of Plants Extracts

The cytotoxicity of the HS001 aqueous root extract of *Ka !nomab* showed 49.70%

viability of the WI-38 cell line at an IC_{50} value of 12 µg/ml as depicted in figure 38

(A) below . While the cytotoxicity assay for the ethanol extract of *Acanthosicyos*

naudinianus   showed an IC_{50} value of 83.34µg/ml against the WI-38 cell line as can

be seen in figure 39 (B) below. Whilst the percentage WI-38 cell viability ranged

between 37.55 % and 108.48% derived from the IC_{50} values of 100µg/ml and 50µg/ml

of the HS001 *Acanthosicyos   naudinianus*   plant extract

(A)   **(B)**
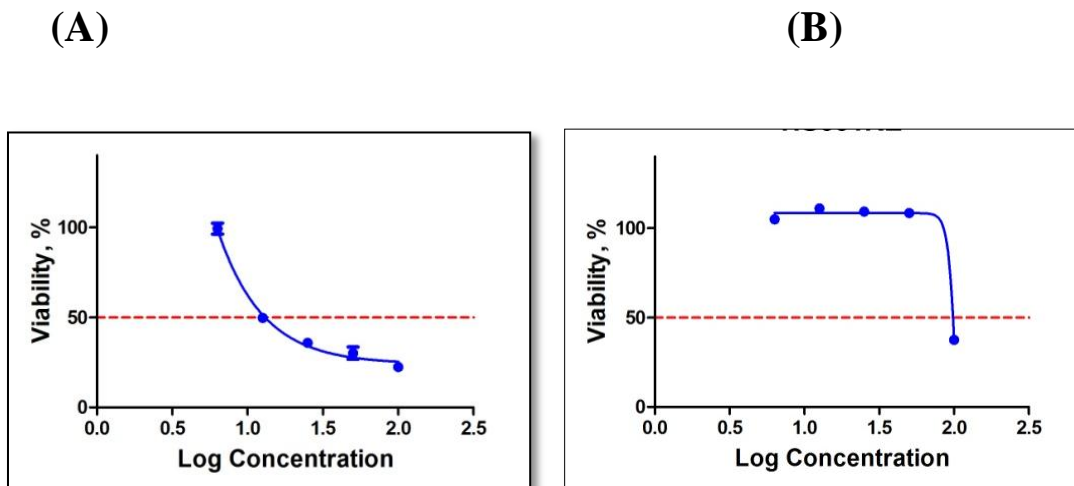

Figure 38 :The viability of WI-38 cell line after treatment with (A) aqueous root extract

of *Acanthosicyos   naudinianus*   and (B) root extract of *Acanthosicyos   naudinianus*

The cytotoxicity assay for the aqueous whole plant extract of *Fockea angustifolia* the

WI-38 cell lines showed an IC50 value of above 100µg/ml,as shown in the figure 40 (A)

below. Hence it also exhibited 71.35 % cell viability at 100µg/ml IC50 value.The ethanol whole plant extract of *Fockea angustifolia* showed and IC_{50} value that was above100µg/ml, similarly to the while the cell viability was 94.48% at100µg/ml, as depicted in the figure 39 (B) below.
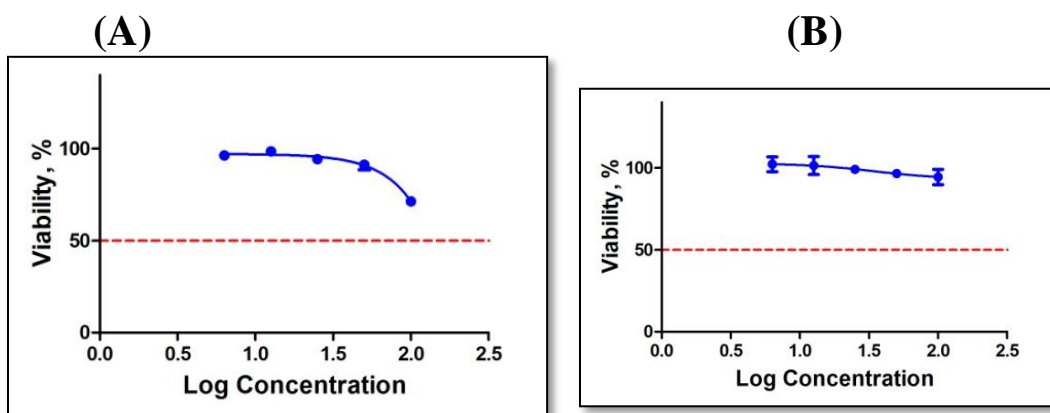

Figure 39: The viability of WI-38 cell line after treatment with (A) aqueous whole plant extract of *Fockea angustifolia* and the (B) ethanol whole plant extract of *Fockea angustifolia*

The aqueous tuberextract of *Fockea angustifolia* as seen in the figure 41 (A) showed cytotoxicity at an IC50 value above 100μg/ml. Furthermore at an IC50 value of 100μg/ml the cell viability of the WI-38 cell line was 72.87%. While the graph in figure 40 (B) above in figure indicates the outcome of the cytotoxicity assay for the ethanoltuberextract of *Fockea angustifolia* which shows that the WI-38 cell lines had an IC50 value of the plant extract was above 100μg/ml, whist the graph also depicts 81.18% cell viability at 100μg/ml of the plant extract.

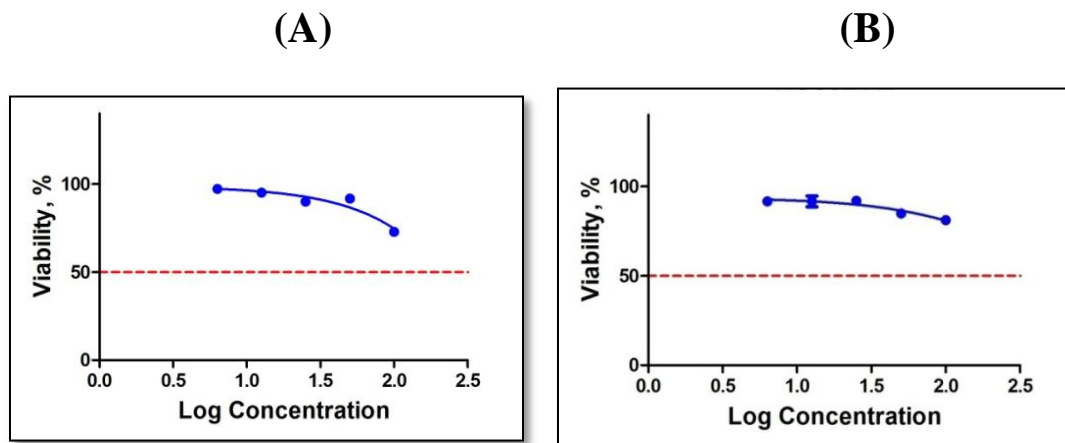**(A)**   **(B)**

Figure 40: The viability of WI-38 cell line after treatment with (A) aqueous tuber extract of *Fockea angustifolia* and the (B) ethanol tuber extract of *Fockea angustifolia*

The graph in figure 42(A) below shows evidence that the $\neq Nu \neq khoeb$ aqueous plant extract, against the WI-38 cell line, for the cytotoxicity assay indicated IC_{50} value above 100µg/ml. Since at 100µg/ml concentration of the *cf Salvadora persia* aqueous plant extract the WI-38 cell viability was 72.58%. However the cyotoxicity assay for the ethanol plant extract of*cf Salvadora persia* showed IC50 of37.88µg/ml against the WI-38 cell lines with a percentage cell viability between 17.76% -75.80% as can be seen in figure 41 (B) .
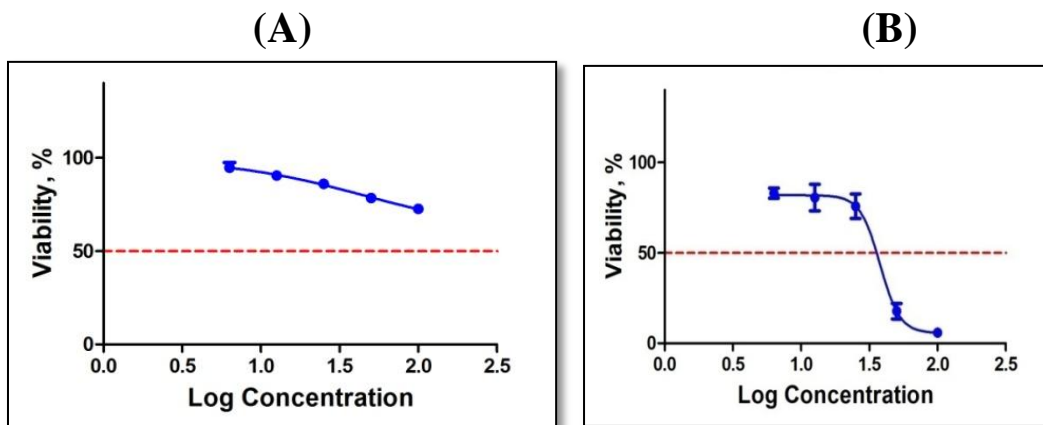
(A)   **(B)**

Figure 41: The viability of WI-38 cell line after treatment with (A) aqueous root extract *cf Salvadora persia* and the (B) ethanol root extract *cf Salvadora persia*

The aqueous plant extract of *Nymania capensis* root exhibited an IC_{50} value above 100µg/ml seen in figure 42 (A) below against the WI-38 cell lin. Whereas the percentage WI-38 cell viability for the IC_{50} value of 100µg/ml was 72.93 %. While the screen for the cytotoxicity of the ethanol root extract of the *Nymania capensis* plant against the WI-38 cell line showed an IC_{50} value above 100µg/ml in figure 42 (B) below. However at an IC50 of 100µg/ml the percentage cell viability of the WI-38 cell lines was 55.10%.
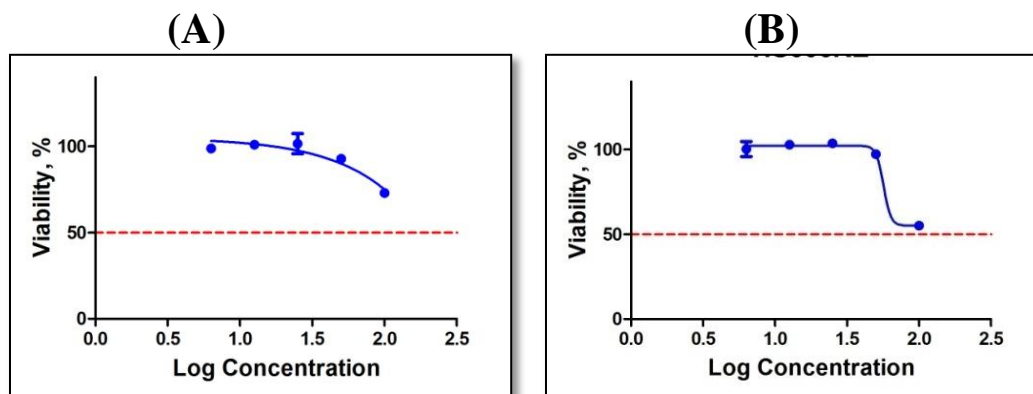

Figure 42: The viability of WI-38 cell line after treatment with (A) aqueous root extract *Nymania capensis* and the (B) ethanol root extract *Nymania capensis*

Appendix FIVE: Two-Sample Assuming Unequal Variances

Statistical evaluation of the antioxidant activity revealed the P values were determined using two-tailed test, for the plant extracts of *Acanthosicyos naudinianus , Fockea angustifolia cf Salvadora persia*and *Nymania capensis* plant.

Table 15: t-Test: Two-Sample Assuming Unequal Variances *Acanthosicyos naudinianus*

t-Test: Two-Sample Assuming Unequal Variances	*Acanthosicyos naudinianus*	*Control*
Mean	0.165	30.53
Variance	4.616E-05	0
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	-9993.678813	
P(T<=t) one-tail	3.0076E-16	
t Critical one-tail	2.131846782	
P(T<=t) two-tail	6.01519E-16	
t Critical two-tail	2.776445105	

Table 16: t-Test: Two-Sample Assuming Unequal Variances

for the *Fockea angustifolia*

t-Test: Two-Sample Assuming Unequal Variances		
	Fockea angustifolia	*Control*
Mean	17.91231	30.53
Variance	9.769973	0
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	-9.02648	
P(T<=t) one-tail	0.000417	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.000834	
t Critical two-tail	2.776445	

Table 17: t-Test: Two-Sample Assuming Unequal Variances cf *Salvadora persia*

t-Test: Two-Sample Assuming Unequal Variances		
	cf Salvadora persia	*Control*
Mean	17.8403	30.53
Variance	18.69028	0
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	-6.56339	
P(T<=t) one-tail	0.001394	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.002788	
t Critical two-tail	2.776445	

Table 18: t-Test: Two-Sample Assuming Unequal Variances of *Nymania capensis*

t-Test: Two-Sample Assuming Unequal Variances		
	Nymania capensis	*Control*
Mean	16.20048	30.53
Variance	4.306986	0
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	-15.4394	
P(T<=t) one-tail	5.14E-05	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.000103	
t Critical two-tail	2.776445	

Table 19: t-Test: Two-Sample Assuming Unequal Variances *Acanthosicyos naudinianus*

t-Test: Two-Sample Assuming Unequal Variances		
	Acanthosicyos naudinianus	*Control*
Mean	16.29934721	30.79
Variance	15.80783108	0
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	-8.149609689	
P(T<=t) one-tail	0.000616867	
t Critical one-tail	2.131846782	
P(T<=t) two-tail	0.001233733	
t Critical two-tail	2.776445105	

Table 20: t-Test: Two-Sample Assuming Unequal Variances *Fockea angustifolia*

t-Test: Two-Sample Assuming Unequal Variances	*Fockea angustifolia*	*Control*
Mean	14.38722	30.79
Variance	12.36483	0
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	-10.4306	
P(T<=t) one-tail	0.000239	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.000477	
t Critical two-tail	2.776445	

Table 21: t-Test: Two-Sample Assuming Unequal Variances *cf Salvadora persia*

t-Test: Two-Sample Assuming Unequal Variances	*cf Salvadora persia*	*Control*
Mean	13.18180016	30.79
Variance	19.83607604	0
Observations	5	5
Hypothesized Mean Difference	0	
Df	4	
t Stat	-8.840403309	
P(T<=t) one-tail	0.000451924	
t Critical one-tail	2.131846782	
P(T<=t) two-tail	0.000903848	
t Critical two-tail	2.776445105	

Table 22: t-Test: Two-Sample Assuming Unequal Variances *Nymania capensis*

t-Test: Two-Sample Assuming Unequal Variances		
	Nymania capensis	*Control*
Mean	11.47722	30.79
Variance	19.01928	0
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	-9.90222	
P(T<=t) one-tail	0.000292	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.000584	
t Critical two-tail	2.776445	

Appendix SIX: Post Normalization

Post Normalization the Total Area intensity of all conditions (Cntrl, 5µg/ml and10µg/ml) as well as their respective replicates align well (Figures below) The total Area intensity normalisation for allconditions and their respective replicates runs 1-3: Control 1-3; 4-6: 5µg/ml1-3; 7-9:10µg/ml 1-3.
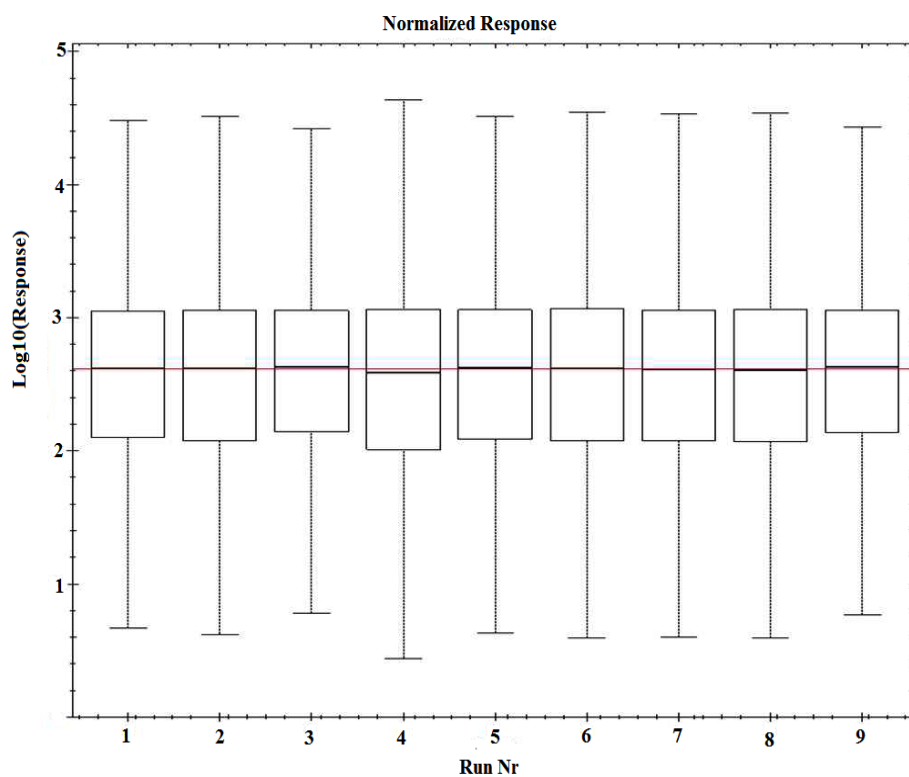
Figure 43: Total Area intensity normalization

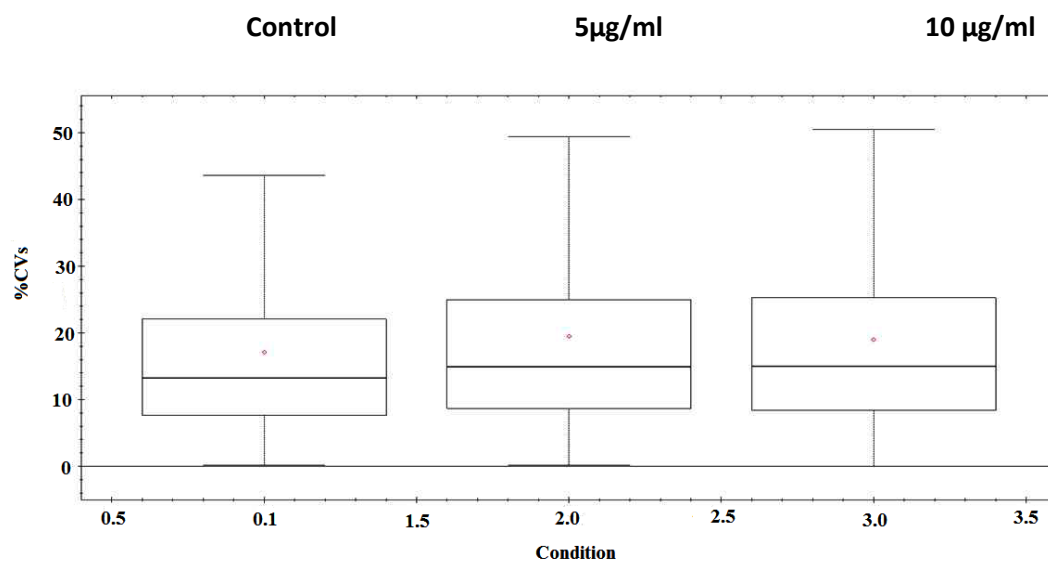Approximately 75% of the quantified peptides had a CV ≤ 25% between replicate runs see (Figure below).

Figure 44: : % CV distribution for Cntrl, 5μg/ml and 10μg/ml replicates

The sum quantity of P09493 protein at 5µ/ml in three replicates, alongside the controls to the sample is   illustrated in figure 46. The two of the replicates of 5µ/ml namely R1 and R3 have sum quantity in the range approximately at $25x10^3$ while R2 the second replica had a sum quantity of protein P09493 at approximately $20x10^{3.}$ However the protein P09493 sum quantity in the control sample remains below $15x10^3$ for all three replicates, control R1, R2 and R3,
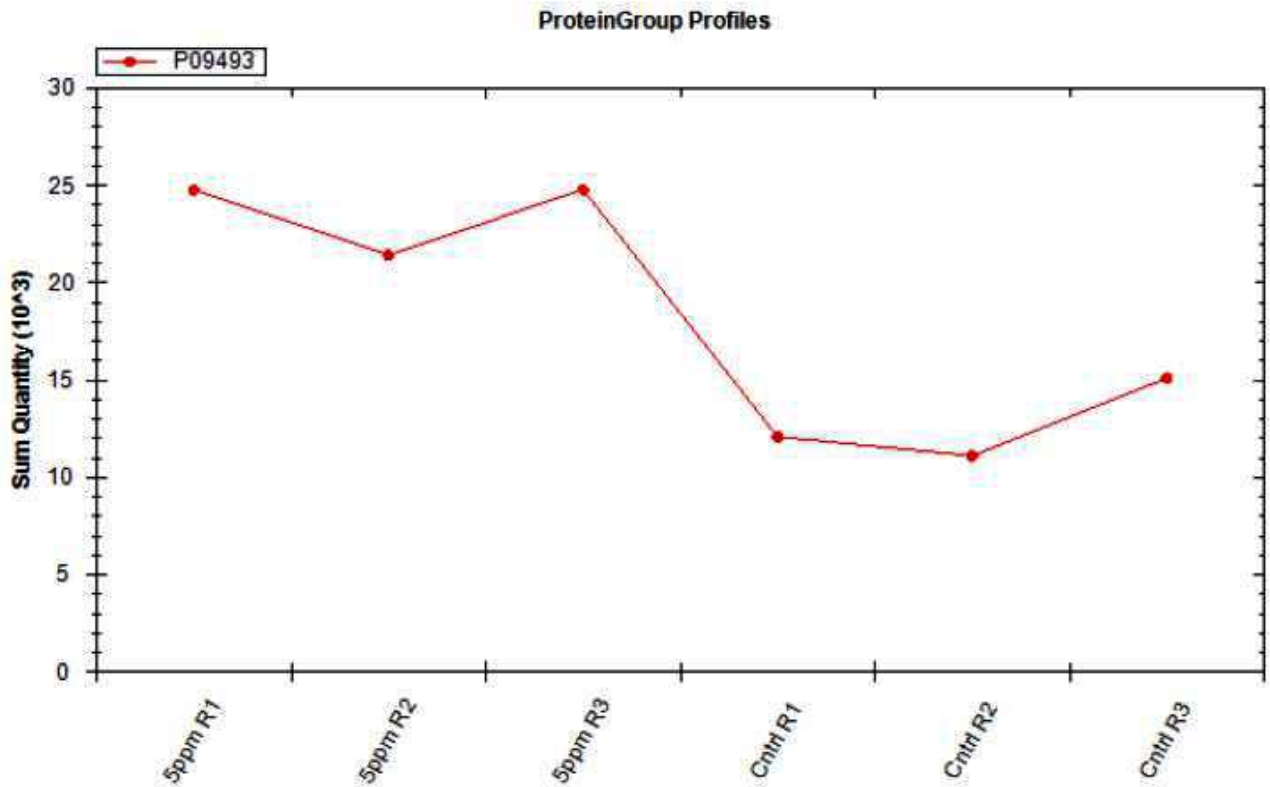
Figure 45: Sum quality for the P09493 protein profile

The sum quantity of P09493 protein at 10μg/ml in three replicates runs, including the sample control, is illustrated in the graph below. The 10μ/ml of the sample and the samples control also has three replicate runs seen in figure 47. The 10μ/ml concentration of the sample has the highest sum quantity at $28x10^3$ since two of the replicate runs namely R1 and R3 fall within this range . While the second replicate run for 10μ/ml concentration R2 had a sum quantity of protein P09493 at approximately $25x10^3$. However the replicate runs for the control sample for profiling protein P09493 the sum quantity   remains below $15x10^3$, with one of the control R3 falling in the range of $15x10^3$ and the remaining two replicates runs for the control R1,and R2 at a quantity of about $10x 10^3$.
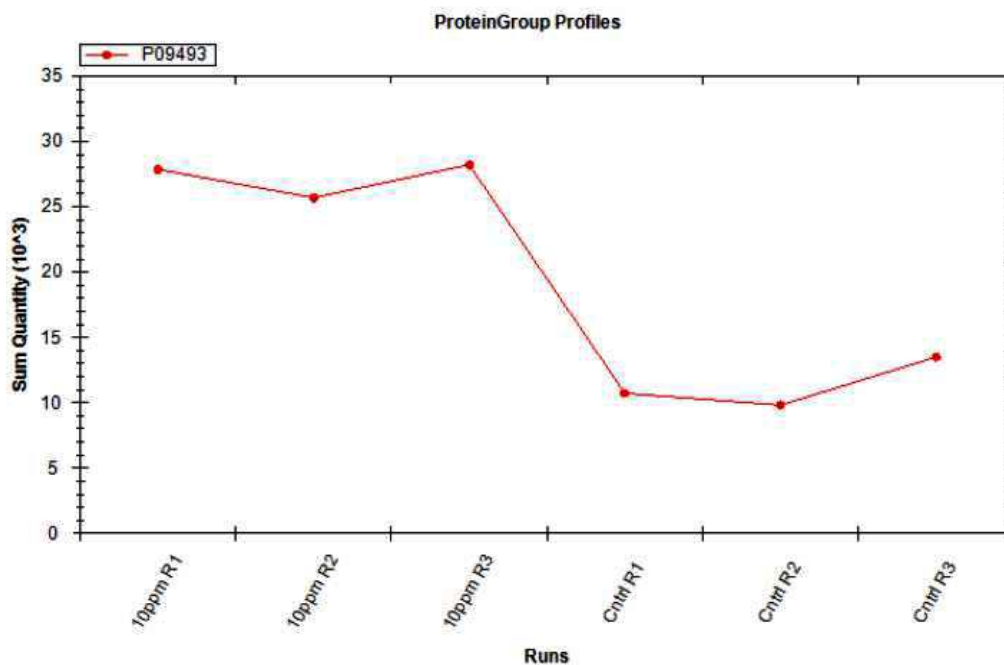

Figure 46:Sum quality for the P09493 protein profile

The graph in figure 48 demonstrates the sum quantity of the Q562R1protein at 5µ/ml in three replicates, alongside the controls to the sample. The first two of the replicates of 5µ/ml namely R1 and R2 shows the sum quantity of the protein ranging approximately at $25x10^2$ while the third replicate R3 had a sum quantity of protein at approximately $30x10^2$ higher quantity then the first two runs of 5µ/ml concentration f the sample . The protein Q562R1 sum quantity in the control sample runs remains below $15x10^2$ for two of the replicates, the control replicate one and two while three exhibited a protein quantity higher than the first two replicates Control R1 and R2 and a protein sum quantity of about $23x10^2$.
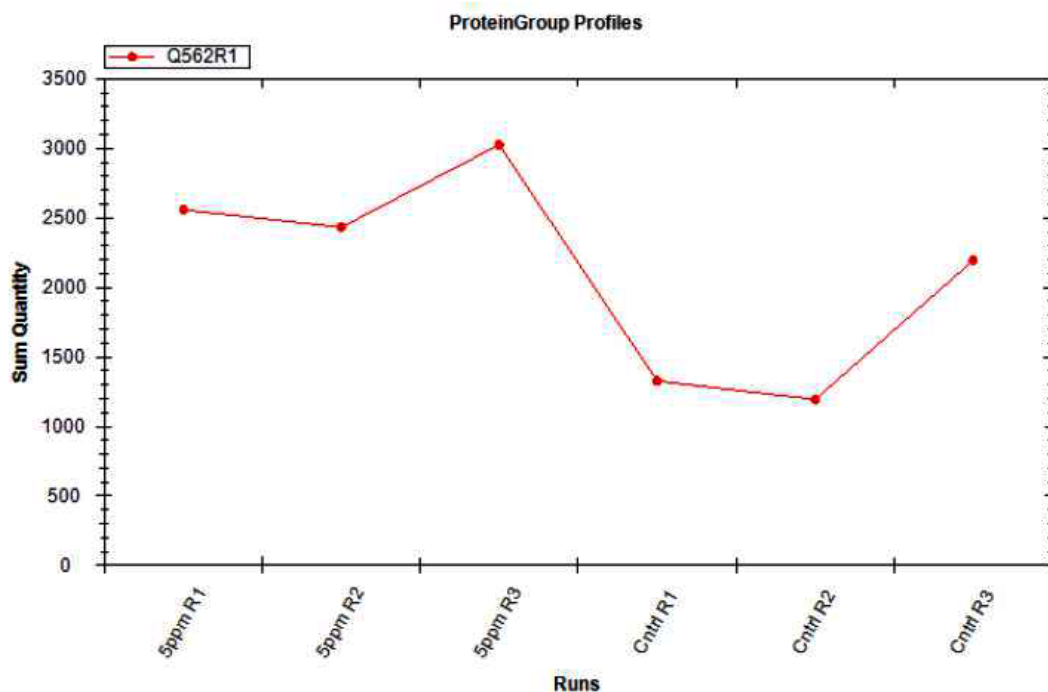

Figure 47: Sum quality for the Q562R1protein profile

The graph in figure 49 demonstrates the sum quantity of the Q562R1protein at 10µ/ml in three replicates, alongside the controls to the sample. The replicates of 10µ/ml R1had a sum quantity of the protein ranging approximately at $25x10^2$ whileR2 shows a sum quantity of $28x10^2$. The third replicate R3 had a sum quantity of protein at approximately $32x10^2$ higher quantity then the first two runs of 10µ/ml concentration of the sample . The protein Q562R1 sum quantity in the control sample runs remains below $15x10^2$ for the first two of the replicates. While run three exhibited a protein quantity higher than the first two replicates Control R1 and R2 and a protein sum quantity of about $23x10^2$.
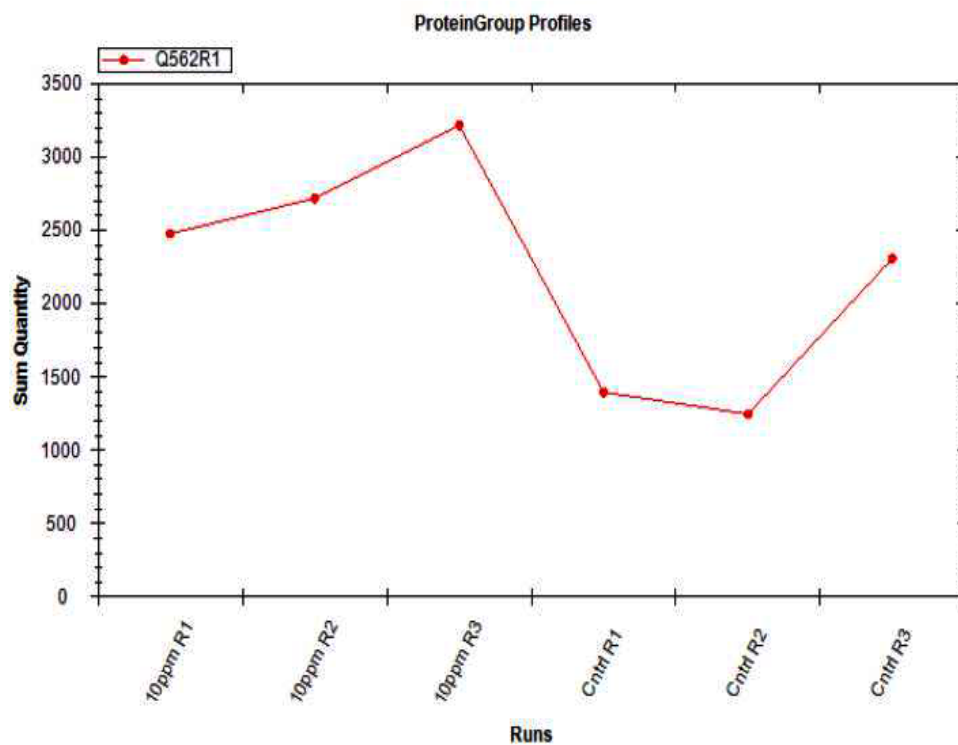

Figure 48: Sum quality for the Q562R1 protein profile

The protein group profile on the Q5TZA2, as can be seen in figure 50 shows the quantity of the protein in the 5μg/ml concentration of the sample in three replicate runs. The sum quantity of the protein ranged between 32×10^2 and 36×10^2. While all three of the control sample replicate runs showed a higher protein sum quality compared to the 5μg/ml concentration .One of the control replicates R2 has a Q5TZA2 protein quantity of   about 54×10^2 while the first and the third (R1 and R3) replicate runs for the control showed a protein sum quantity of about $58 \times 10^{2.}$
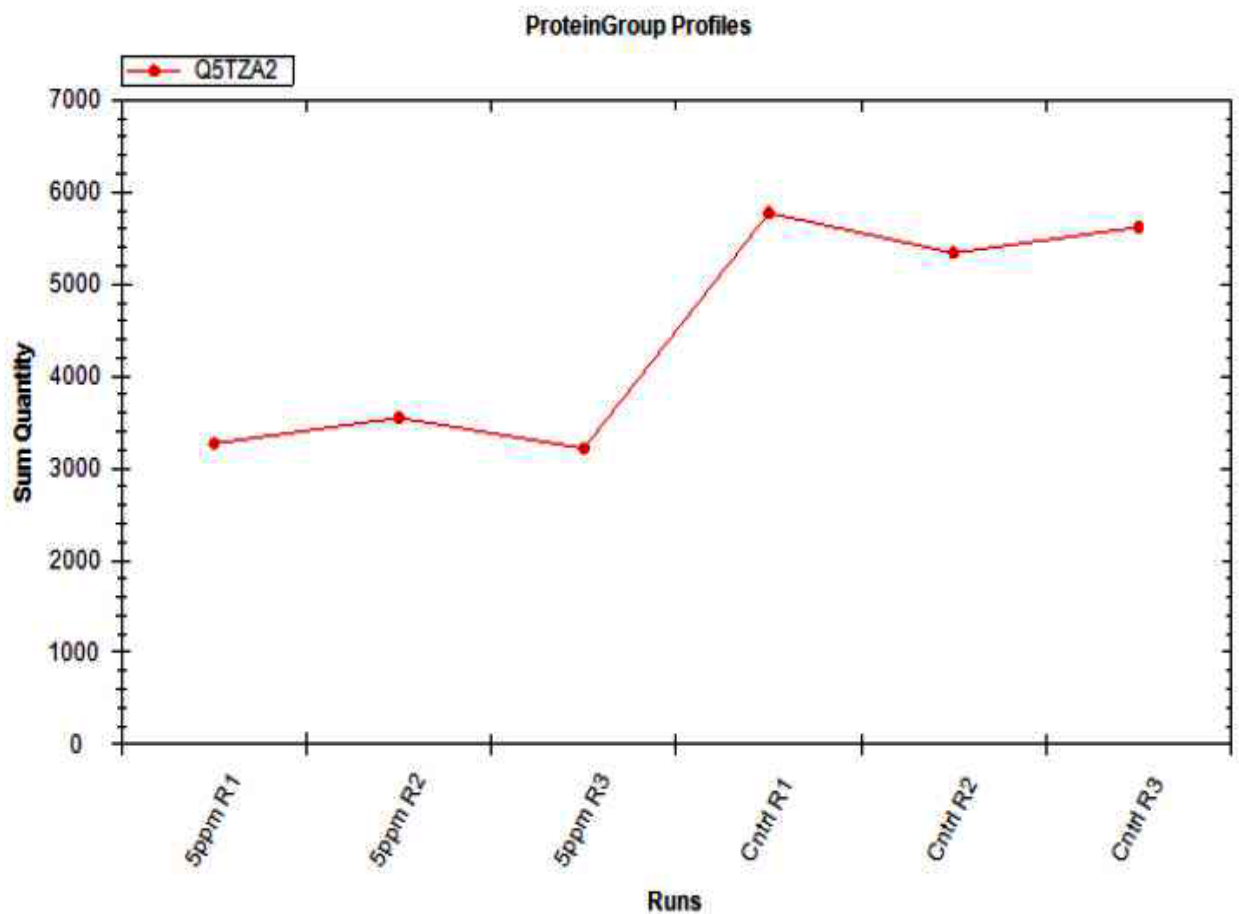

Figure 49: Sum quality for the Q5TZA2 protein profile

The average sum quantity of the P84550 protein at 5µ/ml in three replicates, alongside the controls is demonstrated in figure 51. The 5µg/ml sample run had an average sum quantity of about $25x10^3$, while the 10µg/ml had a sum quantity of protein at approximately $30x10^3$ higher quantity then the first concentration of 5µ/ml of the sample The protein P84550 sum quantity in the control sample runs was below at about $20x10^3$.
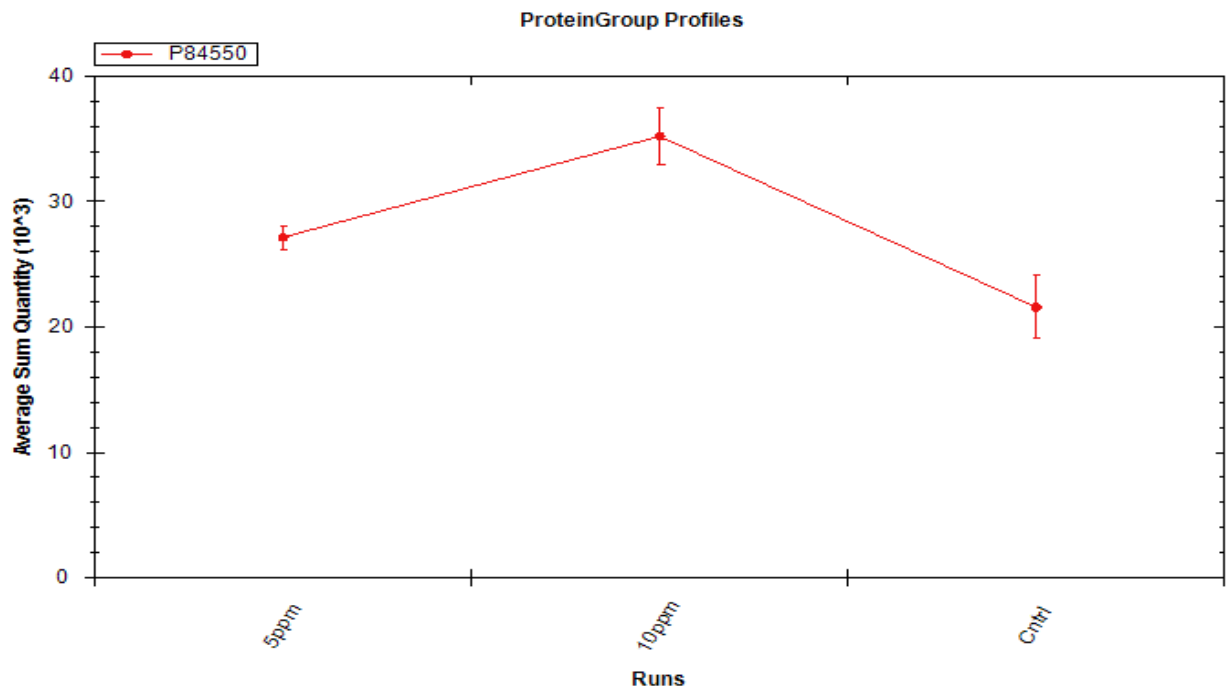

Figure 50: Sum quality for the P84550 protein profile