EVALUATION OF PHARMACOLOGICAL PROPERTIES OF TRADITIONAL MEDICINAL PLANTS USED FOR THE TREATMENT OF CANCER BY SOUTH AFRICAN AND LESOTHO COMMUNITIES

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May 2019

DECLARATION OF INDEPENDENT WORK

DECLARATION WITH REGARD TO

INDEPENDENT WORK

I, POLO-MA-ABIELE HILDAH MFENGWANA, identity number and student number , do hereby declare that this research project submitted to the Central University of Technology, Free State for the Degree DOCTOR OF HEALTH SCIENCE: BIOMEDICAL TECHNOLOGY, is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself of any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

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Evaluation of pharmacological properties of traditional medicinal plants used for the treatment of cancer by South African and Lesotho communities

SUMMARY

The aim of this study was to assess *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* which are used to treat cancer as antibacterial, antioxidant, anti-inflammatory, anticancer and antimutagenicity agents. Medicinal plants extracts were prepared through maceration with methanol, water, Dichloromethane (DCM), methanol: dichloromethane and hexane as solvents. Secondary metabolites were assessed qualitatively, while antibacterial activities were determined using the disk diffusion assay, and the 2, 2- diphenyl-1-picrylhydrazyl (DPPH) assay was used to evaluate antioxidant activity. The lipopolysaccharide (LPS) stimulated RAW 264.7 mouse macrophage *in vitro* model was used to evaluate the anti-inflammatory activity of these three plants, with resveratrol used as a positive control. All extracts were tested *in vitro* for growth inhibitory effects against human prostate cancer (PC3), human breast cancer (MCF-7) and the non-cancerous African green monkey kidney (Vero) cell lines using the MTT assay. Cell cycle analysis was performed on two extracts (methanol and dichloromethane) with selective anticancer properties to elucidate their mechanism of action. Mutagenic and antimutagenic properties were determined by the Comet assay using hepatic cells, and Vitotox test using *Salmonella typhimurium* genotoxicity (*rec*N2-4) and cytotoxicity *(pr1)* strains, with and without S9 metabolic activation. To describe distinctive chemical features of *Asparagus laricinus* and *Senecio asperulus* crude extracts, Liquid chromatography-mass spectrometry (LC-MS) analysis was performed.

Asparagus laricinus inhibited bacterial growth of all selected micro-organisms. However, *Staphylococcus* species were resistant even at 500 µg/ml. Methanol extracts of *Senecio asperulus* inhibited microbial growth at the concentration of 50 µg/ml. The dichloromethane extract of *Senecio asperulus* was active on most bacteria with MICs between 50 µg/ml and 500 µg/ml. Nonetheless, the water and methanol extracts of *Gunnera perpensa* had no activity against all organisms tested. Methanolic

and aqueous extracts of *Asparagus laricinus* showed good antioxidant activity. Aqueous extracts of *Senecio asperulus* and *Gunnera perpensa* showed free radical scavenging activity yielding EC_{50} values of 100 µg/ml and 25 µg/ml, respectively. The aqueous extracts of *Senecio asperulus* showed moderate anti-inflammatory activity from 50 to 200 µg/ml, while the methanol extracts were active at 200 µg/ml and with no cytotoxicity. *Asparagus laricinus* showed weak anti-inflammatory activity when compared with resveratrol. No anti-inflammatory activity was observed from all *Gunnera perpensa*, suggesting that this species may be using other mechanisms for anti-inflammatory activity.

Asparagus laricinus methanol and *Senecio asperulus* dichloromethane extracts exhibited cytotoxicity activity against breast cancer cells with IC_{50} values of 97.6 µg/mL and 69.15 µg/mL, respectively. *Gunnera perpensa* DCM extract also showed cytotoxicity on prostate and breast cancer cells, with less activity on Vero cells. Cell cycle analysis suggested that *Asparagus laricinus* methanol extract induced cell death selectively through apoptosis observed from Annexin V-FITC and PI stain. Cell cycle analysis also showed that *Senecio asperulus DC.* dichloromethane extracts induced breast cancer cells death through cell arrest at the synthesis phase and G2 phase. *Senecio asperulus* dichloromethane extracts further showed cytotoxicity activity against prostate cancer cells with IC_{50} values of 69.25 µg/mL due to cell arrest at the G2 and early mitotic (G2/M) phase. The results obtained from the Vitotox test for *Asparagus laricinus, Senecio asperulus,* and *Gunnera perpensa* correlated well with results obtained from NRU and Comet assays. All the tested extracts had no toxicity and genotoxicity *in vitro*, except the dichloromethane extracts from *Senecio asperulus* and *Gunnera perpensa* which were found to be cytotoxic but not genotoxic after S9 metabolic activation. None of the tested extracts had antigenotoxic properties.

Gunnera perpensa extracts showed more polyphenols than *Asparagus laricinus* and *Senecio asperulus*. The LCMS results of these plant extracts showed the presence of twelve known compounds such as *Luteolin 7-O-rutinoside,* and *Rutin, and* twenty-eight unknown compounds from *Asparagus laricinus. Senecio asperulus* had nineteen unknown compounds and two known compounds; *chlorogenic acid isomer,* and *dicaffeoyl quinic acid*.

Deductions made from the anticancer analysis propose that the methanol extract of *Asparagus laricinus* is a suitable aspirant for future breast cancer chemotherapeutic drug, due to its selective cytotoxicity on cancer cells and not on non-cancerous cells. Furthermore, the anticancer activities of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* observed were not from the protection against genotoxicity as none of these plants had genotoxic properties. Moreover, the negative genotoxicity highlighted the safe use of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* as medicinal plants. Results from this study were encouraging; especially for *Asparagus laricinus* as a potential anti-breast cancer agent, despite the need for clinical studies to confirm the pharmacological activities of these plants and toxicological effects *in vivo*.

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LIST OF ABBREVIATIONS

µL: Microlitre

µM: Micromolar

ATCC: American Type Culture Collection

CO_2: Carbon dioxide

COX-2: Cyclooxygenase 2

DCM: Dichloromethane

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

DPPH: 2,2-diphenyl-1picrylhyrazyl

EC_{50}: Half maximal effective concentration

IF-γ: Interferon gamma

IL-1: interleukin-1

iNOS: induced nitric oxide synthesis

LP: lipoxygenase

LPS: lipopolysaccharides

MeOH: Methanol

MIC: minimum inhibition concentration

mM: Millimolar

MTT: 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide

nm: Nanometre

NOS: Nitric oxide

NSAIDs: Non-steroidal anti-inflammatory drug

ROS: reactive oxygen species

TCA: Trichloroacetic acid

w/v: Weight per volume

LIST OF APPENDICES

PUBLICATIONS FROM THIS THESIS

- Mfengwana PH, Mashele SS, Manduna IT. 2019. Cytotoxicity and cell cycle analysis of *Asparagus laricinus Burch.* and *Senecio asperulus DC.* on breast and prostate cancer cell lines. Heliyon. 5: e01666. https://doi.org/10.1016/j.heliyon.2019.e01666

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- Genotoxicity and anti-genotoxicity evaluation of *Asparagus laricinus Burch., Senecio asperulus DC.,* and *Gunnera perpensa L.* (Submitted to Archives of Toxicology)

CONFERENCE PRESENTATIONS

- *Presentation of a paper (National):* Anticancer screening of Asparagus laricinus and Senecio asperulus plant extracts, PH Ntsoelinyane and SS Mashele, The IPUF 2018 conference that took place from 01 July - 04 July 2018.

- *Presentation of a paper (National):* Potential anti-cancer activities and mechanisms of Asparagus laricinus and Senecio asperulus on prostate and breast cancer cell lines, PH Mfengwana and SS Mashele, Faculty of Health and Environmental

Sciences Prestige Research Day that took place at the Central University of Technology, Free State from 30 October 2018.

- *Presentation of e-poster (International): In vitro* Investigation of Genotoxic and Antigenotoxic Properties of *Gunnera perpensa* Roots Extracts, PH Mfengwana, L Verschaeve, R Anthonissen, IT Manduna. SS Mashele and IT Manduna, the 2nd World Academy of Science, Engineering and Technology held in London, the UK from 14-15 February 2019.

CHAPTER 1

"General background"

1.1 INTRODUCTION

The alliance between humans and plants originates from the beginning of the earth when plants supplied oxygen, food, shelter and medicine needed by higher life forms. Over time, human learned to categorize plant materials for life necessities such as their use as medicine for healing and prevention of diseases (Fabricant and Farnsworth, 2001). Moreover, through personal experience and knowledge passed down for generations, indigenous people have learned which species of plants may help alleviate certain ailments such as toothaches, induce labor, or cure malaria (Moteete and van Wyk, 2011). Even though plants have been in existence and been used for thousands of years, they still continue to provide mankind with new remedies due to their limitless possession of therapeutic properties. Also, to be noted, although medicinal plants have been used for ages, their healing power was considered as un-codified data as the knowledge was passed verbally from generation to generation without proper recording and their activity not being scientifically proven.

However, in 1994, the Dietary Supplement Health and Education Act (DSHEA) was passed which allowed any substance that can be found naturally to be sold as a "dietary supplement" regardless of its concentration or potential hazards (WHO, 2008). Although some authors have proven some of the therapeutic properties from plants to be erroneous, the use of plants nevertheless continues to increase because people believe they are safe as they are very close to nature. Unfortunately, many laities and physicians are unaware of the toxic potential of many plant species and therefore, they may be endangering their health instead of promoting it with the use of medicinal plants. Thus, not all-natural medicinal plants are safe as their users assume because some of their active ingredients such as diester diterpene alkaloids can be potentially toxic (Xu et al., 2005).

Africa has rich plant biodiversity, with over 20 000 different species which have not been explored and is a great source of interest to the scientific community (Cherry, 2005). Currently, around the world, interest in traditional medicinal plants has been revived with new enthusiasm to explore the hidden truths and to fully utilize the healing properties of medicinal plants. Recent scientific validation of traditional medicinal plants has changed the view of scientists about the effects of natural medicinal plants and has led to the establishment of guidelines for the assessment of medicinal plants with an aim of defining standard basic criteria for the evaluation of quality, safety and efficacy of medicinal plants (WHO, 2011). These assessment guidelines include the evaluation of the effect of the crude extract, the preparation of extracts, and biological activity of extracts (Kamboj, 2000). These studies help with the propagation of traditional medicine systems and drug development processes. Thus, there is a need to investigate the pharmacological activities, safety, and efficacy of medicinal plants.

1.2 LITERATURE REVIEW

1.2.1 Usage of traditional medicinal plants

Traditional medicine is defined as the entirety of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures which incorporates plant-based medicine therapy, used for the maintenance of health, prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2006). Traditional use of medicinal plants refers to the historical use of medicinal plants. In some Asian and African countries, up to 80% of the population relies on traditional medicine for their primary health care needs, particularly in rural areas, where it is the only available, accessible and affordable source of the health care system (Mohamoodally, 2013). Practices known as traditional medicines include Ayurveda, Siddha medicine, Unani, ancient Iranian medicine, Islamic medicine, traditional Chinese medicine, traditional Korean medicine, acupuncture, and traditional African medicine. Of which traditional African medicine is defined as an alternative medicine discipline that involves indigenous herbalism and African spirituality. Traditional African medicine comprises of several types of traditional healthcare practitioner which include: diviners, bone throwers, seers, veterinarians, the

sangomas (traditional healers), midwives, and herbalists (Moteete and van Wyk, 2011).

In the past, traditional healers or traditional medicine knowledge holders were not willing to share their knowledge on medicinal plants with strangers due to ritual and religious controls. The knowledge would be verbally passed from generation to generation passively to family members only or through a healer's initiation school accessed via spiritual calling. This lack of documentation led to the loss of valuable information when the knowledge holder passes on. Healers would hold on to the information and share very little information about medicinal plants. However, in recent years, there have been so many ethnobotanical studies that have been successfully completed in Africa. Thus, more information on the use of medicinal plants is now available in accredited journal articles and even on blogs with attempts to provide accurate sources of information regarding botanical medicines and their uses. Many African countries have now developed national herbal pharmacopoeias to document medicinal plants that have been found to be effective and to further ensure their safety, efficacy, and quality (Brendler et al., 2010).

In many developing countries, especially Lesotho, a large proportion of the population rely on plants for their primary health care with about 35,000 to 70,000 species being used as medicaments (WHO, 2011). In Africa, although modern medicine exists side-by-side with traditional practice, traditional medicines often maintained their popularity for historical and cultural reasons. Thus, even urban area populations continue to use traditional medicinal plants as they form part of their culture. Willcox and Bodeker (2000), have reported less frequent adverse effects in patients using traditional medicinal plants when compared with patients using allopathic medicine. However, the remaining mutual doubt between allopathic and traditional practitioners in Africa has continuously hampered and opposed the process of integration between traditional and modern medicines (Nevin, 2001).

The 1994 DSHEA Act allows traditional medicine to be sold as dietary supplements and such products have become more widely available commercially, especially in developed countries (WHO, 2006). In this modern setting, however, some ingredients are sometimes marketed for uses that were never contemplated in the traditional

healing systems from which they emerged. This is because one medicinal plant can have a variety of phytochemicals which may have other pharmacological activities besides those known by the community of which the plants traditional medicinal indications information emerged from. Thus, with modern diseases, and plant evolution (Hart, 2005), some active ingredients can be active against conditions which were not recorded ethnobotanically. In some instances, plants, such as *Plantago ovata* can be used to treat diarrhea and that same plant can also be used to relieve constipation. Moreover, in the oldest Indian medicine system focusing on the treatment of chronic diseases (Siddha medicinal system), poisonous plants such as *Abrus precatorius,* are used to cure some diseases (Narayanaswamy et al., 2014; Tamilselvan et al., 2014) as they have compounds which can act as potential therapeutic agents.

1.2.2 Medicinal plants as a source of drugs

One of the important characteristics of African countries flora is the abundance of medicinal plants. Besides the use of natural plants as food and shelter by humans, plants have been used as medicine to alleviate and treat diseases. Plants are also used as flavors, insect deterrents, ornamentals, fumigants, spices, and cosmetics (Pieroni et al., 2004). As humanity evolved, they invented fire using woods from plants, learned how to make alcohol using plants (such as *Raphiocname hirsuta* in Lesotho), developed religions, and made technological breakthroughs, and learned to develop novel drugs from plants (Hart, 2005). Moreover, natural products have evolved over millions of years and now have unique chemical varieties that result in diversity in their biological activities and drug-like properties. Those products have become the most important resources for new lead compounds in drug development. However, these vast and diverse varieties of organic compounds (phytochemicals) are originally synthesized by plants during their metabolism process for protection against damaging and rapidly changing external environment conditions and not for drug development.

Plants are a rich source of phytochemicals which are also known as secondary metabolites and possess unique therapeutic properties. Secondary metabolites are intermediates and products of plant metabolism. Furthermore, plants produce different classes of secondary metabolites which are restricted to specific plants groups.

Metabolites have numerous functions to the plant which include: signaling, defense, fuel, structure, stimulatory and inhibitory effects on enzymes, the catalytic activity of their own enzymes (usually as a cofactor to an enzyme), and interactions with other organisms. However, humans exploit these secondary metabolites for their benefits in different fields including medicinal properties. When medicinal plants are used for new drug development, they have their unparalleled advantages, such as their unique diversity of chemical structures and unique biological activities. Thus, current research in drug discovery from medicinal plants involves a multi-layered approach which combines botanical, phytochemical, biological and molecular techniques. Moreover, current research findings on secondary metabolites highlight the variation in metabolites compositions in plants. Thus, showing the overlapping of boundaries of different research fields like biochemistry, molecular biology, plant physiology and ecology (Kala et al., 2006).

Numerous biological active molecules such as anticancer, antimicrobial, anti-inflammatory and antiviral compounds have been discovered and developed from traditional medicinal plants. Novel plant-derived compounds from plants have been used as drugs either in their original form or semi-synthetic form. Natural products play a leading role in drug discovery and are undergoing incessant use towards meeting the crucial need to develop novel effective drugs (Taiz and Ziegler, 2006). According to Cragg and Newman (2000), over 50% of the drugs in clinical trials are isolated from natural sources. More than 20 000 different species used in traditional medicine throughout Africa have not been explored (Cherry, 2005). Thus, they are a great source of interest to the scientific community as they form an enormous pool of potential for new drugs. Chemical synthesis can be performed to provide compounds or derivatives with similar uses besides direct extraction from plants. This approach is used to resolve the shortage of supply due to the low yield of compounds from plants.

Several commercial drugs have been derived from medicinal plants since 1805 when the first pharmacologically-active compound morphine, which is a very potent painkiller was isolated from *Papaver somniferum*. Since then, more drugs have been derived from medicinal plants in the past. Tables below will be highlighting chemicals or drugs with different pharmacological activities and all were derived from medicinal plants (Balunas and Kinghorn, 2005; Cragg et al 2005). In **Table 1.2.2.1** are drugs used for

cancer and in **Table 1.2.2.2** is a list of drugs used for inflammation and related conditions. Drugs listed in both tables are derived from plants.

Table 1.2.2.1: *Drugs derived from medicinal plants and used to treat cancer and malaria*

Drug/Chemical name	Plant source	Clinical uses/action
Betulinic acid	*Betula alba*	Anticancerous
Camptothecin	*Camptotheca acuminata*	Anticancerous
Colchiceine amide	*Colchicum autumnale*	Antitumor agent
Colchicine	*Colchicum autumnale*	Antitumor, antigout
Demecolcine	*Colchicum autumnale*	Antitumor agent
Etoposide	*Podophyllum peltatum*	Antitumor agent
Lapachol	*Tabebuia species*	Anticancer, antitumor agent
Monocrotaline	*Crotalaria sessiliflora*	Topical antitumor agent
Podophyllotoxin	*Podophyllum peltatum*	Antitumor, anticancer agent
Taxol	*Taxus brevifolia*	Antitumor agent
Teniposide	*Podophyllum peltatum*	Antitumor agent
Topotecan	*Camptotheca acuminata*	Antitumor, anticancer agent
Vinblastine	*Catharanthus roseus*	Antitumor, Antileukemic agent
Vincristine	*Catharanthus roseus*	Antitumor, Antileukemic agent
Quinine	*Cinchona ledgeriana*	Antimalaria agent

Table 1.2.2.2: *Drugs derived from medicinal plants and used for inflammation and related conditions*

Drug/Chemical name	Plant source	Clinical uses/action
Aescin	*Aesculus hippocastanum*	Anti-inflammatory
Allantoin	*Symphytum officinale*	Vulnerary
Allyl isothiocyanate	*Brassica nigra*	Rubefacient
Asiaticoside	*Centella asiatica*	Vulnerary
Borneol	*Cinnamomum camphora*	Antipyretic, analgesic, anti-inflammatory
Bromelain	*Ananas comosus*	Anti-inflammatory, proteolytic
Camphor	*Cinnamomum camphora*	Rubefacient
Codeine	*Papaver somniferum*	Analgesic
Ephedrine	*Ephedra sinica*	Sympathomimetic, antihistamine
Menthol	*Mentha species*	Rubefacient
Methyl salicylate	*Gaultheria procumbens*	Rubefacient
Morphine	*Papaver somniferum*	Analgesic
Nordihydroguaiaretic acid	*Larrea divaricata*	Antioxidant
Palmatine	*Coptis japonica*	Antipyretic, detoxicant
Rotundine	*Stephania sinica*	Analagesic
Salicin	*Salix alba*	Analgesic
Tetrahydropalmatine	*Corydalis ambigua*	Analgesic

Despite the listed drugs compounds derived from medicinal plants prior to the year 2001, there were some ongoing clinical trials for other new drugs for the potential treatment of various diseases and six novel compounds listed in **Table 1.2.2.3** were developed and approved by the FDA (WHO, 2006). Moreover, ten newly developed drugs as listed in **Table 1.2.2.4** are still undergoing clinical trials and not yet approved.

Table 1.2.2.3: *New developed and approved drugs*

Drug/Chemical name	Plant source	Clinical uses/action
Artemether	*Artemisia annua*	Antimalaria
Apormophine	*Papaver somniferum*	Parkison's disease
Galantamine	*Galanthus woronowii*	Alzheimer's
Nitisinone	*Callistemon citrinus*	Tyrosinaemia
Tiotropium	*Atropa belladonna*	Chronic obstructive pulmonary disease
Varenicline	*Cytisus laburnum*	Smoking cessation

Table 1.2.2.4: *Newly developed drugs still undergoing clinical trials*

Drug/Chemical name	Plant source	Clinical uses/action
Betulinic acid	*Betula pubescens*	Anticancer agent
Bevirimat	*Betula pubescens*	Antiviral agent
Caphuperzine	*Huperzia squarrosa*	Alzheimer's disease
Capsaicin	*Capsicum frutescens*	Relieve pain in osteoarthritis
Ceflatonin	*Cephalotaxus hainanesis*	Chronic myeloid leukemia
Celgosivir	*Brugmansia hybrids*	Chronic Hepatitis C virus infection
Combretastatin A4 phosphate	*Combretum caffrum*	Anaplastic thyroid cancer
Huperzine A	*Huperzia squarrosa*	Acetylcholinesterase inhibitory activity
Ingenol 3-angelate	*Euphorbia peplus*	Antitumor agent
Protopanaxadiol	*Panax ginseng*	Anticancer agent

From all listed compounds derived from plants, it is apparent that one plant can possess a variety of new active compounds with diverse pharmacological activities, for example, *Papaver somniferum*. It should also be noted that in the past 16 years, a

small number of new compounds have been developed and reached the clinical trial stage (Mahomoodally, 2013; WHO, 2006). There is a necessity for the screening of more traditional medicinal plants for biologically active compounds as drug discovery should remain a vital component in the search for new medicines.

Thus, pharmacological investigation of traditionally used medicinal plants is valuable as it will lead to the discovery of novel active agents for current drug-resistant conditions, be a source of potential chemotherapeutic agents, and be used to assess the safety of the continuous use of medicinal plants.

1.3 RESEARCH OBJECTIVES AND OUTCOMES

Problem statement: Current anticancer drugs have dissatisfactory results due to their toxic side effects and dose dependency. Another problem is the decreased quality of life of relapsed cancer patients due to adverse chemotherapeutic effects or chemoresistance of tumors. Consequently, there is a continuous need for cost-effective cancer therapy protocols which can be approved by world health authorities. Research for preclinical pharmacological and molecular investigations, with purified fractions and the evaluation of their effects in chemoprevention or cancer treatment, is of utmost importance for the development of *Asparagus laricinus Burch. (A. laricinus), Gunnera perpensa L.* (*G. perpensa*) and *Senecio asperulus DC. (S. asperulus)* formulations into therapy for cancer and other diseases. This thesis was targeted at the evaluation of pharmacological properties of three medicinal plants used by South African and Lesotho communities, which are expected to be cheap, readily available and less toxic, but with novel active compounds.

1.3.1 The aim of the study

The aim of the present research is to evaluate the pharmacological properties of traditional medicinal plants used by South African and Lesotho communities for the treatment of cancer.

1.3.2 The objectives of this project were as follows:

- To review ethnobotanical and scientific pharmacological evidence of the selected medicinal plants
- To screen for phytochemicals with pharmacological properties present in *A. laricinus, G. perpensa,* and *S. asperulus* plant extracts
- To investigate the antimicrobial, antioxidant and anti-inflammatory activity of *A. laricinus, G. perpensa,* and *S. asperulus*.
- To determine the anticancer activity of *A. laricinus, G. perpensa* and *S. asperulus* plant extracts.
- To determine the antimutagenic and mutagenic effects of *A. laricinus, G. perpensa* and *S. asperulus* plant extracts.
- To identify active compounds using Liquid Chromatography-Mass Spectrometry (LC-MS/MS).
- To provide scientific evidence to translate ethnomedical reports on potential anticancer effects of *A. laricinus, G. perpensa* and *S. asperulus* plant extract agents or compounds into new, safe and effective anticancer treatment.

1.4 METHODOLOGY

1.4.1 Plant material selection

Mashele and Kolesnikova (2010) observed case reports describing the unexpected improvement of patients who have been terminally ill due to advanced prostate cancer after using *A. laricinus* roots. Further research on the roots of this plant was and still is under investigation and so far, all findings have been promising (Mashele and Fuku, 2011). However, not much has been done on other parts of the plant, as different parts of the plant can possess different active compounds. Moreover, in the holy bible; Ezekiel 47:12, it is stated that “The fruit thereof shall be for meat and leaf thereof for medicine”. This supports that the power of the plant is in the leaves of the plant. Thus, this plant was chosen in order to explore the pharmacological properties of the leaves/cladodes if there are any. Ntsoelinyane and Mashele (2014), has conducted some preliminary screening tests on the leaves of *A. Laricinus* and it was demonstrated that the leaves/cladodes extract has some antioxidant activity,

phytochemicals, and antibacterial activity. It was, therefore, essential to continue to investigate this part of the plant even further, hence it was selected for this study.

In Africa, *Gunnera perpensa* is widely known for its high medicinal importance in several traditional medicine systems. Koduru et al., (2007) recorded the use of *G. perpensa* rhizomes as cancer (type was not specified) treatment in Eastern Cape, South Africa. Ethnomedicinal uses of *G. perpensa* as a herbal tonic (as it is mostly used as a mixture with other plants) vary among African countries and communities too. In both South Africa and Lesotho, the most used plant part from *G. perpensa* are the roots. Leaves and rhizomes are also used, but to treat few infirmities. The roots are used to prepare decoctions and infusion for the treatment of endometritis both in humans and animals, to regulate the menstrual cycle, treat impotence, to induce labor and expel the after birth, to treat stomachache, to ease period pains and for the relief of colic in pregnancy (Van Wyk and Gericke, 2000; Ngwenya et al., 2003; Moteetee and Van Wyk, 2011; Maroyi, 2016).

Moreover, in South Africa, decoctions using the leaves of this plant are also used to treat impotence, stomachache, for the management of several inflammatory disorders, to remove excess fluid from the body, to relieve dysuria, to relieve rheumatic pains, for dyspepsia, for colds and to treat urinary complaints (Maroyi, 2016), while the roots decoctions are used to treat stomach ulcers, prostate cancer and cervical cancer in Lesotho. However, in both countries, leaves are used as hot poultices for boils, they can be burnt, crushed and smoked for treatment of headaches (Moteetee and Van Wyk, 2011; Maroyi, 2016). The leaves can further be used for ulcers and as a tick or other parasites repellent, only in South Africa (Maroyi, 2016).

Senecio asperulus is ethnomedicinally used in Lesotho to treat back pain, swollen feet (Mugomeri et al., 2014), for colic, flu, old, sore throat, mouth ulcers, treatment of sore joints and to improve blood circulation (Moteetee and Van Wyk, 2011). There have been claims in a community of Mohale's Hoek, Lesotho, that there were significant improvements observed by people who were diagnosed with prostate cancer, breast cancer, and cervical cancer who had been using the decoction of *G. perpensa* and *S. asperulus* roots for 3-4 weeks prepared by a certain herbalist in Lesotho. This led us to visit the herbalist and asked for the sharing of the concoction preparation knowledge

so that the efficacy and safety of the plants used in that decoction can be investigated and claims are verified. Hence these two plants, *G. perpensa* and *S. asperulus*, were selected for this study. The layout plan of the study covered in this thesis is demonstrated in **Figure 1.3**.

1.4.2 Plant material collection

The plant materials, *Asparagus laricinus* (MAS001), *Gunnera perpensa* (PHM01) and *Senecio asperulus* (PHM02) were collected in Mpharane village in Mohale's Hoek district, Lesotho (28° 56' 13.2" South, 27° 56' 12.3" East) and from Random harvest nursery and Phuthaditjhaba, Qwa Qwa, South Africa. Plant materials collected both in Lesotho and South Africa were authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa. The collected materials were washed, dried at room temperature, pulverized by mechanical mills and weighed. They were then stored in a cool place until analysis.

1.4.3 Extraction method

The extraction was done using maceration. Plant material (15 g of the dried plant parts of interest) were weighed, pulverized and soaked in methanol, methanol: dichloromethane, dichloromethane, hexane, and water for 72 hours, with occasional stirring. The extracts were filtered, and the new solvent was added again for more extraction until the solvents remained clear. The organic extracts were concentrated by using a rotator evaporator while aqueous extracts were concentrated with a freeze dryer.

1.4.4 Study layout

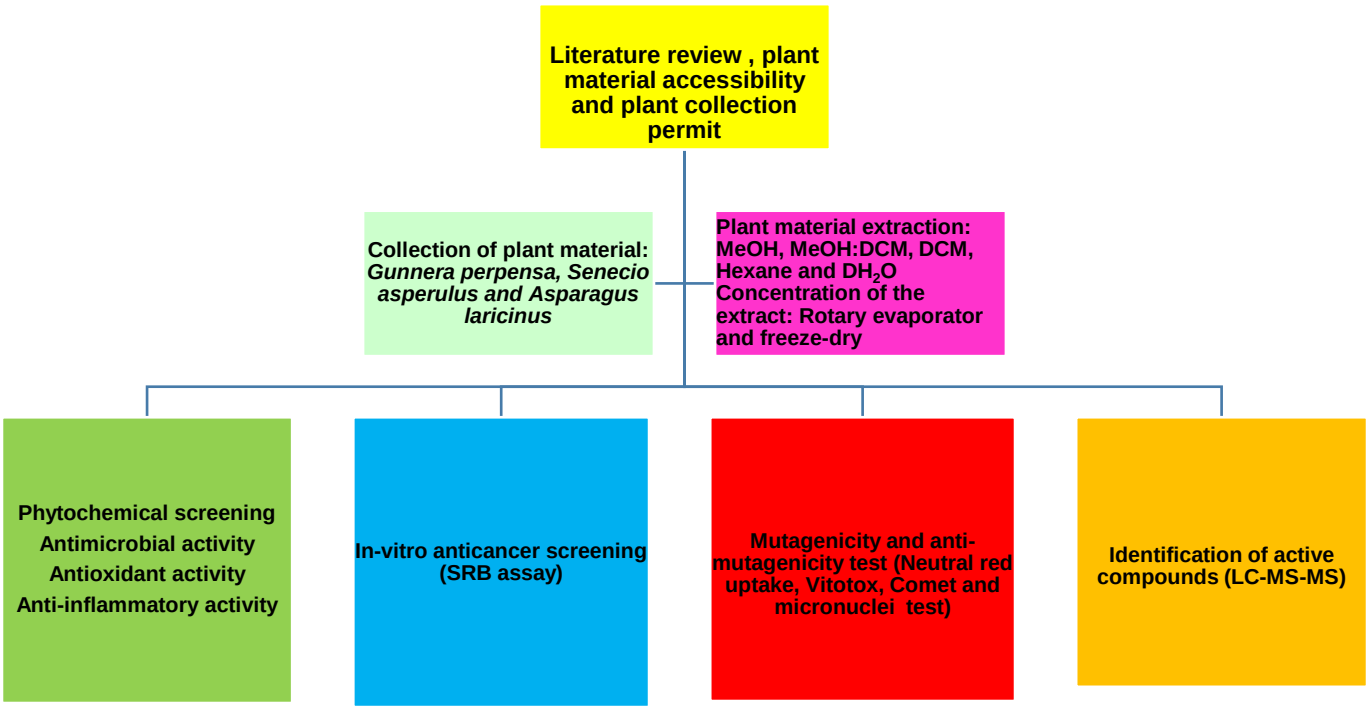


Figure 1.3: A diagrammatic summary of steps in the study of the investigation of the pharmacological activity of *Asparagus laricinus, Gunnera perpensa,* and *Senecio asperulus* plant extract.

1.5 STATISTICAL ANALYSIS

Tests were carried out in triplicate and data reported as mean ± SD. The data obtained from the study were analyzed statistically by analysis of variance (ANOVA) using Graph pad prism version 5.0 software and Mann-Whitney was used for all genotoxicity analysis. Data from the test groups form each experiment was compared with controls and $P<0.05$ was accepted and considered to be statistically significant.

1.6 STUDY OVERVIEW

Chapter 1 demonstrates the importance of medicinal plants in developing countries and the potential of traditional medicinal plants as a source of novel drug development is demonstrated. The evidence of increasing dependence on traditional medicine has led to the development of drugs derived purely from plants. The chosen plants are traditionally used for the treatment of various diseases, but their effectiveness against cancer and their safety has never been explored before. The study objectives were

set out to report the selection of plants used for this study, and on their pharmacological activities with an emphasis on medicinal plants as potential anticancer and chemo-preventative agents (**Chapter 3-6**). The focus was on the validation of ethnobotanical uses of these selected medicinal plants used for cancer, especially breast and prostate cancer. **Chapter 2** provides a comprehensive review of medicinal plants investigated and reported in this thesis. This chapter discusses the morphological, cultivation, phytochemistry and pharmacological activities of selected medicinal plants used for cancer. Gaps in the existing body of knowledge regarding these selected plants were highlighted and some of these gaps will be addressed in the ensuing chapters. **Chapter's 3-5** report on studies that evaluated the *in vitro* antibacterial, antioxidant, anti-inflammatory and anticancer properties of selected medicinal plant extracts. **Chapter 7** focuses on the determination of active compounds present in two plants that have not to be characterized before from *Asparagus laricinus* cladodes/leaves and *Senecio asperulus* roots. **Chapter 6** reports on the evaluation of the mutagenic and antimutagenic effects of *Asparagus laricinus*, *Gunnera perpensa* and *Senecio asperulus* as they have been proven to have promising pharmacological properties. Based on the overall study findings, **Chapter 8** provides a conclusion and recommendations, respectively.

1.7 REFERENCES

Balunas, M.J., Kinghorn, D.A. 2005. Drug discovery from medicinal plants. Review article. Life Sci. 78(5):431-441.

Brendler T, Eloff J.N, Gurib-Fakim A, Phillips L.D. 2010. African Herbal Pharmacopoeia Eds. Association for African Medicinal Plants. Available from <http://cms.herbalgram.org/heg/volume8/files/AfrHPexcerpt.pdf>. Accessed 20 April 2018.

Cherry M. 2005. South Africa — serious about biodiversity science. Public Library of Science Biology. 3:145, DOI:10.1371/journal.pbio.0030145.

Cragg G.M, Newman DJ. 2000. Antineoplastic agents from natural sources: Achievements and future directions. Expert Opinion on Investigational Drugs. 9:1–15.

Cragg, G.M., Newman, D.J. 2005. Biodiversity: A continuing source of novel drug leads. Pure Appl. Chem. 77(1):7-24.

Fabricant, D.S., Farnsworth, N.R. 2001. The value of plants used in traditional medicine for drug discovery. Environ. Health Perspect. 109:69-75.

Hart B.L. 2005. The evolution of herbal medicine: behavioural perspectives. Animal Behaviour. 70:975–989.

Kala C.P, Dhyani P.P, Sajwan B.S. 2006. Developing the medicinal plant's sector in northern India: challenges and opportunities. Journal of Ethnobiology and Ethnomedicine. 91(2-3):331-44.

Kamboj V.P., 2000. Herbal medicine. Curr. Sci., 78:35-39.

Koduru S, Grierson D.S, Afolayan A.J. 2007. Ethnobotanical information of medicinal plants used for the treatment of cancer in the Eastern Cape Province. South Afr Curr Sci. 92(7):906–8.

Mahomoodally M.F. 2013. Traditional Medicines in Africa: An Appraisal of Ten Potent African Medicinal Plants. Evidence-Based Complementary and Alternative Medicine. Available from <http://dx.doi.org/10.1155/2013/617459>. Accessed 20 April 2018.

Maroyi A. 2016. From Traditional Usage to Pharmacological Evidence: Systematic Review of Gunnera perpensa L. Evid Based Complement Alternat Med. 17:14-25.

Mashele, S.S., Kolesnikova, N., 2010. In vitro anticancer screening of asparagus laricinus extracts. Pharmacologyonline 2:246-252.

Moteetee A, Van Wyk B. 2011. The medical ethnobotany of Lesotho: a review. Bothalia. 41(1):209-228.

Mugomeri E, Chatanga P, Hlapisi S, Rahlao L. 2014. Phytochemical characterization of selected herbal products in Lesotho. Lesotho Med Asso J. 12:38-47.

Narayanaswamy T, Thirunavukkarasu T, Prabakar S, Ernest D. 2014. A review on some poisonous plants and their medicinal values. Journal of Acute Disease. 85-89. DOI: 10.1016/S2221-6189(14)60022-6.

Nevin C. 2001. The Environmental Impacts of ECommerce, Proceedings of Sustainability in the Information Society, 15th International Symposium on Informatics for Environmental Protection, Zurich. pages 41-52. Available from <http://enviroinfo.eu/sites/default/files/pdfs/vol103/0041.pdf>. Accessed 21 June 2017.

Ngwenya M.A., Koopman A, Williams R. 2003. Zulu botanical knowledge: an introduction. National Botanical Institute, Durban.

Ntsoelinyane P.H, Mashele S.S. 2014. Phytochemical screening, antibacterial and antioxidant activities of Asparagus laricinus leaf and stem extracts. Bangladesh Journal of Pharmacology. 9:10-14.

Pieroni A, Quave C.L, Villanelli M.L, Mangino P, Sabbatini G, Santini L, et al. 2004. Ethnopharmacognistic survey on the natural ingredients used in folk cosmetics, cosmeceuticals and remedies for healing skin diseases in the inland Marches, Central-Eastern Italy. Journal of Ethnopharmacology. 91:331–344.

Taiz L, Ziegler E. 2006. Plant Physiology. 3rd Edition, Sinauer Associates, Inc. Publishers, Sunderland, MA, USA.

Tamilselvan N, Thirumalai T, Shyamala P, David E. 2014. A review on some poisonous plants and their medicinal values. Journal of Acute Disease 3:85–89.

Van Wyk B-E, Gericke N. 2000. People's plants: a guide to useful plants of southern Africa. Briza Publications, Pretoria.

WHO (World Health Organization). 2006. Traditional medicine strategy 2002-2005. Available from <http://apps.who.int/gb/archive/pdf_files/WHA56/ea5618.pdf>. Accessed 21 June 2017.

WHO (World Health Organization). 2011. The World Traditional Medicines Situation, in Traditional medicines: Global Situation, Issues and Challenges. Geneva 3:1–14.

Willcox M.L, Bodeker G. 2000. Plant-based malaria control: research initiative on traditional antimalarial methods. Parasitol Today. 16(6):220-1.

Xu T.X., Liang X.L, Lu Z. 2005. Prevention and management of Aconitum poisoning. Henan Trad. Chin. Med. 25:65.

CHAPTER 2

"Review of plants"

ABSTRACT

This review summarizes the medicinal properties of *Gunnera perpensa, Asparagus laricinus* and *Senecio asperulus*. There is evidence that the chosen species have tremendous potential to improve human health. Vast information is available for *Gunnera perpensa* only regarding its active compounds and biological activities, and extremely limited information is available for *Asparagus laricinus* and *Senecio asperulus*. However, the existing literature on *Gunnera perpensa* still lacks information about the mechanism of action of various constituents of this plant, and its relation to other plant compounds in poly-herbal formulations (since it's being used as an infusion with *Senecio asperulus*) and long-term use and safety. More in-depth studies are still needed for active compounds and biological activities of *Asparagus laricinus* and *Senecio asperulus*. Consequently, uncountable opportunities and possibilities for investigation are still available in novel areas for these three plants, especially *Asparagus laricinus* and *Senecio asperulus*.

2.1 INTRODUCTION

Historically, plants were used for numerous purposes for mankind in general, *inter alia*, feeding and catering, culinary spices, medicine, various forms of cosmetics, symbols of worship and for a variety of ornamental goods. They are still being used for these purposes. The traditional medicines are sold in market places and prescribed by traditional healers at their homes (Von Maydell, 1996), particularly in the rural areas where herbal medicine is the main part of the healthcare system. South Africa is blessed with a vast variety of plants since it has such a large diversity of more than 20 000 types of species. The research and scientific community find this to be a great source of interest (Cherry, 2005). Since the 1990s, greater interest is being shown in plants which can be used as important sources of new medicines and herbs which have become mainstream throughout Africa (Pavithra et al., 2010).

It is estimated that three-quarters of the world of mankind relies on herbal and traditional medicine as a basis for primary healthcare (Efferth and Kaina, 2011). It was discovered that between 12 and 15 million South Africans still rely on more than 700 indigenous types of plants for the supply of their traditional herbal medicines (Meyer et al., 1996). Up to 60% of the South African population consults one of an estimated 200 000 traditional healers in rural areas (Liu, 2011). These herbal medicines which are extracted from plants often result in acute toxicity. For example, it is estimated that between 8 000 and 20 000 people die every year in South Africa due to the fact that these medicinal plants are used incorrectly (Shaw et al., 2012; Statssa, 2018). The Food and Drug Administration [FDA] indicates that both serious and moderate adverse events from many botanical and others traditional medicinal products are underreported and that the annual number of such cases is at least 50 000 each year (Hazell and Shakir, 2006).

Different research studies to elucidate and validate the ethnobotanical value of medicinal plants have been conducted and reported on by different investigators worldwide, with findings that were established from the use of various methods, and under diverse conditions. In this chapter, three native plant species used in Africa for the treatment of different ailments, more especially cancer, were evaluated for their historical, etymological, morphological, phytochemical and pharmacological aspects.

These species include *Asparagus laricinus* belonging to the Fabaceae family, *Senecio asperulus* from the Asteraceae family and *Gunnera perpensa* from the Halopagaceae family. The findings of this review study are summarized, and the medicinal properties of the chosen Asparagus, Senecio and Gunnera species were documented in this chapter.

2.2 METHODOLOGY

2.2.1 Search criteria

Original articles, research papers published in journals and in Pub med central, Google scholars on the plants of interest (*Asparagus laricinus*, *Senecio asperulus* and *Gunnera perpensa*), and medicinal uses were studied, and related articles and papers were also taken into consideration. *Asparagus laricinus* was the first choice as our laboratory has already investigated its phytochemical constituents, antioxidant and antibacterial activities, while the other two plants of interest were selected due to their use as a special infusion used to treat cancer by Lesotho herbalists.

2.2.2 Data analysis

All the literature was especially studied for historical, etymological, morphological, phytochemical and pharmacological aspects of *Asparagus laricinus*, *Senecio asperulus*, and *Gunnera perpensa*. Priority was given to ethnobotanical reports, laboratory work and clinical trials carried out on all the three plants.

2.3 *ASPARAGUS LARICINUS Burch.*

Vernacular names

English: Wild asparagus; Afrikaans: Bergkatbos, Bergkatdoring, Fynkatbos, Katdoring, Langbeenkatdoring; Sotho: Lerara tau, Zulu: Ibutha, Setswana: Lesitwana (Oketch-Rabah et al., 1997).

Synonym: *Protasparagus laricinus (Burch.) Oberm.*

Scientific classification

Kingdom: plantae; Order: Asparagales; Family: Asparagaceae; Subfamily: Asparagoideae; Genus: Asparagus; Species: *Asparagus laricinus Burch*.

2.3.1 Historical aspects

Asparagus laricinus is a very hardy, evergreen, shrubby Asparagus with fine, feathery foliage and silvery, zigzag branchlets. It has myriads of tiny white, nectar-rich flowers that develop during spring and summer. These flowers are fragrant and attract insects and birds. It grows in sun or semi-shade and is a very useful plant for a security hedge as it is extremely spiny. It is fire-resistant and, if the stems burn, it shoots quickly from the base. The new shoots can be eaten as a vegetable. It grows in sun or shade and in all types of soil except water-logged soils. It can grow up to 1.5m. This plant species is used medicinally to treat tuberculosis, sores, red water, uterine infection, general alignments, umbilical cord inflammation, and serve as a diuretic.

Distribution: *Asparagus laricinus* is native to Botswana and South Africa, Lesotho and Swaziland.

Cultivation: The shrubs prefer a half-shady situation in moist soil. The substrate should be sandy-loamy or gritty-loamy soil. They tolerate temperatures down to -7°C.

2.3.2 Morphological aspects

They are scrambling shrubs or climbers which grow up to 2.5 m. Stems are whitish with short hard spines; cladodes (leaf-like modified branchlets) grow in clusters of up to 60, 2.5-3.5 cm long branchlets; flowers are white on the outside of cladode clusters (**Figure 2.1**); fruits are green and turn red when ripe.

Figure 2.1: *Asparagus laricinus Burch* cladodes, flowers, and fruits.

2.3.2.1 Leaves

Asparagus laricinus is evergreen. The leaves are alternate and resemble the larch.

2.3.2.2 Stem

Branches are ribbed. Shrubs are spiny and 1– 3 m high, erect or climbing or sometimes trailing; young stems and branches are whitish, ribbed, minutely hispidulous, turning brown and smooth with age; spines are short, hard, straight or slightly curved, on stems and below branches at 3.5– 8 mm. Below cladode are spines which are 1.5– 2 mm long.

2.3.2.3 Fruits

The shrubs carry red berries which are 6– 8 mm in diameter, and 1-seeded.

2.3.2.4 Flowers

Flowers number 1–8, on the outside of cladode fascicles; bracts are overlapping, membranous, ovate, 1.5× 1 mm and, rounded at apex; the pedicel is 5– 6 mm long, articulated in the lower half, below the middle. Tepals are 2.5– 4 mm long, white; stamens with red or orange anthers; ovary 3-locular with 5– 6 ovules in each locule; style is short, ± 1 mm long (incl. stigma) with 3 short and spreading stigmas. *Asparagus laricinus* produce clusters of white six-stellate flowers.

2.3.3 Phytochemical active principles

Roots and leaves of *Asparagus laricinus* have tannins, saponins, terpenes, steroids. However, only roots showed the presence of alkaloids (Fuku et al., 2013), while leaves were devoid of alkaloids (Ntsoelinyane and Mashele, 2014). The leaves further had flavonoids, glycosides, steroids, and carbohydrates. The stem was rich in saponins, tannins, and flavonoids, with a lack of steroids, glycosides, and carbohydrates (Ntsoelinyane and Mashele, 2014). The *Asparagus laricinus* aqueous roots extract contained 4.2 g/l GAE total phenolic content, while leaves and stem aqueous extract showed the phenolic concentration of 0.572 mg/GAE and 0.277 mg/GAE, respectively. It was apparent that leaves had more phenolic content than the stem, and this was supported by the number of active phytochemicals identified from both parts of the

plant. Fuku et al., isolated and identified three compounds from the *Asparagus laricinus* roots: indole-3-carbinol, α-sitosterol and ferulic acid (Fuku et al., 2013).

2.3.4 Pharmacological actions

Secondary metabolites produced by plants for plant protection do not only benefit plants, but they also have health benefits for human beings. These compounds result in antimicrobial medicines (Briskin, 2000), anti-inflammatory drugs, anticancer drugs, and plant-based anti-oxidants. Phytochemical screening was performed on the leaves and roots of *Asparagus laricinus*, and parts had tannins, saponins, terpenes, and steroids. However, only roots showed the presence of alkaloids (Fuku et al., 2013). The flavonoids in this plant may contribute to its effect as an antibacterial and antioxidant agent. The leaf extract was also positive for steroids which are very important compounds, especially due to their relationship with compounds such as sex hormones. Both leaves and stem extracts were revealed to contain saponins, which are known to produce an inhibitory effect on inflammation (Just et al., 1998). This tends to justify the use of *Asparagus laricinus* in traditional medicine. *In vitro*, anti-inflammatory activity studies of this plant are being conducted in the Unit for Drug Discovery Research, CUT.

Tannins are found in almost every plant part: bark, wood, leaves, fruits, and roots, and can be toxic to filamentous fungi, yeasts and bacteria (Scalbert, 1991). Alkaloids were not detected in this study. Plant and studies on *Asparagus* species showed no evidence of alkaloids in the Asparagaceae family. Leaf extracts further showed positive antibacterial activity on *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Enterobacter cloacae* and *Bacillus subtilis*. Inhibition of *Staphylococcus* aureus by the *Asparagus laricinus* plant extract demonstrates the huge potential for the use of this plant extract in the treatment of microbial infections, especially in the light of the growing antibiotic resistance in micro-organisms. The presence of phenols correlates with the antibacterial and antioxidant activities of the leaf extract of *Asparagus laricinus*, as demonstrated by Ntsoelinyane and Mashele (2014).

Many antioxidant-based drug formulations are used for the prevention and treatment of complex diseases such as atherosclerosis, strokes, diabetes, Alzheimer's disease and cancer (Mosquera et al., 2009). Recently, interest has increased considerably in

finding naturally occurring antioxidants to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Sasaki et al., 2002). Free radical scavenging molecules such as flavonoids, tannins, alkaloids, quinones, amines, vitamins, and other metabolites possess anti-inflammatory, anti-carcinogenic, antibacterial and antiviral activities (Sala et al., 2002). *Asparagus laricinus* aqueous extracts of roots and leaves showed positive antioxidant activity with DPPH assay (Ntsoelinyane and Mashele, 2014). Flavonoids have anion radicals and inhibit membrane-bound enzymes (Li, 2003; Li et al, 2011). This may explain the mechanisms of the antioxidative action of *Asparagus laricinus* leaf extract. The antioxidant in the plant extract may also be due to polyphenols as phenolics, which are the largest group of phytochemicals, and most antioxidant activity of plants or plant products have been associated with phenols (Thabrew et al., 1998). The aqueous leaves extract of *Asparagus laricinus* showed significant activity as an antioxidant, and this could be due to the presence of ferulic acid as it is a known strong antioxidant. *Asparagus laricinus* extract used was crude and the comparison to the ascorbic acid was not on the basis of molar concentration. Therefore, *Asparagus laricinus* aqueous extract has the ability to protect cells from oxidative stress.

Mashele and Fuku evaluated the mutagenic and antimutagenic properties of the aqueous roots extracts of this plant using the Ames test on *Salmonella typhimurium* strains: TA97, TA98, TA100, and TA102 without metabolic activation (Mashele and Fuku, 2011). The extract was non-mutagenic toward all strains, had a moderate inhibitory effect on TA100, and had low inhibitory effects on TA102 and TA97 (Mashele and Fuku, 2011). Root aqueous extract showed an indirect mutagenic effect towards TA102 after metabolic activation, but not in TA97, TA98, and TA100 (Mashele and Fuku, 2011). The Ames test without S9 metabolic activation can only detect direct mutagens, while S9 metabolic activation allows the detection of indirect mutagens, often caused by conjugation reactions of metabolic oxidation systems. Cytotoxicity activity on Vero cells was also elucidated. The cytotoxicity tests indicated no cytotoxic effect below 500 µg/ml concentration of the *Asparagus laricinus* aqueous extract. Therefore, this plant can be considered promising, if the therapeutic benefit would have increased substantially before the 500 µg/ml dose could be reached *in vivo* studies to be done in the future.

The phytoconstituents detected from *Asparagus laricinus* could be responsible for the cytotoxic activity, though their exact mode of action is poorly understood at present. Only a few compounds were isolated from the roots of *Asparagus laricinus*: indole-3-carbinol, α-sitosterol, and ferulic acid. β-Sitosterol has a number of therapeutic and chemo-preventive uses in the medical field (Zak et al., 1990; Baskar et al., 2010). Indole-3-carbinol is used in prostate cancer (Garikapaty et al., 2005). Anticancer activity on the breast (MCF7), renal (TK10) and melanoma (UACC62) using roots aqueous and ethanol extracts were done by Mashele & Kolesnikova (2010). Their findings revealed that ethanol extracts were very active while aqueous extracts were weakly active. However, ethanol roots extract only showed the presence of tannins while the aqueous roots extract showed a number of active phytochemicals (Fuku et al., 2013). These results should be investigated further to elucidate the aforementioned difference. It may be that the presence of other active compounds somehow affects the ability of tannins by neutralizing their activity in the aqueous root's extracts. Or, it may be that the presence of other active compounds from the ethanol roots extract was missed during the phytochemical screening of this plant.

Mokgawa evaluated the possible toxic effects of the dried roots, stem, and leaves of *Asparagus laricinus* extracts using Sprague Dawley rats as animal models (Mokgawa, 2016). Histological evaluation could not reveal any pathological changes in both aqueous and ethanolic extracts across all levels of dosages. Full blood count results could not point in the direction of toxicity, adverse effects or hazards as indicated by statistically similar results between the exposed and unexposed groups, using both aqueous and ethanol extracts at different concentrations (Mokgawa, 2016). According to results obtained by Mokgawa, histological assessment has proven that both aqueous and ethanolic extracts of *Asparagus laricinus* had no detrimental or adverse effects on vital organs of Sprague Dawley rats (Mokgawa, 2016). Tissue damage, lesions or inflammation were not observed on the kidney, liver or spleen of treatment groups as compared to the control group. The pattern was observed across increasing doses of aqueous and ethanolic extracts. It was, therefore, concluded that toxicological evaluation of *Asparagus laricinus* extracts may be considered relatively free of toxicity when given orally, because it did not cause death, damage or inflammation to tissues, nor did it produce any remarkable biochemical and hematological adverse effects in both male and female Sprague Dawley rats

(Mokgawa, 2016). Further studies may also be conducted to demonstrate *in vivo* efficacy against cancer as studies to date were done using cell lines (*in vitro* studies).

2.4 *SENECIO ASPERULUS DC.*

Vernacular names

Sotho: Moferefere, Letapisa, Letapisoana

Synonym: *Senecio pearsonii Hutch*.

Scientific classification

Kingdom: Plantae; Order: Asterales; Family: Asteraceae; Genus: Senecio.; Species: *Senecio asperulus DC*

2.4.1 Historical aspects

Senecio asperulus is a very aromatic plant with yellow flowers when young which turns to wooly flowers as the plant gets old. It has been used to treat a variety of ailments by laypeople of Lesotho. Their medicinal uses in South Africa has not yet been documented, thus the only available literature covering this plant is reported by Lesotho researchers.

Distribution: Widely spread in Eastern mountains of South Africa, thus very common in the Eastern Cape province, KwaZulu-Natal, and Cape-Town province. In the kingdom of Lesotho, *Senecio asperulus* it is abundant and widely spread across the country.

Cultivation: The shrubs prefer Grassland, Nama Karoo, Savanna, Succulent Karoo habitats.

2.4.2 Morphological aspects

Senecio asperulus is a very flakey plant which is between 15 cm to 55 cm by height and has rhizomes with many congested leaf rosettes and its involucral bracts are glandular hairy, but differs in being scabrid pubescent (Harvey, 1865; Hilliard, 1977).

It has linear lanceolate radical and cauline leaves and discoid capitula young rootstock. Rootstock thick and woody; stem rigid, 20- 45 cm high, the smaller simple, the larger sparingly branched.

Figure 2.2: *Senecio asperulus DC.* leaves and flowers.

2.4.2.1 Leaves

Senecio asperulus has evergreen radical and lowest cauline leaves that are elongated, acute or acuminate, tapering much at the base and petioled, rigid, callous-denticulate, or serrate, and with reflexed margins. These cauline leaves are congested in the rhizomes appearing above the ground as shown in **Figure 2.2**.

2.4.2.2 Stem

Stem erect, herbaceous, laxly leafy, few-headed.

2.4.2.3 Roots

Root leaves are 3–10 inches long, 2–4 lines wide, varying from minutely callosum-denticulate to somewhat coarsely callous-serrate; the narrow ones almost entire.

2.4.2.4 Flowers

Yellow flowers range from 6 to 12 in number, with 1 to 4 white wooly flowers.

2.4.3 Phytochemical active principles

Phytochemical screening performed on the aqueous leaves and roots mixture of *Senecio asperulus* extract revealed the presence of glycosides and further revealed the presence of flavonoids from methanol and acetone extract with phytosterols from acetone extracts only (Mugomeri et al., 2014). From the book documented by Zdero et al, (1989) on the constituents of *Senecio* species, *Senecio asperulus* was recorded to have several known furocremophilones, few diterpenes, and some pyrrolizidine alkaloids as well.

2.4.4 Pharmacological actions

Senecio asperulus has been used mostly in Lesotho by the Basotho people for treatment of a variety of diseases, either on its own or in combination with other medicinal plants. A decoction prepared from the whole plant is used for colds and flu, for sore throat, mouth infections and to improve circulation. The mixture of *Senecio asperulus, Helichrysum odoratissimum* and *Mentha aquatic or Mentha longifolia* has been reported to be used to treat sore joints, rheumatic and arthritic joints (Moteetee and Van Wyk, 2011). From literature, this plant has been recorded to be used for the treatment of mouth ulcers, herpes sores or ulcers wounds, chest pains, swollen gums, as an antiemetic, anti-inflammatory and as a vasodilating agent (Maliehe, 1997; Moteetee and Van Wyk, 2011). Moreover, an ethnobotanical study conducted by Kose et al (2015), revealed other uses of *Senecio asperulus DC* by the Kingdom of Lesotho lay people which were not recorded before and these included: it's used as an anti-TB treatment, for herpes, syphilis, and itchy feet. Identified phytochemicals from *Senecio asperulus* such as flavonoids, glycosides, and phytosterols have numerous medicinal benefits to humans. Glycosides are known for their anti-diarrheal properties, flavonoids as agents for anti-inflammatory, anti-diarrheal, antiviral, anticancer and antimicrobial activities, while phytosterols have anti-inflammatory, antidiabetic activities and have analgesic effects (Kumar and Pandey, 2013; Mugomeri et al., 2014;

Samec et al., 2016). *Senecio asperulus* infusion has also been used as a remedy for internal poisoning (Quattrocchi, 2016). However, most of these ethnobotanical documented claims have not yet been investigated scientifically, thus the pharmacological activity of this plant species still needs to be investigated.

2.5 *GUNNERA PERPENSA L.*

Vernacular names

English: River pumpkin, Wild rhubarb; Afrikaans: Rivierpampoen, Wilde ramenas; Zulu: Ugobhe, Ugobho; Xhosa: Ighobo, Uxobo, Iphuzilomlambo; Sotho: Qobo; Venda: Ranbola-vhadzimu, Shambola-vhadzimu; Swati: Uqobho

Synonym: *Gunnera calthifolia Salisb*.

Scientific classification

Kingdom: Plantae; Order: Gunnerales; Family: Gunneraceae; Subfamily: Viridiplantae; Genus: Gunnera L.; Species: *Gunnera perpensa L.*

Figure 2.3: *Gunnera perpensa L.* leaves and fruits.

2.5.1 Historical aspects

Gunnera perpensa is an African species belonging to the family Gunneraceae and genus *Gunnera* which is further subdivided into six subgenera. It is a perennial shrubby *Gunnera* with dense, feathery foliage and its growth is restricted to moist and marshy areas such as river banks.

Distribution: Mostly distributed in the southern hemisphere of Africa near marshes and stream banks of the Eastern Cape Province, KwaZulu-Natal Province, Mpumalanga Province, across Lesotho and further to the Eastern tropical African countries (**Figure 2.4**).

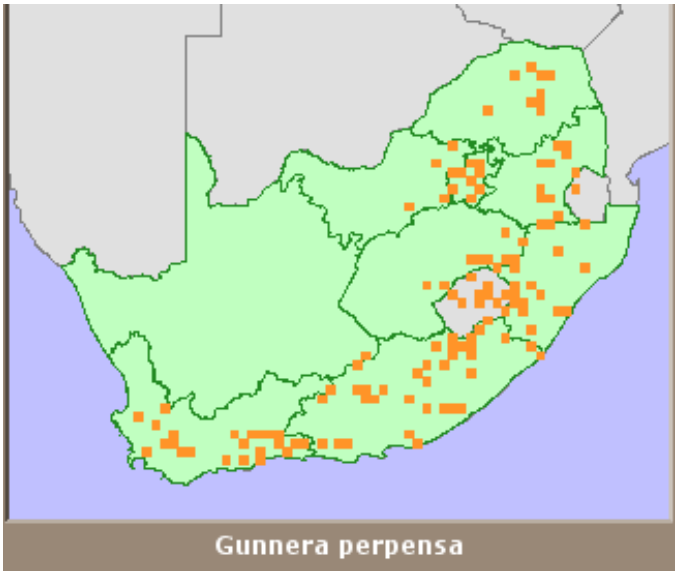


Figure 2.4: Geographical distribution of *Gunnera perpensa L.* (SANBI, 2010)

Cultivation: The shrubs are naturally occurring in black marshy and moist areas. It is compelled permanently in wetland, waterlogged and grazed areas near the edge of the river, close to wells or along streams.

2.5.2 Morphological aspects

Gunnera perpensa is a robust, medium-sized shrub which erects up to a height of 1m with large, round pumpkin-like leaves (**Figure 2.3**) and big fleshy tuberous roots.

2.5.2.1 Leaves

Leaves are large, radiating, dark green, reniform with dentate irregularly toothed margins and coated with hair on both the front and back part. Their size varies from 4 x 6 cm to 25-38 cm area with 15-75 cm long petioles.

2.5.2.2 Stem

Ranges from 15 cm to 1000 cm long, finely hairy and centrally tufted near the rhizome apex just above the soil level.

2.5.2.3 Roots

The roots creep in black muddy soil and may be up to 3 cm thick in diameter with a fleshy yellow-colored inner vascular tissue.

2.5.2.4 Flowers

Flowers are found on the long slender spike which is 2-10 cm long and taller than the leaves. With male, female and bisexual flowers distributed from the top to the base of the spike. These flowers are brown, red and pink but very small and not very noticeable.

2.5.3 Phytochemical active principles

Numerous assortments of active natural compounds are accountable for plants diverse characteristics such as odors (terpenoids), pigmentation (quinines and tannins), and flavors (terpenoids) (Showell et al., 2011). These compounds are beneficial to human health. Notwithstanding the extensive ethnobotanical uses of *G. perpensa*, studies on its chemical constituents are still limited. A few compounds have been identified from *G. perpensa* and those include saponins, phenols, alkaloids, tannins, steroids, cardiac glycosides, flavonoids, proanthocyanins and flavonols (Simelane et al., 2012; Chigor, 2014). Khan et al. (2004), isolated and identified Z-venusol as a major component of *G. perpensa*, however, this was not a novel compound as it was identified already in the 1980s by Proliac et al (1981). However, it was discovered that prolonged exposure of Z-venusol to methanol results into a major compound, Z-methyl lespedezate.

Moreover, other authors have further attempted to investigate and elucidate other active compounds which might be present from *G. perpensa*. Four novel phenolic lactones were isolated from *G. perpensa* roots, and these were 3,3',4'-tri-*O*-methyl ellagic acid lactone, ellagic acid lactone, 1,1'-biphenyl-4,4'-diacetic acid, and p-hydroxybenzaldehyde (Brookes and Dutton, 2007). Mammo et al. (2017) listed other compounds identified from *G. perpensa* such as 3,3′,4′- tri-O-methyl ellagic acid 4-O-β-D-glucopyranoside, punicalagin (main compound of ellagitannins), and β-sitosterol (common phytosterol). In addition to the above-mentioned compounds, sucrose and unidentified sugar were also reported (Peter, 2010).

Drewes et al (2005), additionally isolated three more novel compounds; 2-methyl6-(-3-methyl-2-butenyl) benzo-1,4-quinone, 3-hydroxy-2- methyl-5-(3-methyl-2-butenyl) benzo-1,4-quinone and 6-hydroxy-8-methyl-2,2-dimethyl-2H-benzopyran, and *trans*-phyt-2-enol which was isolated from the aerial parts of this plant and is a known compound. Mtunzi et al. (2012), quantified inorganic elements in *G. perpensa* roots, and it was revealed that this plant has high amounts of magnesium, iron, nickel, zinc, copper, and lead. The latter is known to cause poisoning (both chronic and acute) and poses adverse effects on the liver, kidney, immune and vascular system (Hayes, 1997). However, Mtunzi et al. (2012) concluded that the use of *G. perpensa* roots as herbal medicine will not cause heavy metal toxicity but rather be of good use as supplements for micronutrient deficient people. It should be noted that this untimely conclusion was based on the *in vitro* quantification study of which the exact amount analyzed was not specified. Thus, it will be impossible to know the correct dose and amount of exposure at which *G. perpensa* will remain safe and not pose harmful side effects.

2.5.4 Pharmacological actions

The only *Gunnera* species that have been recorded in Africa is *Gunnera perpensa* which is used in several traditional medicine systems either in monotherapeutic or in synergic application with other medicinal plants. The leaves and roots of this plant have been used in combination with other plants in both South Africa and Lesotho as remedies for the treatment or management of various ailments and conditions (Kose et al., 2015; Mammo et al., 2017). Root decoctions are used in African traditional antenatal practice to initiate labor, for easy delivery and for placenta expulsion in

pregnant women, and also as a remedy for barrenness (Ngwenya et al., 2003; Van Wyk and Gericke, 2000; von Ahlenfeldt et al., 2003). *Gunnera perpensa* exerts these gynecological benefits to both humans and animals. Thus, in both Africa and Europe, it is used in traditional veterinary practice for labor initiation, easy birth, for the removal of excess body fluid or blood, for placenta expulsion and womb clearing after the birth of animals, especially cows (Ngwenya et al., 2003; Watt and Breyer-Brandwijk, 1962).

Gunnera perpensa decoctions are reported to be taken orally for the treatment of dysuria, rheumatic pains, dyspepsia, urinary tract, dysmenorrhoea, stomach bleeding, swelling, sexually transmitted infections (gonorrhea and syphilis) and for diabetes (by Basotho people in Free State) (Buwa and Van Staden, 2006; Gerstner, 1938; Hutchings et al., 1996; Kose et al., 2015; Watt and Breyer-Brandwijk, 1962). This plant species is also used as a menstrual cycle regulator, as a colic remedy, and as a vermifuge. Some of *Gunnera perpensa* uses are contradictory. In Watt and Breyer-Brandwijk, (1962) it is reported that the roots are used to treat barrenness, while Kose et al, (2015) reported the same plant to be used as a contraceptive. Furthermore, the same plant is reported to be used as a stomachic but at the same time as an emetic and as a purgative (Hutchings et al., 1996; Buwa and Van Staden, 2006). This strengthens the need for proper active compound isolation and pharmacological activity investigation of this plant species.

Nevertheless, for external use, the decoction from roots is used as a wound dressing, for the treatment of psoriasis, as insect bite antidote and as tick repellent (Pujol, 1990; Buwa and van Staden, 2006). Moreover, leaves are burnt and crushed and smoked (as snuff) for headaches (Moteetee and Van Wyk, 2011). In the Eastern Cape, warm aqueous infusions and decoctions of *Gunnera perpensa* are administered orally for three to four weeks for the treatment of cancer (Ngwenya et al., 2003; Koduru et al., 2007). With the extensive list of medicinal benefits from the species *Gunnera perpensa*, it is ostensible that novel compounds with promising anticancer agents and other pharmacological importance can be isolated or even derived from this plant. Several studies have been performed for the isolation of some chemical compounds from *Gunnera perpensa* and have demonstrated various biological activities, however, all investigative assays were *in vitro*.

Scientific studies on *G. perpensa* has indicated its wide assortment of bioactive properties from crude roots or a mixture of leaves and stem of this plant species. Water extract of *G. perpensa* demonstrated the ability to inhibit acetylcholinesterase (AChE) enzyme (Ndhlala et al., 2011; Simelane et al., 2012), and also showed uterotonic properties (Kaido et al., 1997; Khan et al., 2004). Thus, this strengthens the use of this plant to induce labor and expel placenta as it inhibits AChE and causes uterus contractility. Ethanol extract of *G. perpensa* showed antibacterial activity against selected Gram-positive micro-organisms, with the water extract being inactive (Steenkamp et al., 2004; Buwa and Van Staden, 2006). However, Nkomo and Kambizi (2009) and McGaw et al., (2000), demonstrated the ability of methanol and water extracts to be having strong antibacterial activity against all Gram-positive strains tested.

Different studies on the antifungal activity screening of *G. perpensa* exhibited ethanol extracts to be having high antifungal activity (Drewes et al., 2005; Buwa and Van Staden, 2006; Maroyi, 2016). Methanol and water extracts showed anti-inflammatory activity (Nkomo et al., 2010; Ndhlala et al., 2011), and Nkomo et al., (2010) further demonstrated the antinociceptive properties of these extracts, thus, supports the use of *G. perpensa* for wound dressing and for the treatment of psoriasis. McGaw et al., (2000) as well as Mwale and Masika (2015), confirmed the anthelmintic properties of *G. perpensa*, thus this corroborates the use of this plant in Lesotho and South Africa for the treatment of gastro-parasites. Water extracts of *G. perpensa* further presented antioxidant activity (Steenkamp et al., 2004; Simelane et al., 2012), anti-tumour (Mathibe et al., 2016), fibroblast growth stimulation ability (Steenkamp et al., 2004; Mabona et al., 2013) and lactogenic (Simelane et al., 2012). The stated lactogenic properties of *G. perpensa* on rats increased milk production validated the traditional use of *G. perpensa* as milk production stimulator on breastfeeding mothers in KwaZulu Natal province, South Africa (Simelane et al., 2012).

Brookes and Smith (2003) showed the non-mutagenic effect of *G. perpensa* water extracts only on human fibroblast and monkey Vero cell with and without S9 metabolic activation. However, most authors found aqueous extracts of *G. perpensa* to be having less bioactivity when compared with organic extracts such as methanol, ethanol, and acetone (Mwale and Masika, 2015; Sanhokwe et al., 2016), thus the toxicity of organic

extracts also require to be investigated and reported. Moreover, *Gunnera perpensa* has a lot of potential as a possible source of pharmaceutical products for the treatment of a wide range of both human and animal diseases and ailments. Nevertheless, *in vivo* studies followed by human clinical trials are needed before *G. perpensa* herbal decoctions and infusions are recommended for different pharmacological applications. Some of the reported pharmacological activities of *Gunnera perpensa* reported in literature correlate with some of its ethnomedicinal uses.

2.6 CONCLUSION

Only preliminary screening of phytochemicals was done on crude extracts of *Asparagus laricinus* and *Senecio asperulus*. Isolation of active pure compounds from *Asparagus laricinus* was only done on roots (three compounds identified) and not on leaves, even though leaves showed so many active compounds. Intensive research on the identification of compounds from both *Asparagus laricinus* and *Senecio asperulus* still needs to be done and testing of them for pharmacological activity is also essential as this has not been done before. Thorough work still needs to be performed regarding the mutagenicity or genotoxicity of all three plants so as to confirm their safety. The toxicological study of the roots of *Asparagus laricinus* confirmed that the plant extract did not cause any harm *in vivo* and can thus be considered as non-toxic. Nonetheless, the *in vivo* anticancer activity of *Asparagus laricinus* root extract has not been done in order to confirm/corroborate the results obtained in the screening study that was conducted. Moreover, not all ethnobotanical claims of *Asparagus laricinus* have been confirmed as the anti-TB activity, anti-inflammatory activity, and its ability as a diuretic still needs to be elucidated. Additionally, pharmacological activity and toxicity investigations of *Gunnera perpensa in vivo* are still outstanding, as only *in vitro* work has been recorded. No scientific evidence has been recorded for the bioactivity of *Senecio asperulus* to corroborate its ethnobotanical use claims which were documented from laypeople of Lesotho. Thus, more research with a focus on the elucidation of mechanisms of action of the active anticancer compounds, their efficacy, toxicity, and clinical relevance of all these three plants is still needed.

2.7 REFERENCES

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

Briskin D. 2000. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. Plant Physiol. 124:507–514.

Brookes K.B, Dutton M.F. 2007. “Bioactive Components of the Uteroactive Medicinal Plant, Gunnera Perpensa (or Ugobo): Research in Action.” South African Journal of Science. 103(5 & 6):187–189.

Brookes K.B, Smith A.N. 2003. Cytotoxicity of pregnancy-related traditional medicines,” South African Medical Journal. 93(5):359–361.

Buwa L.V, Van Staden J. 2006 Antibacterial and antifungal activity of traditional medicinal plants used against venereal diseases in South Africa, Journal of Ethnopharmacology. 103(1):139–142.

Cherry M. 2005. South Africa — serious about biodiversity science. Public Library of Science Biology. 3:145, DOI:10.1371/journal.pbio.0030145.

Chigor C.B. 2014. Development of conservation methods for Gunnera perpensa L.: an overexploited medicinal plant in the Eastern Cape, South Africa. Ph.D. thesis, University of Fort Hare, Alice, South Africa.

Drewes S.E, Khan F, Van Vuuren S.F, Viljoen A.M. 2005. Simple 1, 4-Benzoquinones with Antibacterial Activity From Stems and Leaves of Gunnera Perpensa. Phytochemistry. 66(15):1812–1816.

Efferth T, Kaina B. 2011. Toxicities by herbal medicines with emphasis to traditional Chinese medicine. Current Drug Metabolism. 12(10):989–996.

Fuku S, Al-Azzawi A.M, Madamombe-Manduna I.T, Mashele S. 2013. Phytochemistry and Free Radical Scavenging Activity of Asparagus laricinus. International Journal of Pharmacology. 9(5):312-317.

Garikapaty V.P, Ashok B.T, Chen Y.G, Mittelman A, Iatropoulos M, Tiwari R.K. 2005. Anti-carcinogenic and anti-metastatic properties of indole-3-carbinol in prostate cancer. Oncol. Rep. 13: 89–93.

Gerstner J. 1939. A preliminary check list of Zulu names of plants," Bantu Studies. 13(1):131–149.

Harvey W.H., 1865. 'Compositae' in W.H. Harvey & O.W. Sonder, Flora Capensis. 3: 44–530.

Hayes RB. 1997. The carcinogenicity of metals in humans. Cancer Causes and Control. 8(3):371–85.

Hazell, L., Shakir, S.A.W., 2006. Under reporting of adverse drug reactions: a systematic review. Drug Safety. 29:385–396.

Hilliard, O.M. 1977. Compositae in Natal. University of Natal Press, Pietermaritzburg.

Hutchings A, Scott A.H, Lewis G, Cunningham A.B. 1996. Zulu Medicinal Plants: An Inventory, University of Natal Press, Pietermaritzburg, South Africa.

Just M.J, Recsio M.G, Gner. R.M, Cuellar M.J, Marez S, Bilia A.R, Rios J. 1998. Anti-inflammatory activity of unusual lupane saponins from Buleurum fruiticescens Planta Med. 64(5):404-407.

Kaido T.L, Veale D.J.H., Havlik I, Rama D.B.K. 1997. Preliminary screening of plants used in South Africa as traditional herbal remedies during pregnancy and labor. Journal of Ethnopharmacology. 55(3):185–191.

Khan, Fatima, Xolani K Peter, Rod M Mackenzie, Lynn Katsoulis, Ronette Gehring, Orde Q Munro, Fanie R van Heerden, and Siegfried E Drewes. 2004. “Venusol From Gunnera Perpensa: Structural and Activity Studies.” Phytochemistry. 65(8):1117–1121.

Koduru S, Grierson D.S, Afolayan A.J. 2007. Ethnobotanical information of medicinal plants used for the treatment of cancer in the Eastern Cape Province, South Africa. Current Science. 92(7):906–908.

Kose L.S, Moteetee A, Van Vuuren S. 2015. Ethnobotanical survey of medicinal plants used in the Maseru district of Lesotho. Journal of Ethnopharmacology. 170: 184–200.

Kumar S, Pandey A.K. 2013. Chemistry and Biological Activities of Flavonoids: An Overview. The Scientific World Journal. 1-17.

Li H, Wang Z, Liu Y. 2003. Review in the studies on tannins activity of cancer prevention and anticancer. Zhong-Yao-Cai. 26:444-48.

Li Y.C. 2011. Antioxidant activity of flavonoids from sweet potato vines in vitro. Adv Mater Res. 236:2634–2638.

Liu W.J.H. 2011. *Traditional Herbal Medicine Research Methods*. First Edition. Europe: John Wiley & Sons. p. 111-123.

McGaw L.J, Jäger A.K. Van Staden J. 2000. Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. Journal of Ethnopharmacology. 72(1-2):247–263.

Mabona U, Viljoen A, Shikanga E, Marston A, Van Vuuren S. 2013. Antimicrobial activity of southern African medicinal plants with dermatological relevance: From an ethno pharmacological screening approach, to combination studies and the isolation of a bioactive compound. J. Ethnopharmacol. 148:45–55.

Maliehe E.B. 1997. Medicinal Plants and Herbs of Lesotho (in Sesotho). Mafeteng

Development Project, Lesotho.

Mammo F.K, Mohanlall V, Shode F.O. 2017. Gunnera perpensa L.: A multi-use ethnomedicinal plant species in South Africa. African Journal of Science, Technology, Innovation and Development. 9(1):77-83.

Mashele S.S, Fuku S. 2011. Evaluation of the antimutagenic and mutagenic properties of asparagus laricinus. Med Technol. 2:33-36.

Mashele, S.S., Kolesnikova, N., 2010. In vitro anticancer screening of asparagus laricinus extracts. Pharmacologyonline 2:246-252.

Maroyi A. 2016. From Traditional Usage to Pharmacological Evidence: Systematic Review of *Gunnera perpensa L.* Evid Based Complement Alternat Med. 17:14-25.

Mathibe L.J, Botha J, Naidoo S. 2016. Z-venusol, from Gunnera perpensa, induces apoptotic cell death in breast cancer cells in vitro. South African Journal of Botany. 102:228–233.

Meyer JJM, Afolayan AJ, Taylor MB, Engelbrecht L. 1996. Inhibition of herpes simplex virus type 1 by aqueous extracts from shoots of *Helichrysum qureonites* (Asteraceae). Journal of Ethnopharmochology. 52:41–43.

Mokgawa S.D. 2015. Toxicology of Asparagus laricinus in rats (Thesis). M. Tech: Biomedical Technology, Central University of Technology, Free State. (Accessed on the 14 May 2018). Available from: < http://ir.cut.ac.za/bitstream/handle/11462/1332/Mokgawa%2C%20Sekobane%20Daniel.pdf?sequence=1&isAllowed=y>

Mosquera, O. M., Y. M. Correra, and J. Niño. 2009. Antioxidant activity of plant extracts from Colombian flora. Brazilian Journal of Pharmacology. 19(2A):382–87.

Moteetee A, Van Wyk B. 2011. The medical ethnobotany of Lesotho: a review. Bothalia. 13;41(1):209–228.

Mtunzi F, Muleya E, Modise J, Sipamla A, Dikio E. 2012. Heavy metals content of some medicinal plants from Kwazulu-Natal, South Africa. Pakistan Journal of Nutrition. 11(9):757–761.

Mugomeri E, Chatanga P, Hlapisi S, Rahlao L. 2014. Phytochemical characterization of selected herbal products in Lesotho. Lesotho Med Asso J. 12:38-47.

Mwale M, Masika P.J. 2015. In vivo anthelmintic efficacy of Aloe ferox, Agave sisalana, and Gunnera perpensa in village chickens naturally infected with Heterakis gallinarum. Tropical Animal Health and Production. 47(1):131–138.

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

Ngwenya M.A, Koopman A, Williams R. 2003. Zulu Botanical Knowledge: An Introduction, National Botanical Institute, Durban, South Africa.

Nkomo M, Nkeh-Chungag B.N, Kambizi L, Ndebia E.J, Iputo J.E. 2010. Antinociceptive and anti-inflammatory properties of gunnera perpensa (gunneraceae). African Journal of Pharmacy and Pharmacology. 4(5):263–269.

Nkomo M, Kambizi L. 2009. Antimicrobial activity of Gunnera perpensa and Heteromorpha arborescens var. Abyssinica. Journal of Medical plant Research. 3(12):1051–1055.

Ntsoelinyane P.H, Mashele S.S. 2014. Phytochemical screening, antibacterial and antioxidant activities of Asparagus laricinus leaf and stem extracts. Bangladesh Journal of Pharmacology. 9:10-14.

Oketch-Rabah H, Dossaji S, Christensen SB, Frydenvang K, Lemmich E, Cornett C, et al. 1997. Antiprotozoal compounds from Asparagus africanus. J Nat Prod. 60:1017–22.

Pavithra P.S, Janani V.S, Charumathi K.H, Indumathy R, Potala S, Verma R.S. 2010. Antibacterial activity of plants used in Indian herbal medicine. Int J Green Pharm. 4:22-8

Peter X.K. 2010. Structure and Synthesis of Gunnera Perpensa Secondary Metabolites. D. Tech. School of Chemistry, University of KwaZulu-Natal.

Proliac A, Desage M, Favre-Bonvin J. 1981. Structure of venusol, a novel substance from Umbilicus pendulinus. Tetrahedron Letters. 22:3583-3584.

Pujol J. 1990. Natur Africa: The Herbalist Handbook, Jean Pujol, Natural Journal of Healers Foundation, Durban, South Africa.

Quattrocchi U. 2016. CRC World Dictionary of Medicinal and Poisonous Plants: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology. Boca Raton, Fla: CRC, Taylor & Francis Group.

Sala A, Recio M.D, Giner R.M, Manez S, Tournier H, Schinella G, Rios JL. 2002. Anti-inflammatory and antioxidant properties of *Helichrysum italicum*. J Pharm Pharmacol. 54(3):365-371.

Samec D, Pavlovic I, Salopek-Sondi B. 2016. “White cabbage (Brassica oleracea) botanical, phytochemical and pharmacological overview”. Phytochemical Reviews. 16(1):117–135.

Sanhokwe M, Mupangwa J, Masika P.J, Maphosa, Muchenje V. 2016. Medicinal plants used to control internal and external parasites in goats. Onderstepoort Journal of Veterinary Research. 83(1):7.

SANBI (South African national Biodiversity institute). 2010. Wild Rhubarb. Available from: <http://redlist.sanbi.org/species.php?species=2537-1>

Sasaki K, et al. 2002. In vitro antifungal activity of naphthoquinone derivatives. Biol Pharm Bull. 25(5):669-70.

Scalbert A. 1991. Antimicrobial properties of tannins. Phytochemistry. 30:3875-3883.

Shaw D, Ladds G, Duez P, Williamson E, Chan K. 2012. Pharmacovigilance of herbal medicine. Journal of Ethnopharmacology. 140:513-518.

Showell M.G, Brown J, Yazdani A, Stankiewicz MT, Hart RJ. 2011. Antioxidants for male subfertility. Cochrane Database Syst Rev. 1:CD007411.

Simelane M, Lawal O.A, Djarova T.G, Musabayane C.T, Singh M, Opoku A.R. 2012. Lactogenic activity of rats stimulated by Gunnera perpensa L. (Gunneraceae) from South Africa. African Journal of Traditional, Complementary and Alternative Medicines. 9(4):561–573.

Statssa (Statistics South Africa). 2018. Mortality and causes of death in South Africa, 2016: Findings from death notification. Available from: <http://www.statssa.gov.za/publications/P03093/P030932016.pdf>.

Steenkamp V, Mathivha E, Gouws M.C, van Rensburg C. E. 2004. Studies on antibacterial, antioxidant and fibroblast growth stimulation of wound healing remedies from South Africa. Journal of Ethnopharmacology. 95(2-3):353–357.

Thabrew M.I, Hughes R.D. McFarlane IG. 1998. Antioxidant activity of Osbeckia aspera. Phytother Res. 12:288–290.

van Wyk B, Gericke N. 2000. People's Plants: A Guide to Useful Plants of Southern Africa, Briza, Pretoria, South Africa.

von Ahlenfeldt D, Crouch N.R, Nichols G, et al. 2003. Medicinal Plants Traded on South Africa's Eastern Seaboard, Ethekweni Parks Department and University of Natal, Durban, South Africa.

Von Maydell, H-J. 1996. *Trees and Shrubs of the Sahel*. Weikersheim, Germany: Josef Margraf. p. 562.

Watt J.M, Breyer-Brandwijk M.G. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa, E. S. Livingstone, London, United Kingdom.

Zak D.R, Groffman P.M, Pregitzer K.S, Christensen S, Tiedje J.M. 1990. The vernal dam: plant-microbe competition for nitrogen in northern hardwood forests. Ecology. 71:651-656.

Zdero C, Bohlmann F, Liddell R.J. 1989. Secoeremophilanes and other constituents from South African Senecio species. Phytochemistry. 28:3532–3534.

CHAPTER 3

"Phytochemical analysis of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa*"

ABSTRACT

Medicinal plants are great reservoirs of chemical constituents, phytochemicals, that can be used for the development of novel therapeutic agents. Phytochemicals are chemical compounds formed during the plants' bioactivity as plants protect themselves from harsh environmental conditions and insects. Most phytochemicals have properties that are beneficial to humankind, thus, they have the potential to serve as leads for novel drug discovery and development. The aim of this chapter was to investigate the phytochemical constituents and total phenolic content of *Asparagus laricinus, Senecio asperulus and Gunnera perpensa* extracts using colorimetric assays. The estimated total phenol content values of *Asparagus laricinus, Senecio asperulus and Gunnera perpensa* extracts were analyzed at 1 mg/ml against gallic acid using Folin and Ciocalteu's phenol reagent. *Gunnera perpensa* extracts showed more polyphenols than *Asparagus laricinus* and *Senecio asperulus*. The qualitative phytochemical analysis confirmed the presence of tannins, flavonoids, steroids, terpenoids, alkaloids, and phlobatannins from *Gunnera perpensa* extracts. *Asparagus laricinus* extracts showed saponins, steroids, tannins, terpenoids, and flavonoids while *Senecio asperulus* extract showed the presence of flavonoids, tannins, terpenoids and steroids only. The gallic acid equivalents (GAEs) of the estimated phenolic concentrations for all three-plant species ranged from 0.112 ± 0.009 to 0.067 ± 0.003 mg/GAE. Our results indicate that *Gunnera perpensa*, which is used for almost every ailment by the Basotho in Lesotho, does indeed have various phytochemical compounds when compared to other two plant species.

3.1 INTRODUCTION

The research fundamentals on traditional medicinal plants as potential sources for new drugs development begins with the understanding of their bioactive compounds and traditional therapeutic indications. Phytochemicals are bioactive chemicals of plant origin that are formed during the plants' normal metabolic processes and plants use them to protect themselves (Watson et al., 2001; Ning et al., 2009). Phytochemicals are regarded as secondary metabolites because the plants that manufacture them may have little need for them. They are naturally synthesized in all parts of the plant body: bark, leaf, stem, branch, shoot, root, flower, fruit and seed, therefore any part of the plant body may contain active components (Tiwari et al., 2011). However, the quantity and quality of phytochemicals present in plant parts do differ from one part to another. In fact, there is still a lack of information on the distribution of the biological activity in different plant parts essentially related to the difference in the distribution of active compounds or active principles (Lahlou, 2004). The geographical positioning of the plant does also influence the quantity and quality of present secondary metabolites as soil nutrition also influence plants metabolic processes.

Medicinal plants are of great importance to the healthcare system and as such, they are very beneficial to individuals and communities using them, and to the pharmaceutical industry in general. The value of medicinal plants lies in chemical substances that produce a certain physiological action on the human body. The most important bioactive constituents of plants beneficial to humankind are steroids, alkaloids, triterpenes, tannins, flavonoids, and phenolic compounds. Phenolic compounds possess several biological properties, such as anti-inflammation, anticancer, anti-aging, anti-atherosclerosis and cardiovascular protection (Han et al., 2007; Vaghasiya et al., 2011). There is, therefore, a need to investigate secondary metabolites from medicinal plants which are frequently used for medicinal purposes traditionally, as this will lead to the elucidation of the pharmacological activities of such medicinal plants. Screening of phytochemicals from medicinal plants provides clearer direction for the identification, isolation, and characterization of active compounds that can be added to the potential list of novel drugs. This chapter aims to report the phytochemicals found in *Asparagus laricinus, Senecio asperulus and Gunnera perpensa* plant extracts using colorimetric tests.

3.2 LITERATURE REVIEW

Secondary metabolites possess multiple functionality and bioactivity due to the presence of more than one functional group (Dey and Harborne, 1989). Secondary metabolites are classified based on their chemical structure, composition, their solubility, original synthetic pathways and their biosynthetic pathways (Harbone et al., 1996). There are six major secondary metabolites families which are: flavonoids, steroids, alkaloids, triterpenes, tannins and phenolics. Plants have their own mechanisms of producing secondary metabolites in small to large amounts and concentrations vary. Van Wyk and Wink (2004) reported that there are generally three major groups of secondary metabolites, namely nitrogen-containing compounds (e.g. alkaloids, and terpenoids), phenolics (e.g. flavonoids and tannins) and glycosides.

3.2.1 Flavonoids

Flavonoids are naturally occurring polyphenolic compounds containing two benzene rings linked together with a pyrone ring in the case of flavones or a dihydropyrone ring in the case of flavanones (Giuseppe et al., 2007). Flavonoids represent the most common class of phenolics and can occur as monomers, dimers and oligomers. They are water-soluble phenolic molecules containing 15 carbon atoms and are a group of low molecular weight chemical compounds. Among others, the phenylbenzopyrones, are commonly found in all vascular plants. **Figure 3.1** shows the chemical structure of a typical flavonoid with the basic unit of a ketone. Flavonoids consist of various groups such as quercetin, chalcones, rutinosides, aurones, flavanones, isoflavonoids, flavones, flavonols, leucoanthocyanidins, kaempferols, catechins, and anthocyanins.

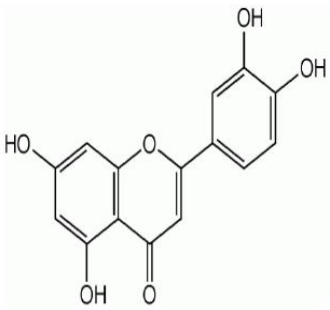


Figure 3.1: The chemical structure of luteolin, a flavonoid.

Flavonoids are normal constituents of the human diet and are responsible for a variety of biological activities. Thus, they are common constituents of fruit, vegetables, nuts, seeds, stems, flowers, tea, wine beverages, herbs and honey (Grange and Davey, 1990). Flavonoids are physiologically active compounds and have been used to treat human diseases (Cushnie and Lamb, 2005). These constituents have many useful medicinal properties, including anti-inflammatory activity, estrogenic activity, enzyme inhibition, antimicrobial activity, anti-allergic activity, antioxidant activity, vascular activity, cytotoxic antitumor activity and anti-inflammatory activity (Havsteen, 1983; Middleton and Chithan, 1993; Harborne and Baxter, 1999; Robards *et al*., 1999; Harborne and Williams, 2000; Middleton *et al*., 2000). However, the molecular mechanisms explaining how flavonoids suppress the response of the latter are still being elucidated (Havsteen, 2002).

3.2.2 Steroids

Steroids are a group of cholesterol derived lipophilic, low-molecular-weight compounds with the fundamental structure of four carbon rings called the steroid nucleus. Plant steroids classes are differentiated based on their chemical structure and their pharmacological activities. Their family includes sterols, several hormones, stigmasterol, α-spinasterol, β-sitosterol and some other hydrocarbons (Sultan and Raza, 2015). Steroids possess many interesting medicinal, pharmaceutical and agrochemical activities like anti-tumor, immunosuppressive, hepatoprotective, antibacterial, plant growth hormone regulator, sex hormone, antihelminthic, cytotoxic and cardiotonic activity (Patel and Savjani, 2015).

3.2.3 Alkaloids

Alkaloids contain a nitrogen-based heterocyclic ring within their molecules and can be categorized as monocyclic, bicyclic or polycyclic alkaloids. Active alkaloids used in drug development include vinblastine, quinine, morphine, atropine, nicotine and many more. Alkaloids are well known for potent pharmacological activities in analgesics, anti-malarial medication, antispasmodics, and products for the treatment of hypertension, mental disorders and tumors (Rajnikant, 2005). Humans have found numerous uses for plant alkaloids, from medicinal (pain relievers, tranquilizers, stimulants, muscle paralyzers) to agricultural (pesticides and herbicides). Some common examples of plant alkaloids include caffeine and cocaine (Robins, 1994).

Previous studies indicate that alkaloids have antitumor potential and among other characteristics have shown *in vivo* activity against various human viruses (Duri *et al*., 1994; Hutchings *et al*., 1996). **Figure.3.2** shows the chemical structure of a typical alkaloid with the basic unit of nitrogen from the amino acid.

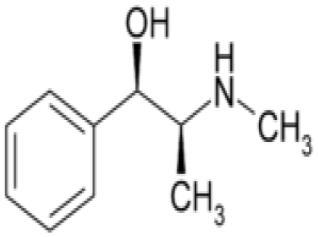


Figure 3.2: The chemical structure of ephedrine, a phenethylamine alkaloid.

3.2.4 Triterpenes

Triterpenes are a unique group of hydrocarbons whose structures may be derived from isoprene (Tiwari and Rana, 2015) and their basic skeleton structure can be divided into three types: oleanane, ursane, and lupane. There are two major triterpenes components: oleanolic acid and ursolic acid and these are known for important activities such as antitumor, anti-inflammatory, antibacterial insecticidal and as insect pollinators (Heads et al., 2017). Terpenoids are derived from triterpenes and acts as toxins and feeding deterrents to many plant-feeding insects and mammals (Taiz and Zeiger, 2006). Many terpenoids play important roles as plant hormones and in the chemical defenses of plants against microbial diseases and insect herbivores (Croteau, 1998). Terpenoids are reported to have medicinal properties such as anti-carcinogenic, anti-malaria, anti-ulcer, antimicrobial and diuretic activity (Aharoni *et al*., 2005). Previous studies reported that plants with terpenes possessed strong antimicrobial activity (Marin *et al*., 2001; Ahmed *et al*., 2005). **Figure 3.3** shows the chemical structure of a typical terpenoid with the basic five-carbon skeleton.

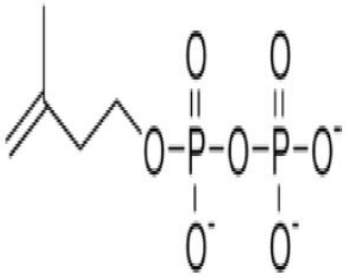


Figure 3.3: Chemical structure of the isopentenyl pyrophosphate, a terpenoid.

3.2.5 Tannins

Tannins are a complex group of plant secondary metabolites that are soluble in polar solutions and are distinguished from other polyphenolic compounds by their ability to precipitate proteins (Silanikove *et al*., 2001). The amount and type of tannins synthesized by plants vary considerably, depending on plant species, cultivars, tissues, stage of development and environmental conditions (Cornell, 2000). Tannins bind to proteins and form an insoluble tannin-protein complex on parts of the plants such as the bark, woody part, fruit, fruit pods, leaves, roots, and plant galls. These secondary metabolites are evenly distributed in all leaf tissues. Plants containing more than 10% tannins may have potentially adverse effects on humans, including upset stomachs, renal damage, hepatic necrosis and increased risk of esophageal and nasal cancer (Kemper, 1999). **Figure 3.4** shows the chemical structure of a typical tannin with the basic unit of phenol groups.

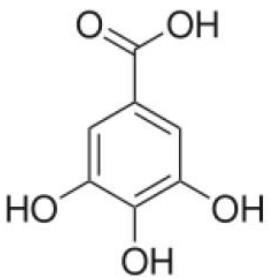


Figure 3.4: Chemical structure of gallic acid, a tannin.

3.2.6 Saponins

Saponins are non-volatile bioactive compounds that are formed naturally in plants. Their name means soap and was derived from the Latin word ‘sapo’, due to their ability to form a soap-like-foams when shaken with water (Hostettmann and Marston, 2005). Saponins originate from terpenes and are subdivided into two classes which are triterpenoid and steroid saponins. These two classes differ structurally based on their carbon skeleton as steroid saponins have 27 carbon atoms while triterpenoid saponins have 30 carbon atoms (Hostettmann and Marston, 2005). According to Chwalek et al. (2006), the aglycone structure and the number of sugar units involved in saponins class influence their biological properties. **Figure 3.5** shows the chemical structure of the two classes of saponins.

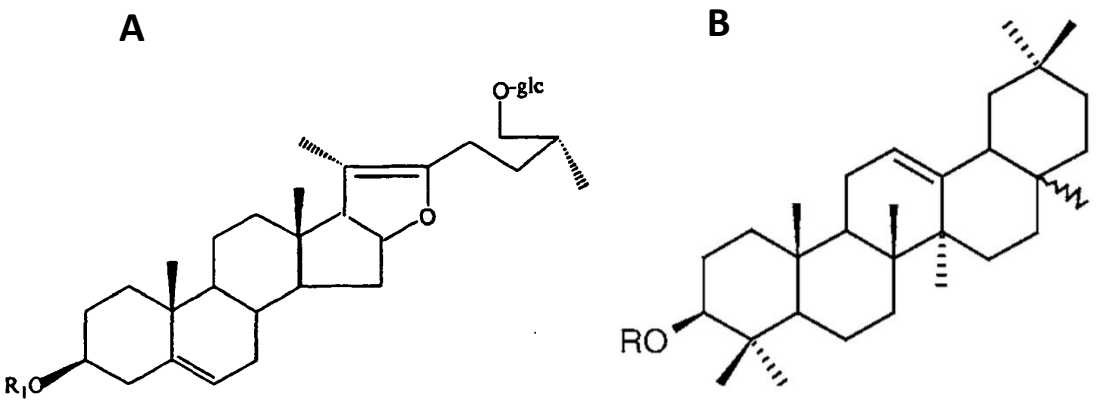


Figure 3.5: Chemical structure of steroid saponin (A) and triterpenoid saponin (B). (Sparg et al., 2004).

Saponins have several diverse applications ranging from its uses in cosmetics, beverages, and pharmaceuticals due to its emulsifying, foaming, sweetness, bitter and medicinal properties (Oda et al., 2000; Kitagawa, 2002; Sparg et al., 2004; Heng et al., 2006). Saponins have several biological effects, some of which are antibacterial, antifungal, antiparasitic, antitumor/cytotoxicity, antiviral and antioxidant activities (Sparg et al. 2004).

3.2.7 Phenolics

Finally, are phenolics, which mainly include phenylpropanoids that can be salvanic acid A, rosmarinic acid, *cis*- and *trans*-caffeic acids and other simple phenylpropanoids (Bai et al., 2013). Phenols can interfere with digestion, block enzyme activity, and cell division or slow growth (Tiwari and Rana, 2015). Phenolic acids are a large and heterogeneous group of biologically active non-nutrients. They are present in plants as hydroxylated derivatives of benzoic and cinnamic acids (Havsteen, 1983; Shahidi and Naczk, 1995). Phenolic compounds are important in the defense mechanisms of plants under different environmental stress conditions such as wounding, infection and excessive light or ultraviolet irradiation (Dixon and Paiva, 1995). Phenolics are not only unsavory or poisonous but also of possible pharmacological value (Strack, 1997). Hydroxybenzoic acids have a general structure derived directly from benzoic acid and variations in the structures of individual hydroxybenzoic acids lie in the hydroxylations and methylations of the aromatic ring (Macheix *et al*., 1990).

Phytochemicals are inexpensive, effective, readily applicable and accessible bioactive compounds that neutralize free radicals causing cell damage. They are directly responsible for different activities such as antioxidant, antimicrobial, antifungal and anticancer ones (Kokate, 1997; Harborne, 1998; Hossain and Nagooru, 2011). The inherent potential of these phytochemicals, especially when used in their natural state for the chemoprevention of cancer cannot be overemphasized, considering their robust safety records when compared with conventional anti-cancer therapies. This has extended the field of research for potential anticancer compounds, as some derived anticancer compounds are already extensively used, such as etoposide, teniposide, vinblastine and vincristine (Lee, 1999; Mans *et al*., 2000). Thus, the aim of this study was to screen for biological compounds present in *Gunnera perpensa, Senecio asperulus* and *Asparagus laricinus* plant extracts.

3.3 METHODOLOGY

3.3.1 Plant material

Roots of *Senecio asperulus* and *Gunnera perpensa* and *Asparagus laricinus* cladodes were collected as discussed in **Chapter 1**, washed, air-dried at room temperature and then grounded into a fine powder using an electric blender and then weighed. They were then stored in a cool place until analysis.

3.3.2 Phytochemical analysis

The qualitative phytochemical screening for the presence of flavonoids, tannins, saponins, terpenoids, and alkaloids was carried out with the following methods by Shanmugam et al., (2010).

Test for Tannins

The dried powdered plant samples (0.5 g each) was boiled in 20 ml of distilled water respectively. A few drops of 0.1% ferric chloride solution were then added to half the volume of filtrate. The appearance of an intense green or brownish green or a blue-black coloration indicated the presence of tannins. This was then confirmed by adding a few drops of iodine in the second half of the filtrate to yield a faint bluish coloration if tannins are indeed present.

Test for Saponins

The dried powdered plant samples (2.0 g) were boiled in 20 ml of distilled water in a water bath and filtered. Thereafter, 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was then mixed with 3 drops of olive oil, shaken vigorously again so that the emulsion could form. The presence of saponins was indicated by the formation of a heavy emulsion.

Test for Flavonoids

The dried powdered plant samples (2.0 g) were heated with 10 ml of ethyl acetate over a steam bath for 3 minutes. The mixture was then filtered, 4 ml of the filtrate was shaken, and 1 ml of dilute ammonia solution was added to it. A yellow coloration indicated a positive test for flavonoids.

Test for Steroids

The dried powdered plant samples (0.5 g) were mixed with 2 ml of acetic anhydride respectively. This was then followed by the addition of 2 ml sulphuric acid. The color change from violet to blue or green indicated the presence of steroids.

Test for Terpenoids (Salkowski test)

The dried powdered plant samples (5.0 g) were mixed in 2 ml of chloroform and thereafter 3 ml concentrated sulphuric acid (H_2S0_4) was carefully added to form a layer. A reddish-brown coloration at the interface indicated the presence of terpenoids.

Test for Alkaloids

The dried powdered plant sample (0.5 g) was mixed with 8 ml of 1% HCl, warmed, and filtered. Thereafter, 2 ml of the filtrate was treated with a few drops of freshly prepared Dragendorffs reagent. The presence of precipitate showed a positive result.

Determination of total phenolic content

Total phenols content in the extracts obtained were estimated by a colorimetric assay based on procedures described by Singleton and Rossi (1965) with some modifications. Briefly, 1 mL of sample was mixed with 1 mL of folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of a saturated sodium carbonate solution was added

to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm wavelength. Gallic acid was used for constructing the standard curve. The results were expressed as mg of GAEs/g of extract.

3.4 RESULTS

Phytochemical screening analysis of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* are reported in **Table 3.1**. A positive and a negative sign was used with (**+**) indicating the presence of the phytochemical and additional **+** sign/s showed the intensity of colour change. While (**-**) Indicated the absence of phytochemicals and no colour change.

Phytochemical screening results showed that *Asparagus laricinus* Burch. is rich in steroids, with tannins only extracted under aqueous conditions (aqueous extracts) and flavonoids from DCM extracts. Aqueous and methanol extracts further showed the presence of saponins and terpenoids. The phenol contents of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* were estimated using the standard curve created with values of gallic acid (**Figure 3.6**).

Table 3.1: Phytochemical screening analysis of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa.*

Plant extract	Flavonoids	Tannins	Saponins	Terpenoids	Steroids	Alkaloids
Asparagus laricinus (H_2O)	-	++	++	-	-	-
Asparagus laricinus (MeOH)	-	-	+	+	+	-
Asparagus laricinus (MeOH: DCM)	+	-	+	+	+	-
Asparagus laricinus (DCM)	+++	-	-	-	++	-
Asparagus laricinus (Hexane)	-	-	-	-	++	-
Senecio asperulus (H_2O)	-	+	+	+	++	+
Senecio asperulus (MeOH)	++	++	-	+	+	-
Senecio asperulus (MeOH: DCM)	-	-	-	+	+	+
Senecio asperulus (DCM)	-	-	-	++	+	+
Senecio asperulus (Hexane)	-	-	-	+	++	-
Gunnera perpensa (H_2O)	+++	+	-	+	-	-
Gunnera perpensa (MeOH)	+	+	+	++	-	+
Senecio asperulus (MeOH: DCM)	+	+	+	+	-	+
Gunnera perpensa (DCM)	+++	-	+	++	-	++
Gunnera perpensa (Hexane)	+	+	+	+	-	+

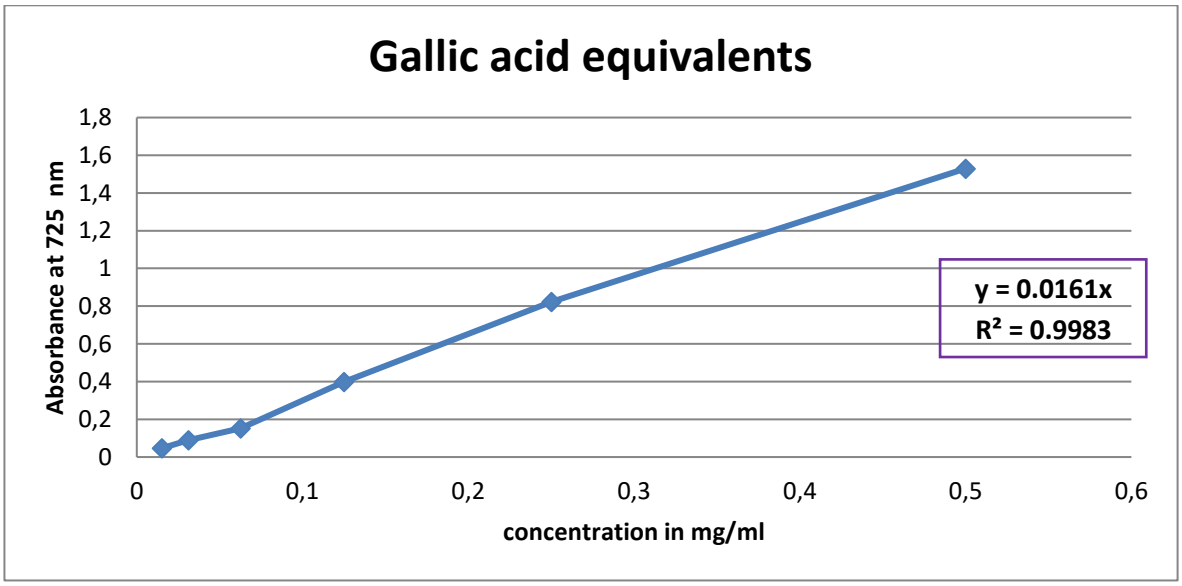


Figure 3.6: Gallic acid standard curve for the estimation of *Asparagus laricinus, Senecio asperulus and Gunnera perpensa* phenol content.

Estimated total phenolic content in this study was measured using the Folin-Ciocalteau assay and phenolic acid (such as gallic acid) to set up a calibration curve (Figure 3.7). Total phenolic content was estimated using the gallic standard curve and expressed as GAE in mg/g extract (Singleton and Rossi, 1965). **Table 3.2** displays the estimated total phenol content values of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa*, which were analyzed at 1 mg/ml against gallic acid.

Table 3.2: Estimated total phenolic content of *Asparagus laricinus, Senecio asperulus and Gunnera perpensa extracts.*

Sample [1 mg/ml]	Absorbance Mean	Polyphenol concentration (mg/GAE)
Asparagus laricinus	**0.114**	**0.112 ± 0.009**
Senecio asperulus	**0.063**	**0.067 ± 0.003**
Gunnera perpensa	**0.083**	**0.085 ± 0.006**

Asparagus laricinus extracts showed more polyphenols than both *Senecio asperulus* and *Gunnera perpensa*. The GAEs of the estimated phenolic concentrations ranged from 0.112 ± 0.009 to 0.067 ± 0.003 mg/GAE.

3.5 DISCUSSION

Phytochemicals are naturally produced by plants for their protection and as a defense mechanism against microbes and insects. These phytochemicals can either be primary compounds such as chlorophyll, proteins and common sugars or be secondary compounds such as terpenoids, alkaloids, flavonoids, reducing sugars, tannins and phenols (Krishnaiah et al., 2007). Plants have their own mechanisms of producing secondary metabolites in small to large amounts and concentrations vary. Many of these secondary metabolites are highly toxic and are often stored in specific vesicles or in vacuoles. Several studies were undertaken by Cragg and Newman (2000) and Kinghorn *et al.* (2003) indicated that this kind of storage functions on the one hand as a detoxification of the plant itself and, on the other hand, it protects the plant against pathogens that are present in the soil. The importance of these compounds to plants at large is usually of an ecological nature, as they are used as defenses against predators, parasites and diseases (Vaghasiya et al., 2011).

The qualitative analysis findings in this chapter agreed with those reported by Ntsoelinyane and Mashele, (2014) on aqueous extracts of *Asparagus laricinus leaves*. Mugomeri et al. (2014) conducted a study on *Senecio asperulus* and revealed the methanol extracts of this species to possess flavonoids. Results from the current study also showed the presence of flavonoids from *Senecio asperulus* methanol extracts and not aqueous extracts. Furthermore, *Senecio asperulus* extracts showed the presence of terpenoids, steroids as well as alkaloids, with flavonoids only extracted by the methanol and total absence of saponins from this plant extracts. Aqueous extracts of *Senecio asperulus* showed the presence of more phytochemicals when compared to organic extracts of the same plant species.

Thus, it was observed that active ingredients from *Senecio asperulus* were extracted better under polar conditions. On the other hand, *Gunnera perpensa* possessed all investigated phytochemicals except for steroids. However, reports from Simelane (2012); Chigor (2014)**;** Mammo et al. (2017), all are in consonance that *Gunnera* perpensa collected from South Africa has steroids. This shows indeed that the geographical effects do lead to variations in secondary metabolites present in the

same species as *Gunnera perpensa* collected from Lesotho showed absence of steroids (**Table 3.1**). Several studies have been conducted on *Gunnera perpensa* stems and leaves from South Africa, for the isolation and identification of active antimicrobial compounds (Grierson and Afolayan, 1999; Drewes et al., 2005). Moreover, root extracts from Eastern Cape and KwaZulu Natal Provinces (South Africa) were investigated for their lactogenic and uterus contractility properties (Simelane 2012; Chigor, 2014). However, not much has been done on the roots from Lesotho. *Gunnera perpensa* water extracts showed only the presence of flavonoids, terpenoids and tannins. However, its methanolic and dichloromethane extracts had all investigated phytochemicals (**Table 3.1**). This discovery indicates that active ingredients from *Gunnera perpensa* are more hydrophobic, thus extracted better with solvents which are non-polar.

Medicinal plants are used by humans in an attempt to restore and/or uphold health, however, their value lies in some important secondary metabolites they possess such as alkaloids, tannins, flavonoids and other phenolics (Hill, 1952). Phytochemicals have a diverse range of pharmacological activities, which may help in protection against diseases which are acute or even chronic. For example, but not limited to, alkaloids and flavonoids protect against chronic diseases due to their capacity to transfer electrons to free radicals, saponins protect against hypercholesterolemia and have antibiotic properties, steroids have analgesic properties as well as triterpenoids, and tannins acting as anti-inflammatory agents (Harbone and Williams, 1992; Okwu, 2004; Mnxati, 2009). Many studies have proved the diverse pharmacological activities of terpenoids as anti-bacterial, anti-oxidant, anti-inflammatory, anticancer, anti-viral, anti-malarial and anesthetic agents (Kappers et al., 2005; Negi et al., 2010; Rabi and Bishayee, 2009; Wagner and Elmadia, 2003). From our results, terpenoids were present from all plant species (Table 3.1) extracted in different solvents, thus supporting the traditional medicinal values of these plants.

Phenolic compounds are commonly found in plants and have multiple biological effects as they possess very strong antioxidant properties (Kähkönen et al., 1999; Li *et al*., 2008). Plants with high levels of phenols can be considered interesting for therapeutic use and are worth further in-depth investigation of their pharmacological

properties. Polyphenols are the most abundant antioxidants in our diet, thus, foods and beverages rich in polyphenols always have great potential in disease prevention. Due to strong antioxidant properties, polyphenolic compounds are effective in the prevention of oxidative stress-related diseases. All plant species showed the presence of flavonoids. Flavonoids are a group of polyphenolic compounds with diverse characteristics and chemical structures. The therapeutic potential of these flavonoids has been determined and they are known to have a number of pharmacological and biochemical properties, namely antibacterial, antiviral, anti-allergic, vasodilatory and anti-inflammatory ones, exhibiting activity against the enzymes cyclo-oxygenase and lipoxygenase (Middleton *et al*., 2000).

The total phenolic content of *Asparagus laricinus* extracts showed a correlation with the antioxidant activity reported by Ntsoelinyane and Mashele (2014). However, there was no correlation between the total phenol content values of *Senecio asperulus* and active biological compounds it comprises. *Senecio asperulus* showed the presence of alkaloids, which are known for their effects as antioxidant agents. However, intensive research is still needed to comprehend the pharmacological effects and the identification of flavonoids and alkaloid categories present in this part of the plant, this will be covered in chapters to follow. Nonetheless, the reported phytochemicals support the ethnobotanical use of *Asparagus laricinus*, *Senecio asperulus* and *Gunnera perpensa* as medicine for numerous ailments in traditional medicine practices, either on their own or mixed with other medicinal plants.

3.6 CONCLUSION

From the present study, plants species screened for phytochemical constituents seemed to have various compounds that are vital for good health, thus, have the potential to act as a source of useful drugs. Because of the presence of various phytochemical constituents such as alkaloids, flavonoids, phenol, terpenoids, saponin, steroids and tannins, it can be concluded that *Asparagus laricinus*, *Senecio asperulus,* and *Gunnera perpensa* have abundant phytochemicals which are produced as secondary metabolites. These compounds could be useful for drug discovery and development of new anticancer drugs in the pharmaceutical industry as they are known to have pharmacological benefits. Our results give basis for the uses of

Asparagus laricinus, *Senecio asperulus* and *Gunnera perpensa* as medicine for numerous ailments in traditional medicine practices. These compounds still need to be investigated further (**chapters 6**), to identify exactly which flavonoids, alkaloids, steroids, tannins, saponins, and terpenoids are present as this could lead to the identification of novel active compounds.

3.7 REFERENCES

Aharoni A, Jongsma M.A, Bouwmeester H.J. 2005. Volatile science? Metabolic engineering of terpenoids in plants. Trends in Plant Science. 10(12):594-602.

Ahmed F, Selim M.S.T, Shilpi J.A. 2005. Antibacterial activity of Ludwigia adscendens. Phytotherapy Research. 76:473-475.

Bai X, Zhang H, Ren S. 2013. Antioxidant activity and HPLC analysis of polyphenol enriched extracts from industrial apple pomace. J Sci Food Agric. 93:2502–2506.

Chwalek M, Lalun N, Bobichon H, Plé K, Voutquenne-Nazabadioko L. 2006. Structure-activity relationships of some hederagenin diglycosides: hemolysis, cytotoxicity and apoptosis induction. Biochimica et Biophysica Acta. 1760(9):1418–1427.

Chigor C.B. 2014. Development of conservation methods for Gunnera perpensa L.: an overexploited medicinal plant in the Eastern Cape, South Africa. Ph.D. thesis, University of Fort Hare, Alice, South Africa.

Cornell. 2000. Tannins: Chemical analysis, Available from http://www.ansci.cornell.edu/plants/toxicagents/tannin.html. Accessed 07 July 2013.

Cragg G.M, Newman D.J. 2000. Antineoplastic agents from natural sources: Achievements and future directions. Expert Opinion on Investigational Drugs. 9:1–15.

Croteau R.B. 1998. The discovery of terpenes. In*: Discoveries in Plant Biology* Vol. I, Kung S-D, Yang, SF (eds). Singapore: World Scientific. p. 112-120.

Cushnie T.P.T, Lamb A.J. 2005. Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents. 26(5):343-356.

Dey, P.M., Harborne, J.B. 1989. Methods in Plant Biochemistry. Plant Phenolics, Academic Press, New York, 1.

Dixon R.A., Paiva N.L. 1995. Stress-induced phenylpropanoid metabolism. Plant Cell. 7:1085–1097.

Duri Z.J, Scovill J.P, Huggins J.W. 1994. Activity of a methanolic extract of Zimbabwean *Crinum macowanii* against exotic RNA viruses *in vitro*. Phytotherapy Research. 8:121-122.

Drewes S.E, Khan F, Van Vuuren S.F, Viljoen A.M. 2005. Simple 1, 4-Benzoquinones with Antibacterial Activity from Stems and Leaves of Gunnera Perpensa. Phytochemistry. 66(15):1812–1816.

Giuseppe G, Barreca D, Gargiulli C, Leuzzi U, Caristi C. 2007. Flavonoids composition of citrus juice. Molecules. 12:1641-1673.

Grange J.M, Davey R.W. 1990. Antibacterial properties of propolis (blue glue). Journal of Royal Society of Medicine. 83:22-26.

Grierson D.S, Afolayan A.J. 1999. An ethnobotanical study of plants used for the treatment of wounds in the Eastern Cape, South Africa. 67(3):327-32.

Han X, Shen T, Lou H. 2007. Dietary polyphenols and their biological significance. Int. J. Mol. Sci. 8:950-988.

Harborne J.B. 1998. Phytochemical methods: A guide to modern techniques of plant analysis. 2nd ed. London: Chapman and Hall. p. 54-84.

Harborne J.B. 1996. The flavonoids. In: *Advances in Research Since 1986*. Vol 1. London: Chapman and Hall. p. 448–478.

Harborne J.B, Baxter H. 1999. The handbook of natural flavonoids. Vol 1& 2. Chichester: John Wiley & Sons. p. 122-13.

Harborne J.B, Williams CA. 1992. Advances in flavonoid research since 1992. Phytochemistry. 55:481.

Havsteen B. 1983. Flavonoids, a class of natural products of high pharmacological potency. Biochem. Pharmacol. 32:1141–1148.

Havsteen B.H. 2002. The biochemistry and medical significance of the flavonoids. Pharmacology and Therapeutics. 96:67–202.

Heads S.W, Miller A.N, Crane J.L, Thomas M.J, Ruffatto D.M, Methven A.S, et al. 2017. The oldest fossil mushroom. PLoS ONE. 12(6):e0178327.

Heng L, Vincken J.P, Hoppe K, van Koningsveld G.A, Decroos K, Gruppen, H, van Boekel M.A.J.S, Voragen A.G.J. 2006. Stability of pea DDMP saponin and the mechanism of its decomposition. Food Chemistry. 99:326–334.

Hill A.F. 1952. Economic Botany. A textbook of useful plants and plant products, New York, McGraw-Hill Book Company Inc. p. 26.

Hostettmann K, Marston A (2005): Saponins. Chemistry and pharmacology of natural products, Cambridge University Press, Cambridge ISBN-10: 0521020174.

Hossain M.A, Nagooru M.R. 2011. Biochemical profiling and total flavonoids contents of leaves crude extract of endemic medicinal plant Corydyline terminalis L. Kunth. Pharmacogn J. 3(24):25-30.

Hutchings A, Scott A.H, Lewis G, Cunningman A.B. 1996. Zulu medicinal plants, An inventory. University of Natal Press; Pietermaritzburg, South Africa. p. 213-215.

Kähkönen M.P, Hopia A.I, Vuorela H.J, Rauha J.P, Pihlaja K, Kujala T.S. 1999. Anti-oxidant activity of plant extracts containing phenolic compounds. Journal of Agricultural and Food Chemistry. 47:3954-3962.

Kappers I.F, Aharoni A, van Herpen T.W, Luckerhoff L.L, Dicke M, Bouwmeester H.J. 2005. Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. Science. 309:2070-2072.

Kemper K.J. 1999. Longwood herbal task force, Available from http://www.mcp.edu/herbal/default.htm. Accessed 07 July 2013.

Kinghorn A.D, Farnsworth N.R, Soejarto D.D, Cordell G.A, Swanson S.M, Pezzuto J.M, Wani M.C, Wall M.E, Kroll N.H, Kramer R.A, Rose W.C, Vite G.D, Fiarchild C.R, Peterson R.W, Wild R. 2003. Novel strategies for the discovery of plant-derived anticancer agents. Pharmaceutical Biology. 41:53–67.

Kitagawa I. 2002. Licorice root: A natural sweetener and an important ingredient in Chinese medicine. Pure and Applied Chemistry. 74:1189–1198.

Kokate K.C. 1997. *Practical pharmacognosy*. 4th ed. Delhi: Vallabh Prakashan. p. 218.

Krishnaiah D, Rosalam S, Awang B. 2007. Phytochemical antioxidants for health and medicine A move towards nature. Biotechnol. Mol. Biol. Rev. 1(4):097-104.

Lahlou M. 2004. Methods to study the phytochemistry and bioactivity of essential oils. Phytotherapy Research. 18(6):435-445.

Lee K.H. 1999. Anticancer drug design based on plant-derived natural products. Journal of Biomedical Science. 6:236-250.

Macheix J-J, Fleuriet A, Billot J. 1990. *Fruit phenolic*s. Boca Raton, USA: CRC Press.

Mammo F.K, Mohanlall V, Shode F.O. 2017. Gunnera perpensa L.: A multi-use ethnomedicinal plant species in South Africa. African Journal of Science, Technology, Innovation and Development. 9(1):77-83.

Mans D.R.A, Da Rocha A, Schwartsmann G. 2000. Anti-cancer drug discovery and development in Brazil: Targeted plant collection as a rational strategy to acquire candidate anti-cancer compounds. The Oncologist. 5:185-198.

Marin P.D, Grayer R.J, Veitch N.C, Kite G.C, Harborne J.B. 2001. Acacetin glycosides as taxonomic markers in *Calamintha* and *Micromeria*. Phytochemistry. 58:943-947.

Middleton E, Chithan K. 1993. The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer. In: J.B. Harborne (ed.), The flavonoids: Advances in research since 1986. London: Chapman and Hall. p. 145-166.

Middleton E. (Jr.), Kandaswami C, Theoharides TC. 2000. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. Pharmacological Reviews. 52(4):673-751.

Mnxati S. 2009. Philenoptera violacea (Klotzsch) Schrire. Available from http://www.plantzafrica.com/plantnop/philenviol.htm. Accessed 07 July 2013.

Mugomeri E, Chatanga P, Hlapisi S, Rahlao L. 2014. Phytochemical characterization of selected herbal products in Lesotho. Lesotho Med Asso J. 12:38-47.

Negi J.S, Singh P, Joshi G.P, Rawat M.S, Bisht V.K. 2010. Chemical constituents of Asparagus. Pharmacogn Rev. 4(8):215–220.

Ning G, Tianhua L, Xin Y, He P. 2009. Constituents in Desmodium blandum and their antitumor activity. Chinese Traditional and Herbal Drug. 40:852–856.

Ntsoelinyane P.H, Mashele S.S. 2014. Phytochemical screening, antibacterial and antioxidant activities of Asparagus laricinus leaf and stem extracts. Bangladesh Journal of Pharmacology. 9:10-14.

Oda K, Matsuda H, Murakami T, Katayama S, Ohgitani T, Yoshikawa M. 2000. Adjuvant and haemolytic activities of 47 saponins derived from medicinal and food plants. Biol. Chem. 381:67–74.

Okwu D.E. 2004. Phytochemicals and vitamin content of indigenous spices of Southern Nigeria. J. Sustain. Agric. Environ. 6(1):30-37.

Patel S.S, Savjani J.K. 2015. Systematic review of plant steroids as potential anti-inflammatory agents: Current status and future perspectives. The Journal of Phytopharmacology. 4(2):121-125.

Rabi T, Bishayee A. 2009. Terpenoids and breast cancer chemoprevention. Breast Cancer Res Treat. 115:223-239.

Rajnikant D.K. 2005. Weak C-H-O hydrogen bonds in alkaloids: An overview. Bulletin of Material Science. 28(3):187-198.

Robins R.I. 1994. Secondary products from cultured cells and organs: Molecular and cellular approaches. In: Plant cell culture. 1st Ed. Dixon RA, Gonzales RA (eds). Oxford, Tokyo: IRL Press. p. 56-58.

Shahidi F, Naczk M. 1995. *Food phenolics, sources, chemistry, effects, applications*. Lancaster, USA: Technomic Publishing Company, Inc. p 45.

Shanmugam S, Sathish K.T, Panneer S.K. 2010. Laboratory handbook on Biochemistry. PHI learning private limited Delhi.

Silanikove N, Perevolotsky A, Provenza F.D. 2001. Use of tannin-binding chemicals to assay for tannins and their negative post-ingestive effects in ruminants. Animal Feed Science and Technology. 91:69-81.

Simelane M, Lawal O.A, Djarova T.G, Musabayane C.T, Singh M, Opoku A.R. 2012. Lactogenic activity of rats stimulated by Gunnera perpensa L. (Gunneraceae) from South Africa. African Journal of Traditional, Complementary and Alternative Medicines, 9(4):561–573.

Singleton V.L, Rossi J.R. 1965. Colorimetric of total phenolics with phosphomolybdic phosphotungstic acid reagents. American Journal of Enology and Viticulture. 16:144–158.

Sparg, S.G, Light M.E, van Staden J. 2004. Biological activities and distribution of plant saponins. Journal of Ethnopharmacology. 94(2–3):219–243.

Strack D. 1997. Phenolic metabolism. In: Dey PM, Harborne JB. (eds). *Plant biochemistry*. London, UK: Academic Press. p. 387–416.

Sultan A, Rauf Raza A. 2015. Steroids: A Diverse Class of Secondary Metabolites. Med chem. 5:310-317.

Taiz L, Zeiger T. 2006. *Plant physiology*. 3rd Edn. Sunderland, Massachusetts: Sinauer Associates, Inc., Publishers. p. 287-290.

Tiwari P, Kumar B, Kaur M, Kaur G. 2011. Phytochemical screening and extraction: A review. International Pharmaceutical sciencia. 1(1): 98-106.

Tiwari R, Rana C.S. 2015. Plant secondary metabolites: A review. International Journal of Engineering Research and General Science. 3(5):661-670.

Vaghasiya Y, Dave R, Chanda. S. 2011. Phytochemical Analysis of Some Medicinal Plants from Western Region of India. Res.J. Med.Plant, 5: 567-576.

Van Wyk BE, Wink, M. 2004. Medicinal plants of the world: An illustrated scientific guide to important medicinal plants and their uses. 1st Edn. Timber Press: Portland, OR, USA. p. 367.

Wagner K.H, Elmadfa I. 2003. Biological relevance of terpenoids: Overview focusing on mono-di and tetraterpenes [abstract]. Ann Nutr Metab. 47:95-106.

Watson A.A, Fleet G.W.T, Asano N, Molyneux R.J, Nash R.J. 2001. Polyhydroxylated alkaloids - natural occurrence and therapeutic applications. Phytochemistry. 56: 265-295.

CHAPTER 4

"*In vitro* antibacterial, antioxidant and anti-inflammatory activity of *Senecio asperulus Gunnera perpensa* and *Asparagus laricinus*"

ABSTRACT

Traditional medicinal plants have been widely used to treat or manage various ailments for centuries in Africa. With increased challenges of multidrug resistance and undesired adverse events from current drugs, there is a need for alternative drugs. In this chapter, we aimed at the investigation of antibacterial, antioxidant and anti-inflammatory effects of *Asparagus laricinus* cladodes*, Senecio asperulus* and *Gunnera perpensa* roots extracted in three solvents which showed to have more phytochemicals as reported in Chapter 3. Antibacterial activity was determined using the disc diffusion method, while antioxidant activity was determined using free radical scavenging of the 2,2-diphenyl-1-picrylhydrazyl assay. The Lipopolysaccharide (LPS) stimulated RAW 264.7 mouse macrophage *in vitro* model was used to evaluate the anti-inflammatory activity of both plants. Resveratrol was used as a positive control.

Asparagus laricinus inhibited bacterial growth of all selected micro-organisms. However, *Staphylococcus spp* were resistant even at the highest tested concentration of 500 µg/ml. Methanol extracts of *Senecio asperulus* inhibited microbial growth even at the lowest concentration of 50 µg/ml. *Senecio asperulus* dichloromethane extract was active on most bacteria with MIC's between 50 µg/ml and 500 µg/ml. However, the water and methanol extracts of *Gunnera perpensa* had no activity against all organisms tested. *Asparagus laricinus* methanolic and aqueous extracts showed good antioxidant activity. Aqueous extracts of *Senecio asperulus* and *Gunnera perpensa* showed free radical scavenging activity yielding EC_{50} values of 100 µg/ml and 25 µg/ml, respectively.

The aqueous extracts of *Senecio asperulus* showed moderate anti-inflammatory activity from 50 to 200 µg/ml. while the methanol extracts were at 200 µg/ml and with no cytotoxicity. *Asparagus laricinus* showed weak anti-inflammatory activity when compared with resveratrol. No anti-inflammatory activity was observed from all

Gunnera perpensa extracts using LPS-induced macrophages, this suggests that this species may be using other mechanisms for anti-inflammatory activity. The antibacterial and antioxidant activities of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* justify the pharmacological uses of these plants. Furthermore, the anti-inflammatory activities observed from water extracts of *Asparagus laricinus* and *Senecio asperulus* support their ethnomedicinal use for the management of inflammation-related diseases. Sadly, this cannot be said with *Gunnera perpensa* as our findings do not support it.

4.1 INTRODUCTION

In traditional herbal practice in Africa, indigenous medicinal plants have been employed in the treatment of several important infections (Taylor et al., 2001). Medicinal plants play a vital role in the African continent's primary healthcare system as they are components of the most diverse and oldest African therapeutic systems. People in Africa use medicinal plants for self-medication and they can diagnose and treat minor ailments without even consulting a traditional doctor or herbalist (Moteetee and Van Wyk, 2011). This occurs particularly in remote rural areas with limited access to health facilities, as Africa comprises of so many developing countries. African populations suffer from chronic diseases whose treatment and follow-up create a major economic problem for them (Konkon et al., 2008).

Of all the alternative modalities, herbal medicine is probably the most popular and the most ubiquitous (Akerele, 1993) as it is easily accessible and less expensive. Interest in medicinal plant research has escalated, with the aim of identifying alternative medicinal therapies to overcome multi-drug-resistance, severe adverse effects and dose limitations of currently available drugs. The common link between carcinogenesis and bacterial infection, as well as carcinogenesis and DNA damage due to reactive unstable oxidants, is inflammation (Li et al., 2012). Inflammation is the protective response to injury of body cells and tissues due to exposure to various factors like infections, chemicals, heat and mechanical injuries. Chronic inflammation could be considered the basis of disease and this is a typical feature of many chronic disorders.

Infections by organisms which are resistant to killing and clearing by the body, tend to cause chronic inflammation. Excessive reactive oxygen species are closely involved in various human diseases such as inflammation, cancer, heart disease, aging, atherosclerosis, rheumatoid arthritis and Alzheimer's disease. It is also known that antioxidants neutralize excessive ROS which can induce the oxidative stress that causes cell damage and culminates in inflammation. Chronic inflammation is a major cause of cancer cells formation. *Asparagus laricinus, Senecio asperulus,* and *Gunnera perpensa* are traditionally used to treat cancer as well as other inflammation-related disorders (as discussed in Chapter 2 of this thesis). Thus, their preventative benefits against bacterial infections, oxidative stress, and inflammation are worth exploring.

4.2 LITERATURE REVIEW

4.2.1 Microorganisms

Microorganisms' that are associated with carcinogenesis have been recognized and documented for over a century now. These include Gram-negative pathogens such as *Escherichia coli* and Gram-positive pathogens such as *Staphylococcus aureus.* De Nunzio et al., (2011), considered inflammation due to *Escherichia coli* toxins to be a new domain in basic and clinical research in patients with prostate cancer and benign prostatic hyperplasia (BPH). *Escherichia coli* have been identified as being responsible for inflammatory response induction through toxins they secrete and have been linked with prostate cancer, while *Staphylococcus aureus* was described as a cancer-producing agent and was associated with breast cancer (Velázquez *et al*., 2010). However, its mechanism of action, the link between *Staphylococcus aureus* and breast cancer, was not investigated properly and reported, and thus still needs to be investigated to support these findings by Velázquez et al., 2010. Nonetheless, *Staphylococcus aureus,* together with type 16 papillomavirus (HPV-16), has also been found in the genome of different bacteria isolated from cervical cancer. According to Ma *et al*. (2009), HPV-16 induces cervical infection that leads to cervical cancer and *Staphylococcus aureus* is used as a vector by this virus. Thus, leading to inflammation and the inflammatory microenvironment favors the survival and proliferation of neoplastic cells (Protti and De, 2012; Li et al., 2012). Therefore, this indicates that the modulation of factors fueling chronic inflammation may have anticancer effects.

4.2.2 Reactive oxidative species

Oxygen is an important source of life and it is needed for some processes to occur in the body. However, an excess amount of oxygen could result in oxidative damage to the DNA of body cells. The damage is not due to the presence of oxygen, but rather to its role in the reduction of certain products to toxic free radicals. Free radicals are reactive oxidative species (ROS), such as superoxide anions, hydroxyl radicals, and hydrogen peroxide. As much as the formation of free radicals is mostly accounted for by external environmental factors (**Figure 4.1**), ROS's are also part of the living cell's normal metabolic processes, thus include, but not limited to; detoxification processes

and immune system defenses mechanisms. However, when these free radicals are excessively expressed, the body's natural antioxidant mechanisms get overpowered and fail to remove them (Larkins, 1999). This phenomenon is called oxidative stress.

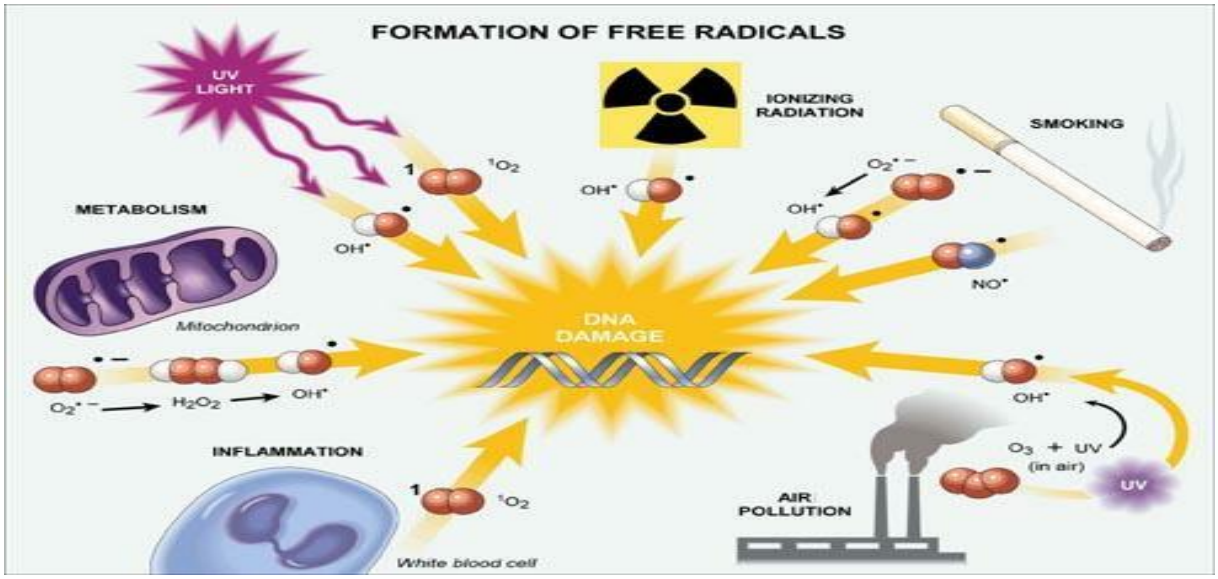


Figure 4.1: Diagram illustrating the formation of free radicals from the environment (Zander, 2013).

When this occurs, the normal cell structure gets disrupted and the DNA of the cell is altered. This then causes the gene mutations leading to carcinogenesis and the malfunctioning of the cell (Kerr et al., 1994). This cellular malfunction contributes to the development of various diseases and may lead to uncontrolled proliferation of abnormal cells. Moreover, it is known that in a state of oxidative stress, ROS can stimulate the release of cytokines and chemokines that drive recruitment and activation of additional inflammatory cells, including activated neutrophils and macrophages, and these play an important role in the pathogenesis of cancer (Auroma, 1998; Michael et al., 2006). Therefore, free radicals are important mediators that provoke inflammatory processes, and are neutralized by antioxidants that exert an anti-inflammatory effect (Filomena et al., 2008).

4.2.3 Inflammation

Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterized by persistent/chronic inflammation ultimately

resulting in tissue dysfunction. The inflammation process triggers the release of mediators; histamine, kinins, and prostaglandins, by damaged tissues which in turn involve cell membrane alterations, vascular permeability and increased protein denaturation (Leelaprakash and Mohan, 2011). Inflammation mediators are released as a result of induced cyclooxygenase 2 (COX-2), nitric oxide (NOS) and lipoxygenase (LP). Most non-steroidal anti-inflammatory drugs inhibit pathways that lead to the generation of inflammation mediators and this is essential for the treatment of inflammation. A good non-steroidal anti-inflammatory drug (NSAIDs) acts through the inhibition of induced nitric oxide synthase (iNOS), which is a key enzyme for the generation of nitric oxide (NO) and cyclooxygenase (COX) isozymes. There is dissatisfaction with existing anti-inflammatory drugs, as they impose toxicity and adverse side effects, particularly affecting the gastrointestinal and cardiovascular systems (Brune and Patrignani, 2015). Moreover, they lead to much dependence on them, as disorders reoccur after treatment has been discontinued. The bad side effects from the synthetic anti-inflammation drugs that are currently available have triggered a global trend of returning to natural sources of medicines. Thus, there is a significant increase in scientific and commercial interest in the discovery of new anti-inflammatory therapeutic and preventative agents from natural product sources. This part of the study was carried out to validate and justify the traditional claims for the use of *Asparagus laricinus, Senecio asperulus and Gunnera perpensa* in the treatment of inflammation-related diseases.

4.3 METHODOLOGY

4.3.1 Sterilization

Autoclavable materials such as agar and broth were aseptically sterilized in an autoclave at 121 ^{o}C for 15 minutes. Petri dishes, beakers, McCartney bottles, pipettes, test tubes, filter papers and other metal apparatus such as spatulas and forceps were sterilized using a hot air oven at a temperature of 160 ^{o}C for one hour. The wire loops were sterilized by heating them on the blue flame of the bunsen burner until red-hot and allowing them to cool and 70% alcohol was used to swab/clean the workbench area to prevent contamination. The process was carried out aseptically.

4.3.2 Extraction method

The powdered *Asparagus laricinus* cladodes, and roots of *Senecio asperulus* and *Gunnera perpensa* were soaked separately with distilled water (DH_2O), methanol (MeOH) and dichloromethane (DCM) for 72 hours with occasional stirring. Extracts were then filtered, and the aqueous extracts were lyophilized, while the organic solvent extracts were concentrated with rocket evaporator.

For anti-inflammatory activity only, extracts were reconstituted in dimethyl sulphoxide (DMSO) to give a final concentration of 100 mg/mL respectively. Stock solutions were sonicated where solubility was an issue. All samples were then stored at 4 ^oC.

4.3.3 Cell culture

The mouse macrophage cell line, RAW 264.7 were cultured and maintained in RAW 264.7 growth medium 1 (RAWGM1) at 37 ^oC in a humidified atmosphere with 5% CO_2. Suspensions of RAW 264.7 monolayer culture were seeded into 96 well microtiter plates at a density of 25 000 cells per well using a volume of 50 µl in each well. The microtiter plates were then incubated at 37 ^oC, 5% CO_2 and 100% relative humidity for 24 h prior to addition of test compounds to allow for cell attachment. The culture medium was then removed, and the samples were added to give final concentrations of 12.5 and 50 µM.

4.3.4 Microorganisms

The American Type Culture Collection (ATCC) microorganisms used in this study were donated from the Pathcare microbiology laboratory in Welkom, South Africa. Four Gram-positive bacteria (*Staphylococcus aureus* ATCC BAA-1026, *Staphylococcus saprophyticus* ATCC BAA-750, *Enterococcus faecalis* ATCC 29212, and *Streptococcus pneumoniae* ATCC 49619) and four Gram-negative bacteria (*Enterobacter cloacae* ATCC 700323, *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 35659, and *Klebsiella pneumoniae* ATCC 700603) were inoculated onto Muller-Hinton (MH) agar, incubated aerobically at 37 ^oC and later used for this study.

To produce bacterial counts that are within an expected range, all bacterial suspensions were respectively prepared using sterile saline to obtain an optical

density comparable to the density of 0.5 McFarland barium sulfate standard (turbidity =108 CFU/mL).

4.3.5 Control drugs

Discs (6 mm filter paper discs) dipped in saline were used as negative controls. Commercial chloramphenicol discs (25 µg) and ampicillin discs (10 µg), were used as positive controls against Gram-negative bacteria and Gram-positive bacteria, respectively.

4.3.6 *In vitro* antibacterial assay

The antibacterial activity was conducted using the disc diffusion method as adopted from Thitilertdecha et al., (2008) and Su et al., (2015). To determine the minimum inhibition concentration (MIC) of each plant extract, selected Gram-positive and Gram-negative micro-organisms were treated with 4 concentrations of plant extracts from serial dilution namely: 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml. After serial dilutions were prepared, 6 mm filter paper discs were impregnated with each of the different dilutions. Extract loaded discs were then placed on the surface of the agar inoculated with different micro-organisms as mentioned previously. Plates were sealed with a sealing tape and incubated aerobically at 37 ^{o}C, and zones of inhibition were measured after 24 hours. The zones of inhibition were measured in mm using a caliper and the lowest concentration at which growth inhibition resulted, where the diameter was more than 11 mm, was considered as minimum inhibition concentration (MIC). The tests were performed in triplicates and the mean was calculated and reported.

Figure 4.2 A: Illustration of sensitivity showing zone of inhibition.

Figure 4.2 B: illustration of resistant organism showing no zone of inhibition

4.3.7 *In vitro* antioxidants assay

The DPPH (2,2-diphenyl-1picrylhyrazyl) free radical scavenging assay was performed using a method by Najafabad and Jamei (2014). Two hundred microliters (200 µL) of 0.1 mM DPPH prepared in methanol was added to 100 µL of the plant extract diluted in different concentrations. The mixture was incubated in the dark at room temperature for 30 minutes. Absorbance was then measured at 517 nm. Ascorbic acid was used as a positive control. The experiments were performed in triplicates and percentage inhibition of the DPPH radical scavenging activity was calculated using the formula:

% Inhibition=[(Ac-A1)/Ac] X 100

Where Ac is the absorbance of the control and A1 is the absorbance of the sample.

4.3.8 Anti-inflammation assay

4.3.8.1 Anti-inflammatory activity in RAW 264.7 macrophages

To assess the anti-inflammatory activity of the plant extracts, 50 µl of Lipopolysaccharide (LPS) containing medium was added to the 96-well plates with RAW 264.7 treated with plant extracts (as mentioned above under cell culture). Cells were then incubated for 20 hours. To quantify nitric oxide (NO) production, 50 µl of the above culture medium with LPS was transferred to a new 96-well plate and 50 µl Griess reagent added. Absorbance was then measured at 540 nm wavelength and the results were expressed relative to the appropriate untreated control. A well-known inhibitor of Inducible nitric oxide synthase (iNOS) expression, resveratrol, was used as a positive control.

4.3.8.2 Toxicity check (MTT assay)

The MTT assay is a colorimetric assay used to reflect the number of viable cells present through the assessment of cell metabolic activity after treatment with test compounds. MTT, a yellow tetrazole, is reduced to purple formazan in living cells (Mosmann, 1983). A solubilization solution is then added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution is quantified spectrophotometrically by measuring light absorbance at a

wavelength of 600 nm. The degree of light absorption depends on the solvent. NAD(P)H-dependent oxidoreductase enzymes in the cytosolic compartment of the cell influences the reduction of tetrazolium dye (Berridge et al., 2005). Thus, MTT reduction depends on the cellular metabolic activity due to NAD(P)H flux.

To confirm that toxicity was not a contributory factor for anti-inflammatory activity, cell viability was assessed using the MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) assay (Van Meerloo et al., 2014). RAW 264.7 cells were seeded in 96 well microtiter plates at a density of 25 000 cells per well using a volume of 50 µl in each well and allowed to adhere for 24 hours at 37 ^{o}C in a carbon-dioxide (CO_2) incubator. Media was then carefully aspirated from adherent cell cultures, discarded and replaced with fresh media. Cells were then treated with various concentrations of the plant extracts and re-incubated for 24 hours at 37 ^{o}C. After incubation, the culture media was aspirated again and replaced with equal volumes of fresh media and MTT working solution (5 mg/mL in phosphate buffer solution). Plates were then incubated further for 4 hours at 37 ^{o}C. The media with MTT was removed and 100µl of DMSO solution was added into each well to solubilize the formed MTT formazan crystals (purple colour). Absorbance was measured at 570 nm wavelength and cell inhibition percentage was determined using the formula:

Percentage Cell Inhibition = [100- Abs (sample)/Abs (control)] X 100.

4.4 RESULTS

Antibacterial, antioxidant and anti-inflammatory activities of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* were investigated using extracts (methanol, aqueous and dichloromethane) that showed more phytochemicals as observed from the qualitative screening test performed and reported in **Chapter 3**. The selection of those three extracts was strengthened by their variation in their polarity levels as the broader conclusion can be made on pharmacological activity differences between hydrophobic and hydrophilic compounds. Numerous studies have shown that secondary metabolites are known to have important medicinal benefits to humans and have diverse pharmacological activities (Wagner and Elmadfa, 2003; Kappers et al., 2005; Krishnaiah et al., 2007; Rabi and Bishayee, 2009; Santhi

and Sengottuvel, 2016). This section, therefore, reports on the antibacterial, antioxidant and anti-inflammatory activities of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa.* To determine the antibacterial activity and minimum inhibitory concentration (MIC) of each plant extract, selected Gram-positive and Gram-negative micro-organisms were treated with different concentrations of plant extracts. The zone of inhibition was measured and compared to that of controls.

Minimum inhibition concentration (MIC) required to inhibit *S. faecalis* was 500 µg/ml for *Senecio asperulus* methanol extract and 50 µg/ml for the aqueous and DCM extracts. Aqueous extracts further showed MIC to be 50 µg/ml for most tested Gram-negative's and Gram-positive's, except for *S. aureus* and *S. saprophyticus*, as their MIC were at 100 µg/ml and 250 µg/ml, respectively (**Table 4.1**). *Senecio asperulus* further showed the MIC to be at 50 µg/ml for *S. faecalis, P. mirabilis,* and *K. pneumoniae* and at 100 µg/ml for *E. coli*. *E. cloacae* resisted the *Senecio asperulus* DCM extract on all other concentrations, besides the highest tested concentration of 500 µg/ml. However, the zone of inhibition was > 16 mm, thus still not significant. *Gunnera perpensa* DCM and all *Asparagus laricinus* extracts were very active with the MIC of 50 µg/ml for most organisms, besides *S. faecalis* for *Gunnera perpensa* and *S. aureus and S. saprophyticus* for *Asparagus laricinus* as there was growth even at the highest concentration of 500 µg/ml (**Table 4.1**).

Table 4.1: Antibacterial analysis of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa.*

Micro-organisms	[µg/ml]	*A1*	*A2*	*A3*	*S1*	*S2*	*S3*	*G1*	*G2*	*G3*	Control A	Control B
S. faecalis	500	++	++	+	+	+	+	-	-	-	++	-
	250	++	+	+	-	+	+	-	-	-		
	100	+	+	-	-	+	+	-	-	-		
	50	+	+	-	-	+	+	-	-	-		
S. pneumoniae	500	+	-	++	-	+	-	-	-	+	++	-
	250	+	-	+	-	+	-	-	-	+		
	100	+	-	+	-	+	-	-	-	+		
	50	+	-	+	-	+	-	-	-	+		
P. mirabilis	500	+	+	+	-	++	+	-	-	++	-	++
	250	+	++	+	-	+	+	-	-	+		
	100	+	+	+	-	+	+	-	-	+		
	50	+	+	+	-	+	+	-	-	-		
K. pneumoniae	500	+	+	+	-	++	+	-	-	++	-	++
	250	+	+	+	-	+	+	-	-	++		

Micro-organisms	[µg/ml]	*A1*	*A2*	*A3*	*S1*	*S2*	*S3*	*G1*	*G2*	*G3*	Control A	Control B
	100	+	+	+	-	+	+	-	-	+		
	50	+	+	-	-	+	+	-	-	+		
S. saprophyticus	500	+	+	-	-	+	-	-	-	+	++	-
	250	+	+	-	-	+	-	-	-	+		
	100	+	+	-	-	-	-	-	-	+		
	50	-	-	-	-	-	-	-	-	+		
S. aureus	500	+	+	-	-	+	-	-	-	+	++	-
	250	+	+	-	-	+	-	-	-	+		
	100	+	-	-	-	+	-	-	-	-		
	50	-	-	-	-	-	-	-	-	-		
E. coli	500	+	++	+	-	+	+	-	-	+	-	++
	250	+	++	+	-	+	+	-	-	+		
	100	+	+	+	-	+	+	-	-	+		
	50	+	-	+	-	+	-	-	-	+		
E. cloacae	500	++	++	+	-	+	+	-	-	++	-	++

Micro-organisms	[µg/ml]	*A1*	*A2*	*A3*	*S1*	*S2*	*S3*	*G1*	*G2*	*G3*	Control A	Control B
	250	**+**	**+**	**+**	-	+	-	-	-	+		
	100	**+**	**+**	**+**	-	+	-	-	-	+		
	50	**+**	**+**	**+**	-	**+**	-	-	-	**+**		

Abbreviations: ***A1=*** *Asparagus laricinus* methanol extract, ***A2=*** *Asparagus laricinus* aqueous extract, ***A3=*** *Asparagus laricinus* dichloromethane extract, ***S1=*** *Senecio asperulus* methanol extract***, S2=*** *Senecio asperulus* aqueous extract***, S3=*** *Senecio asperulus* dichloromethane extract***, G1***= *Gunnera perpensa* methanol extract***, G2=*** *Gunnera perpensa* aqueous extract***, G3***= *Gunnera perpensa* dichloromethane extract**, [++]** = sensitive with zone of inhibition ≥ 16 mm**, [+]** = sensitive with zone of inhibition ≤ 16 mm, **[-]** = resistant with no zone of inhibition. **Control A**= Ampicillin, **Control B**= Chloramphenicol. The MIC of each active extracts is presented with "**Bold**" cross signs (**+**) and yellow colouring.

The DPPH assay was used to investigate the free radical-scavenging activities of *Asparagus laricinus, Senecio asperulus,* and *Gunnera perpensa*. Activities were expressed as percentages in order to determine the plant extract concentration required to achieve a 50% DPPH scavenging activity (EC_{50}). The dose-dependent antioxidant activity of the tested extracts is summarized in **Figures 4.3-4.5**.

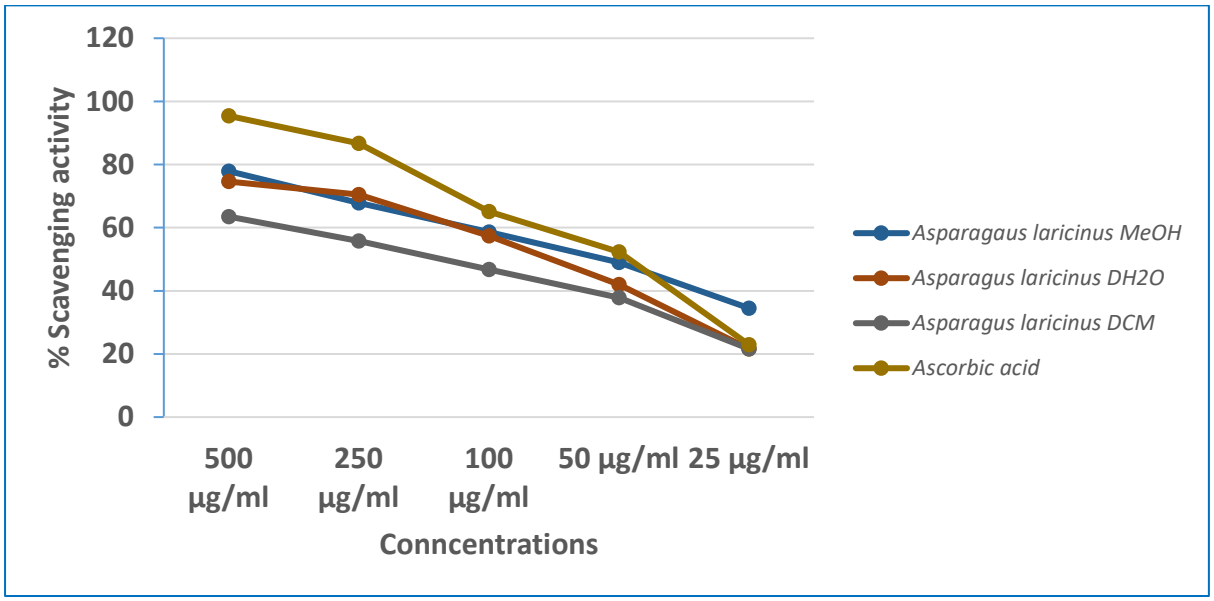


Figure 4.3: % Scavenging activity of *Asparagus laricinus* cladodes extracts versus ascorbic acid.

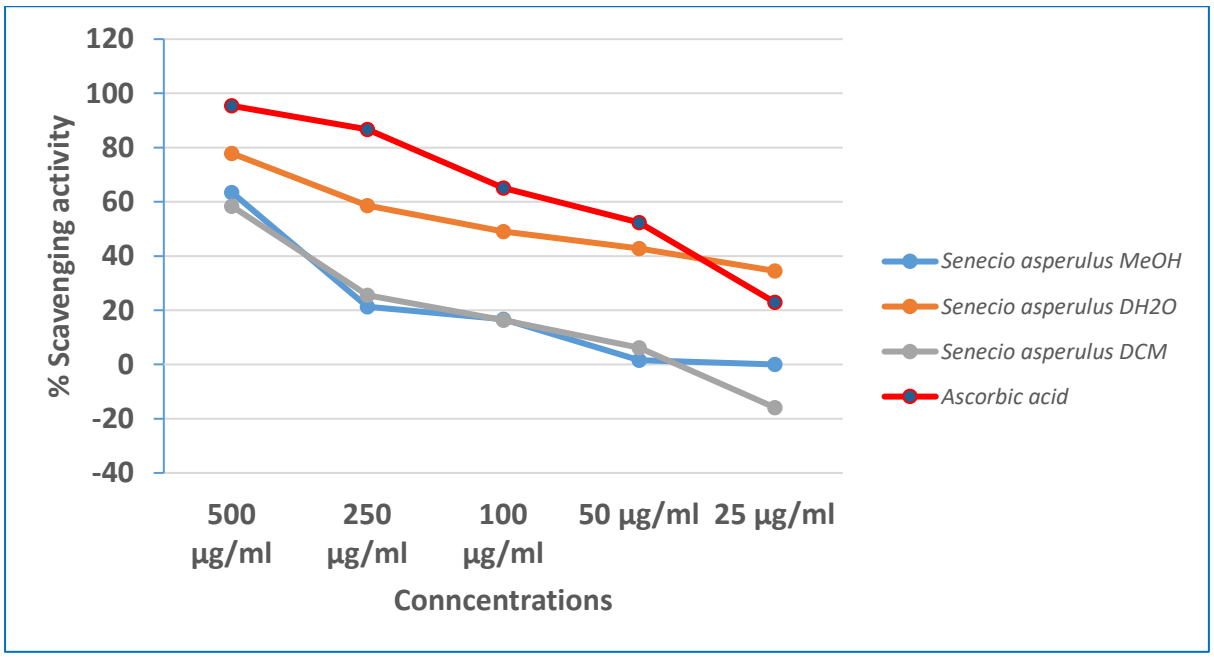


Figure 4.4: % Scavenging activity of *Senecio asperulus roots* extracts versus ascorbic acid.

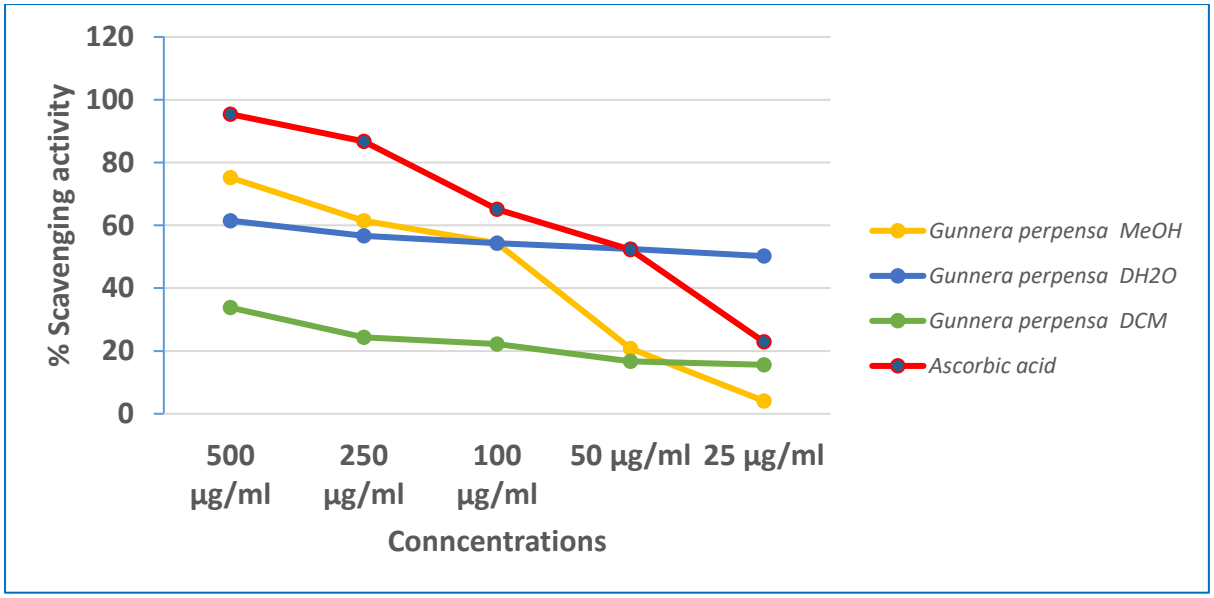


Figure 4.5: % Scavenging activity of *Gunnera perpensa roots* extracts versus ascorbic acid.

Changes in NO production were determined by measuring the levels of nitrate in the culture medium. **Figure 4.6** illustrated the levels of nitrate macrophage cells produce, nitrite and nitrate in response to LPS formed after macrophages were exposed to plant extracts. No anti-inflammatory activity was observed from all *Gunnera perpensa* extracts using LPS-induced macrophages even at the highest concentration of 200 µg/ml, this suggests that this species may be using other mechanisms for anti-inflammatory activity. However, at the same concentration of 200 µg/ml *Asparagus laricinus* methanolic and aqueous extracts showed minimum activity, which is not clinically significant.

Senecio asperulus methanol and aqueous extracts exhibited anti-inflammatory potential, that was moderate when compared with that of the positive control, resveratrol. There was a very high observed anti-inflammatory activity resulting from *Senecio asperulus* DCM extract, even when compared with the activity of the control. *Senecio asperulus* showed an ability to suppress NO production by down-regulating the expression of iNOS in RAW 264.7 macrophages.

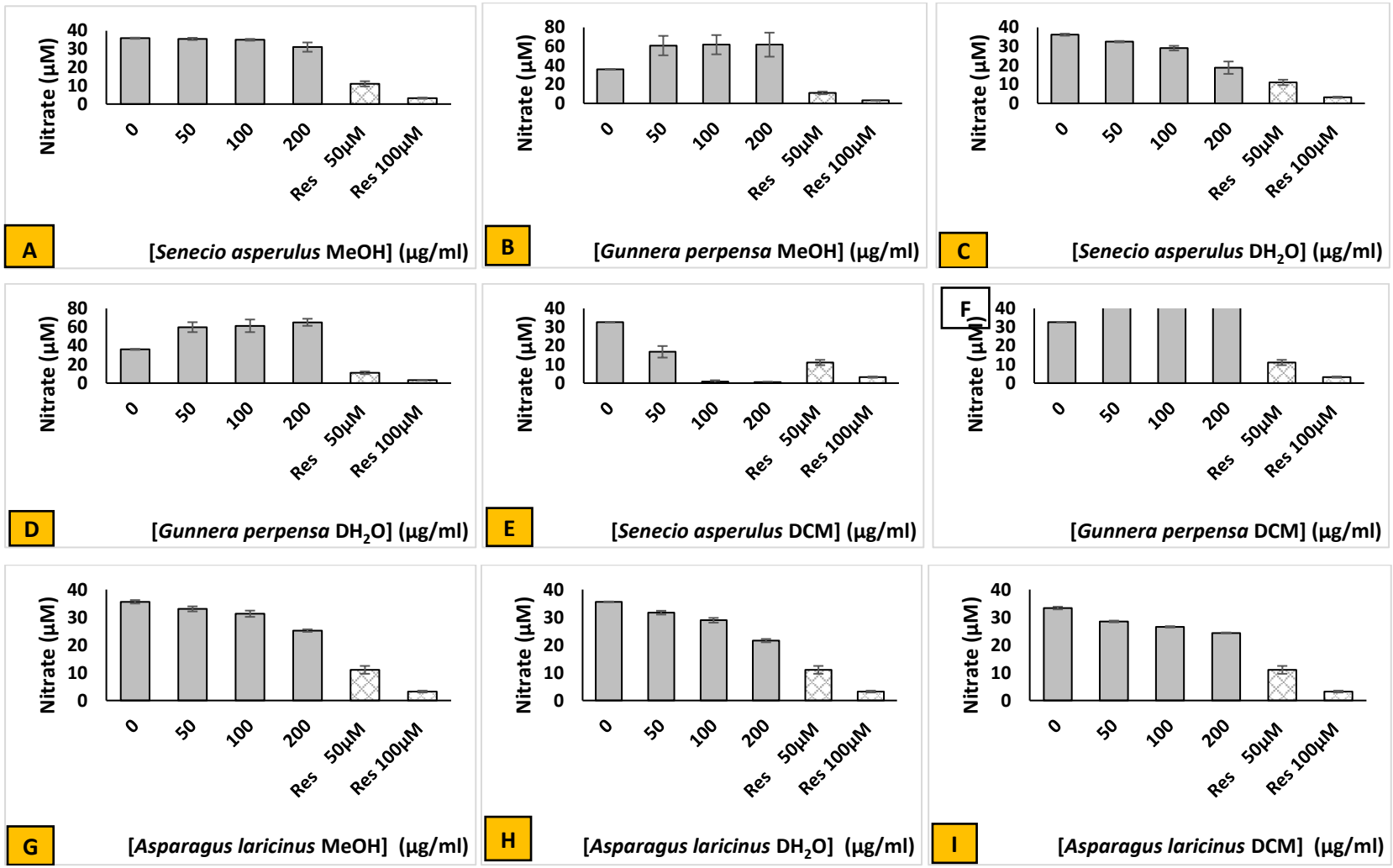
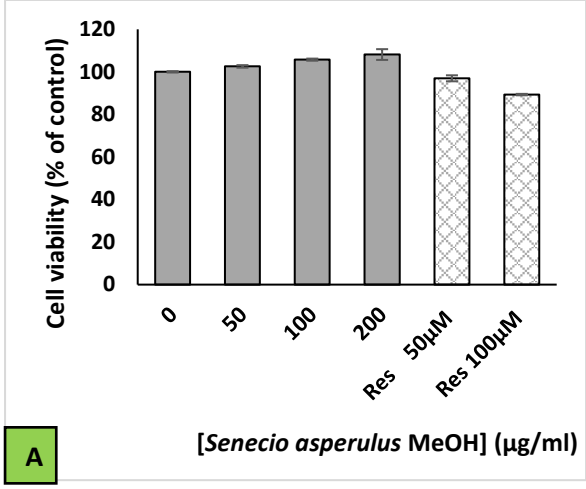
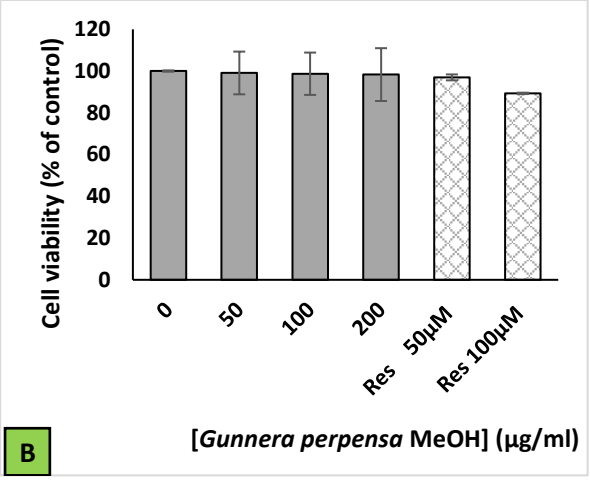
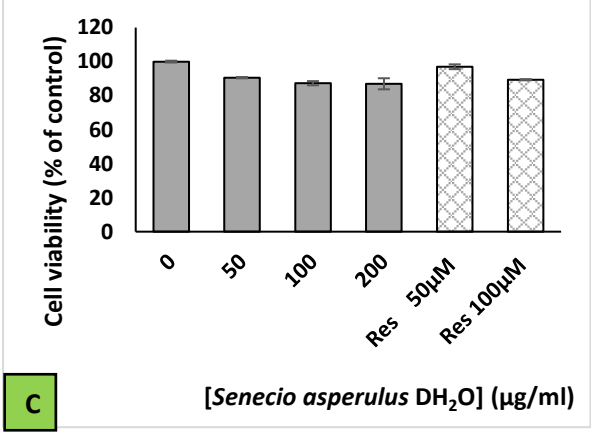
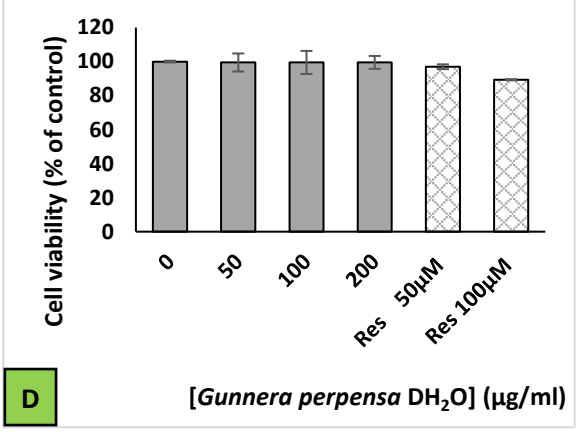


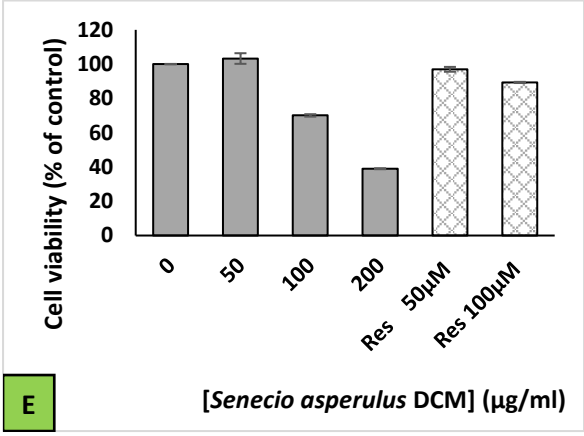
Figure 4.6 A-I: Nitric oxide production in LPS activated macrophages treated with different concentrations of *Asparagus laricinus*, *Senecio asperulus* and *Gunnera perpensa* extracts compared with resveratrol.

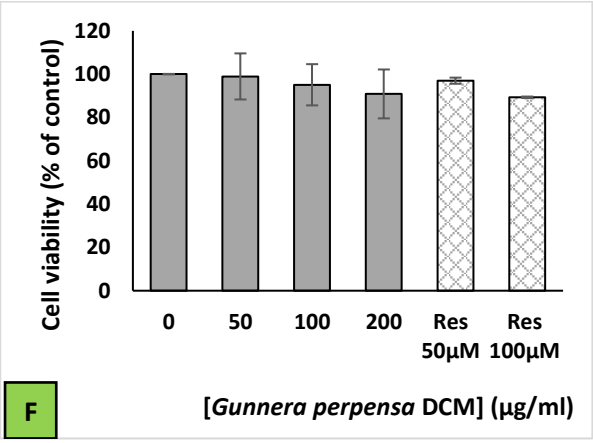












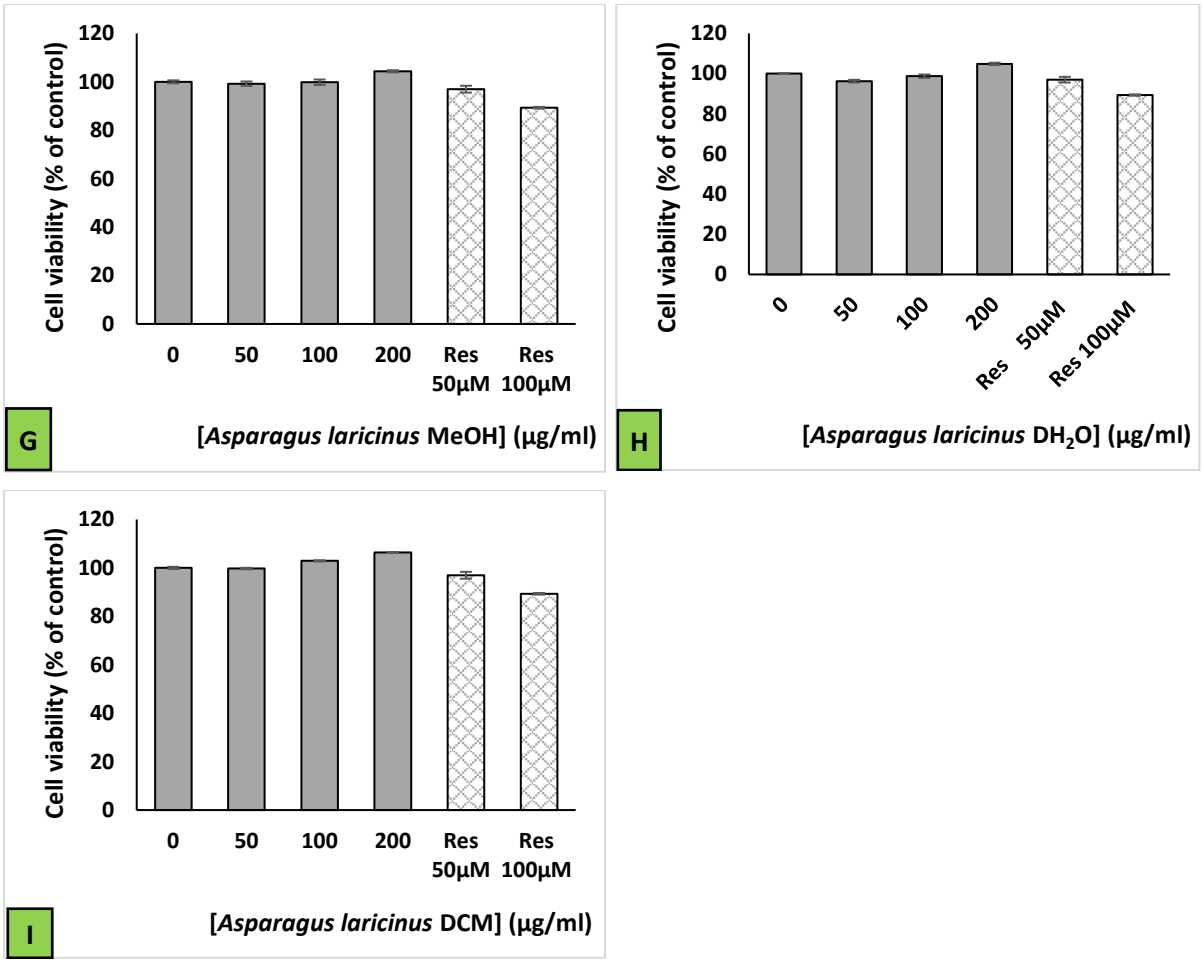


Figure 4.7 A-I: Cytotoxicity evaluation of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* different extracts in a macrophage cell line, RAW 264.7.

To confirm the absence of toxicity as the anti-inflammation results contributory factor, cell viability was assessed using MTT and reported in **Figure 4.7**.

4.5 DISCUSSION

4.5.1 Antibacterial activity

Both aqueous and methanolic extracts of *Asparagus laricinus* inhibited bacterial growth of all Gram-negatives and Gram-positives investigated in this study (**Table 4.1**). This shows that *Asparagus laricinus* is rich in secondary metabolites that this plant uses to protect itself from microorganisms as it shows good antibacterial activity. We can't, however, overlook the fact that *S. saprophyticus* and *S. aureus,* which both

belong to the genus *Staphylococcus*, were resistant to the dichloromethane extracts of this plant species. It has been reported that *Staphylococcus* strains could develop resistance and have waves of resistance as their virulence factors interfere with bacterial killing (Chambers and DeLeo, 2009). These strains have an ability to produce agents that hydrolyze antimicrobial agents, and their resistance is often a result of horizontal gene transfer that occurs when spontaneous mutations and positive selections develop (Chambers and DeLeo, 2009; Pantosti et al., 2007). Thus, this could be the reason for this observed resistance to even high concentrations of *Asparagus laricinus* plant extracts.

The methanolic extracts of both *Senecio asperulus* and *Gunnera perpensa* failed to inhibit the growth of the Gram-positive and negative microorganisms selected for this study even at higher concentrations of 500 µg/ml, except for *S. faecalis* (**Table 4.1**). However, both these plants showed the presence of most phytochemicals that have antimicrobial activities. A study by Ngo et al., (2017) revealed that organic solvents used for extraction do affect the levels of yielded phytochemicals contents from plants. Thus, this inactivity could be that flavonoids which were extracted by methanol solvent were either in small quantities or not active against selected micro-organisms. The most active plant extract was *Senecio asperulus* aqueous extract as it managed to inhibit the growth of all strains tested against. These findings were not peculiar as the same plant extract showed to possess most secondary metabolites (**Table 3.1, Chapter 3**) when compared with other plant extracts and our phytochemical analysis results agreed with findings by Mugomeri et al., (2014). Moreover, this plant is traditionally extracted with water for its reported ethnobotanical uses, thus, these results support its traditional medicinal applications. *Gunnera perpensa* dichloromethane extracts also inhibited most microorganisms and only one organism (*S. faecalis*) was resistant even at the highest concentration. The antibacterial activity of *Senecio asperulus* aqueous extract and *Gunnera perpensa* dichloromethane extract was related to the presence of diverse phytochemical contents observed in **Table 3.1, Chapter 3**.

4.5.2 Antioxidant activity

Antioxidant properties produced by plant species have a full range of applications in human healthcare. However, knowledge of the potential antioxidant compounds present in a plant species does not necessarily indicate its antioxidant capacity, as the total antioxidant effect may be greater than the individual antioxidant activity of one compound, owing to synergism between different antioxidant compounds in the same plant. Total antioxidant capacity can be assessed and measured through the use of different assays such as: DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), FRAP (ferric reducing antioxidant power), PFRAP (Polarized Fluorescence Recovery After Photobleaching) assay, CUPRAC (Cupric Reducing Antioxidant Capacity), Hydroxyl Radical Antioxidant Capacity (HORAC) and ORAC (Oxygen Radical Absorbance Capacity), to name a few. All the previously mentioned assays are easy to conduct; however, DPPH is mostly used especially for natural products such as plant extracts and food. DPPH assay results can either be reported with the supporting results from any of the other antioxidant activity assays or even on their own due to the accuracy, sensitivity and validity of this assay (Kedare and Singh, 2011). This was further corroborated by Sah and Modi (2015), as they found DPPH assay to be the most preferred method as it can be rapidly performed and has high reproducibility when compared to other antioxidant capacity assays.

This section reports on the antioxidant activities of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* plant extracts *in vitro*. *Asparagus laricinus* water and DCM extracts EC_{50} was at 100 µg/ml and that of the methanolic extract at 50 µg/ml, thus, showing that the methanol extract had strong antioxidant potential. This was unexpected as this extract did not show the presence of either flavonoids or tannins, but only showed the presence of terpenoids. This could imply that levels of terpenoids in this extract were very high and stabilized an unstable DPPH free radical. The aqueous extract of S*enecio asperulus* had an EC_{50} of 100 µg/ml and managed to scavenge about 78% DPPH at the highest concentration of 500 µg/ml. *Gunnera perpensa* aqueous extract EC_{50} was observed at the lowest concentration of 25 µg/ml, whereas ascorbic acid EC_{50} was only at 50 µg/ml, which is two-fold. This was very impressive as it suggests *Gunnera perpensa* to be a very strong antioxidant agent

when compared to the standard. There was a directly proportional relationship between the presence of both flavonoids and tannins and the antioxidant activity of *Asparagus laricinus, Senecio asperulus* methanol and *Gunnera perpensa* aqueous extracts. Thus, indicating that these phenols; flavonoids and tannins, were the leading contributors to the observed antioxidant activities.

4.5.3 Anti-inflammatory activity

Cell walls of gram-negative bacterial have lipopolysaccharides (LPS), which are endotoxins that can cause inflammation. The host response to LPS is known to be mediated by reactive oxygen species (ROS) as they lead to Rac activation and IL-1 expression, which are required to induce inflammation response (Hsu and Wen, 2002; Antonicelli et al., 2004; Hsu et al., 2004). The mouse macrophage cell line, RAW 264.7, is a well-characterized and popular model to investigate the anti-inflammatory potential of test samples. When macrophage cells are activated, various highly reactive oxidants including nitric oxide (NO), a product of the catalysis of arginine by the enzyme inducible nitric oxide synthase (iNOS) are produced (Sangwon and Prem, 2000). Changes in NO production reported in this chapter were determined by measuring the levels of nitrate in the culture medium.

According to Chen *et al* (2011), non-lectin glycoprotein (PCP-3A) inhibits LPS-induced production of NO through the down-regulation of iNOS via a transcription factor NF-JB mechanism. Thus, this could suggest that anti-inflammatory activity observed from *Senecio asperulus* (**Figure 4.6**) may be through the mechanism involving the transcription factor NF-kB. However, this remains to be elucidated through further investigations. Furthermore, it is known that LPS induces the expression of iNOS through redox-sensitive signaling pathways where superoxide anions act as second messengers. Antioxidants inhibit redox-active signaling by blocking radical producing enzymes (Jedinak et al., 2011). Thus, the antioxidant potential of this *Senecio asperulus* and *Asparagus laricinus* could be responsible for the observed anti-inflammatory activity. The lipopolysaccharide-induced anti-inflammatory activity assay was carried out simultaneously with the evaluation of cell viability (MTT assay) to confirm the absence of cytotoxicity of the test samples as this can be mistaken with the production of nitrate from the anti-inflammation activity of the plant.

Asparagus laricinus and *Gunnera perpensa* showed no evidence of cytotoxicity on RAW 264.7 cells. Nonetheless, methanol and aqueous extracts of *Senecio asperulus* showed no significant toxicity towards RAW 264.7 cells (**Figure 4.7, A and C**). However, the toxicity of the DCM extract of *Senecio asperulus* did override the meaningful prediction of anti-inflammatory potential from **Figure 4.6, E**. Thus, re-evaluation at lower concentrations below 50 µg/ml may provide more conclusive results as toxicity remains a potential risk for continual use of any medicine, not excluding natural medicinal plants.

4.6 CONCLUSION

Phytochemicals reported in **chapter 3** showed that *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* are rich in phytochemicals especially flavonoids, tannins, saponins, and alkaloids. These phytochemicals are known for their pharmacological activities such as anti-oxidant, antidiabetic, antibacterial, antiviral, anti-inflammatory, anti-diarrheal, hepatoprotective and radioprotective effects, amongst others. Thus, based on our antibacterial, antioxidant, anti-inflammatory and cytotoxicity results, we suggest that *Asparagus laricinus* and *Senecio asperulus* aqueous and methanol extracts could be good candidates for potent anti-inflammatory, antibacterial and novel antioxidant drugs development, as they had no cytotoxicity effects on tested cells. Furthermore, our results validated the Basotho traditional medicinal use of *Senecio asperulus* and *Gunnera perpensa* and South African uses of *Asparagus laricinus* and *Gunnera perpensa* for the treatment of inflammation-related conditions and for the treatment of wounds (due to their antibacterial, antioxidant and anti-inflammatory activity). However, further studies for the purification of bioactive compounds (so as to synthesize these active compounds in order to promote their conservation) and *in vivo* studies are recommended for the evaluation of these active extracts as an effective anti-inflammatory, antibacterial and antioxidant agents.

4.7 REFERENCES

Akerele O. 1993. Nature's medicinal bounty: Don't throw it away. World Health Forum. 14:390-395.

Antonicelli F, Brown D, Parmentier M, Drost E.M, Hirani N, Rahm D.K, MacNee W. 2004. Regulation of LPS-mediated inflammation *in vivo* and *in vitro* by the thiol antioxidant nacystelyn. Am. J. Physiol. Lung Cell Mol. Physiol. 286:1319-1327.

Auroma O.I. 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. Journal of the American Oil Chemists Society. 75:199-212.

Berridge M.V, Herst P.M, Tan A.S. 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol Annu Rev. 11:127-152.

Brune, Patrignani. 2015. New insights into the use of currently available non-steroidal anti-inflammatory drugs. J Pain Res. 8:105-118.

Chambers H.F, DeLeo F.R. 2009. Waves of Resistance: Staphylococcus aureus in the Antibiotic Era. Nat Rev Microbiol. 7(9):629–641.

Chen J.N, De Mejia E.G, Wu J.S.B. 2011. Inhibitory effect of a glycoprotein isolated from golden oyster mushroom (Pleurotus citrinopileatus) on the lipopolysaccharide-induced inflammatory reaction in RAW 264.7 macrophage. J Agric Food Chem. 59(13):7092-7097.

De Nunzio C, Kramer C, Marberger M, Montironi R, Nelson W, Schröder F, Sciarra A, Tubaro A. 2011. The controversial relationship between benign prostatic hyperplasia and prostate cancer: The role of inflammation. European Urology. 60:106-117.

Filomena C, Silvio S, Mariangela M, Federica M, Giancarlo A.S, Dimitar U, Aurelia T, Francesco M, Roberto D.L. 2008. In vivo anti-inflammatory and in vitro antioxidant activities of Mediterranean dietary plants. Journal of Ethnopharmacology. 116:144-151.

Hsu H.Y, Wen M.H. 2002. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. J Biol Chem. 277(25):22131-9.

Hsu B.G, Yang F.L, Lee R.P, Peng, T.C, Harn, H.J, Chen H.I. 2004. N-Acetylcysteine Ameliorates Lipopolysaccharide-Induced Organ Damage in Conscious Rats. J Biomed Sci. 11:152-162.

Jedinak A, Dudhgaonkar S, Wu Q, Simon J, Sliva D. 2011. Anti-inflammatory activity of edible oyster mushroom is mediated through the inhibition of NF-B and AP-1 signaling. Nutr J. 10:52-59.

Kappers I.F, Aharoni A, van Herpen T.W, Luckerhoff L.L, Dicke M, Bouwmeester H.J. 2005. Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. Science. 309:2070-2072.

Kedare S.B, Singh R.P. 2011Genesis and development of DPPH method of antioxidant assay. J Food Sci Technol. Aug; 48(4): 412–422.

Kerr J.F.R, Winterford C.M, Harmon B.V. 1994. Apoptosis — its significance in cancer and cancer therapy. Cancer. 73:2013-2026.

Konkon N.G, Adjoungoua A.L, Manda P, Simaga D, N'Guessan K.E, Kone B.D. 2008. Toxicological and phytochemical screening study of Mitragyna inermis (willd.) O ktze (Rubiaceae), anti-diabetic plant. Journal of Medicinal Plants Research. 2:279-284.

Krishnaiah D, Rosalam S, Awang B. 2007. Phytochemical antioxidants for health and medicine A move towards nature. Biotechnol. Mol. Biol. Rev. 1(4):097-104.

Larkins N.J. 1999. Free radical biology and pathology. Journal of Equine Veterinary Science. 19:84-89.

Leelaprakash G.S, Mohan D.S.M. 2011. *Invitro* anti-inflammatory activity of methanol extract of *Enicostemma axillare*. Int. j. drug dev. res. 3(3):189-196.

Li S, Wang N, Brodt P. 2012. Metastatic cells can escape the proapoptotic effects of TNF-alpha through increased autocrine IL-6/STAT3 signaling. Cancer Research. 72:865–875.

Ma Z, Liu L, Zhang F, Yu M, Wang K, Luo J, et al. 2009. Human papillomavirus type 16 exists in bacteria isolated from cervical cancer biopsies. J Int Med Res. 37:1065 74.

Michael K.M, Lawrence T, Nizet V. 2006. Innate immunity gone awry: Linking microbial infections to chronic inflammation and cancer. Cell. 124:823–835.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65(1–2):55–63.

Moteetee A, Van Wyk B. 2011. The medical ethnobotany of Lesotho: a review. Bothalia. 41(1):209–228.

Mugomeri E, Chatanga P, Hlapisi S, Rahlao L. 2014. Phytochemical characterization of selected herbal products in Lesotho. Lesotho Med Asso J. 12:38-47.

Najafabad A.M, Jamei R. 2014. Free radical scavenging capacity and antioxidant activity of methanolic and ethanolic extracts of plum (*Prunus domestica L*.) in both fresh and dried samples. Avicenna J Phytomed. 4(5):343–353.

Ngo T.V, Scarlett C.J, Bowyer M.C, Ngo P.D, Vuong Q.V. 2017. Impact of Different Extraction Solvents on Bioactive Compounds and Antioxidant Capacity from the Root of Salacia chinensis L. Journal of Food Quality. Article ID 9305047, 8 pages https://doi.org/10.1155/2017/9305047.

Pantosti A, Sanchini A, Monaco M. 2007. Mechanisms of antibiotic resistance in Staphylococcus aureus. Future Microbiol. (3):323-34.

Shah P, Modi H.A. 2015. Comparative Study of DPPH, ABTS and FRAP Assays for Determination of Antioxidant Activity. International Journal for Research in Applied Science & Engineering Technology (IJRASET). 3(6):636-641.

Protti M.P, De M.L. 2012. Cross-talk within the tumor microenvironment mediates Th2-type inflammation in pancreatic cancer. Oncoimmunology. 1:89-91.

Rabi T, Bishayee A. 2009. Terpenoids and breast cancer chemoprevention. Breast Cancer Res Treat. 115:223-239.

Sangwon K, Prem P. 2000. Effects of Interferon-g and Lipopolysaccharide on Macrophage Iron Metabolism Are Mediated by Nitric Oxide-induced Degradation of Iron Regulatory Protein 2. J Biol Chem. 275(9):6220–6226.

Santhi K, Sengottuvel R. 2016. Qualitative and Quantitative Phytochemical analysis of *Moringa concanensis* Nimmo. Int.J.Curr.Microbiol.App.Sci. 5(1):633-640.

Su P, Yang C, Yang J, Su P, Chuang L. 2015. Antibacterial Activities and Antibacterial Mechanism of Polygonum cuspidatum Extracts against Nosocomial Drug-Resistant Pathogens. Molecules. 20(6):11119-11130.

Taylor J.L.S, Rabe T, McGraw L.J, Jager A.K, van Staden J. 2001. Towards the scientific validation of traditional medicinal plants. Plants Growth Regulation. 34:23-37.

Thitilertdecha N, Teerawutgulrag A, Rakariyatham N. 2008. Antioxidant and antibacterial activities of *Nephelium lappaceum L.* extracts. LWT-Food Sci Technol. 41(10):2029-2035.

Van Meerloo J, Kaspers G.J.L, Cloos J. 2014. Cell sensitivity assays: The MTT assay. In: Cree IA, editor. Cancer cell culture: Methods and protocols. New York: Springer Science + Business. pp. 237-45.

Velázquez E, Peix Á, Gómez-Alonso A. 2010. Microorganisms and cancer: Scientific evidence and new hypotheses. CIR ESP. 89(3):136-144.

Wagner K.H, Elmadfa I. 2003. Biological relevance of terpenoids: Overview focusing on mono-di and tetraterpenes. Ann Nutr Metab.47:95-106.

Zander A. 2013. Human aging: Free radical formation theory. Available from http://alex-zander.hubpages.com/hub/Free-Radical-Theory. Accessed 03 January 2014

CHAPTER 5

"Anticancer activity and cell cycle analysis of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa*"

ABSTRACT

Medicinal plants play an important role in our African communities for treatment and prevention of various diseases including cancer. This chapter was aimed at evaluating the anticancer activities of *Asparagus laricinus Burch., Senecio asperulus DC.* and *Gunnera perpensa L. In vitro* anticancer screening was carried out using fluorescent cellular stains on human prostate cancer (PC3), human breast cancer (MCF-7) and the non-cancerous African green monkey kidney (Vero) cell lines. The cells were imaged with the ImageXpress Micro XLS Widefield fluorescent microscope, and the acquired images were analyzed using the MetaXpress software and the Multi-Wavelength cell scoring application module. Melphalan was used as a positive control in all experiments. *Asparagus laricinus* methanol and *Senecio asperulus DC.* dichloromethane extracts exhibited cytotoxicity activity against breast cancer cells with IC_{50} values of 97.6 µg/mL and 69.15 µg/mL, respectively. *Gunnera perpensa* DCM extract also showed cytotoxicity on prostate and breast with less activity on Vero cells. Cell cycle analysis suggested that *Asparagus laricinus* methanol extract induced cell death selectively through apoptosis observed from Annexin V-FITC and PI stain. Cell cycle analysis also showed that *Senecio asperulus DC.* dichloromethane extracts induced breast cancer cell death through cell arrest at the synthesis phase and G2 phase. *Senecio asperulus DC.* dichloromethane extracts further showed cytotoxicity activity against prostate cancer cells with IC_{50} values of 69.25 µg/mL due to cell arrest at the G2 and early mitotic (G2/M) phase. We, therefore, propose that the methanol extract of *Asparagus laricinus* is a suitable aspirant for future breast cancer chemotherapeutic drug, due to its selective cytotoxicity on cancer cells and not on non-cancerous cells.

5.1 INTRODUCTION

Cancer is a serious public health problem and it continues to be the leading cause of mortality and morbidity worldwide (Lee et al., 2014; Ogbole et al., 2017). In the African region, the most common cancers are breast, cervical, liver and prostate cancer (WHO, 2017). Prostate cancer is leading cancer in males: with nearly 1 in 5 men to be diagnosed with this illness during their lifetime and more than 4000 men being diagnosed with prostate cancer every year in South Africa (Sylla and Wild, 2012; Siegel et al., 2018). Breast cancer is also a growing health problem in sub-Saharan Africa (Akarolo-Anthony et al., 2010; Jemal et al., 2012; Sylla and Wild, 2012) and has now surpassed cervical cancer as the leading cause of death in many countries, with 94 378 new cases of breast cancer diagnosed annually (Akarolo-Anthony et al., 2010; Siegel et al., 2018). Additionally, the number of other types of cancer cases and death are estimated to increase over the next two decades (WHO, 2017), despite current advancements in scientific knowledge.

Several chemotherapeutic agents are available and in use for the management of cancer, nevertheless, the problem of indiscriminate toxicity and serious adverse events still exist (Ogbole et al., 2017). Thus, there is a significant increase in scientific and commercial interest in the continued discovery of novel anticancer agents from natural product sources. Herbal medicine has always been one of the main components of healthcare systems for ages. However, most ethnobotanical claims have not yet been investigated scientifically. The investigation of traditionally used medicinal plants is valuable as a source of potential chemo-preventative and chemotherapeutic agents. Plant-derived natural products provide an interesting source for isolating and screening potent molecules to combat a variety of ailments, including the modern disease “cancer”. Thus, the anticancer analysis of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* were performed against breast cancer cell line (MCF-7), prostate cancer cell line (PC3) and non-malignant African green monkey kidney cell line (Vero).

5.2 LITERATURE REVIEW

Natural products are an excellent source of compounds with a wide variety of biological activities and there exist numerous mechanisms by which phytochemicals aid in the prevention of cancer. These mechanisms of phytochemical chemo-preventive agents are either blocking agents or suppressing agents (Ugbogu *et al*., 2013). The preventative action most probably results from the additive or synergistic effects of several phytochemicals present in a certain part of the plant. Cancer is a multi-step process thus phytochemicals' chemo-preventive mechanism for an effective anticancer agent may include anti-inflammatory activity, free radical scavenging activity, antiproliferative activity, cell-cycle arresting activity, induction of apoptosis, and the activation of enzyme cofactors (Manson *et al*., 2000; Juge *et al*., 2007). As much as medicinal plants are rich in biologically active secondary metabolites, there are only four structural classes of plant anticancer agents which are currently available, and these are constituted by the Catharanthus (Vinca), alkaloids (vinblastine, vincristine, vinorelbine), the epipodophyllotoxins (etoposide, etoposide phosphate, teniposide), the taxanes (paclitaxel and docetaxel), and the camptothecin derivatives (irinotecan and topotecan) (Cragg et al., 1997).

Secondary metabolites with many biochemical and pharmacological properties, including cancer-preventative effects are flavonoids (Williams and Grayer, 2004; Moon et al., 2006). Biochemical interferences produced by flavonoids are associated with their capacity to control cell growth through certain mechanisms. Flavonoids can prevent carcinogenesis by interfering with different steps of benign tumor cells formation that may progress to malignant tumors as illustrated in **Figure 5.1.** These mechanisms include their free radical scavenging ability, the modification of enzymes to activate or detoxify carcinogens and the inhibition of the induction of the transcription factor activator protein activity by tumor promoters (Canivence-Lavier *et al*., 1996; Shih *et al*., 2000; Moon *et al.*, 2006).

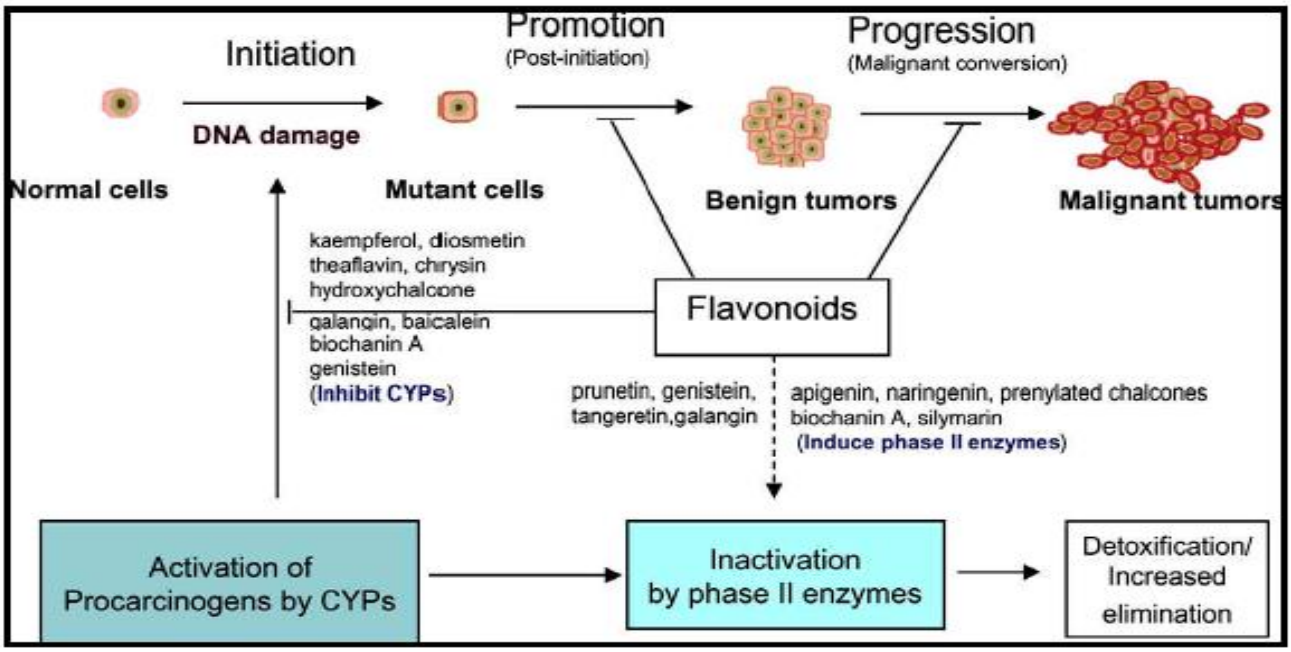


Figure 5.1: Flavonoids blocking and suppressing multi-stage carcinogenesis (Moon *et al.,* 2006).

5.3 METHODOLOGY

5.3.1 Sample preparation

The plant extracts were reconstituted in dimethyl sulphoxide (DMSO) to give a final concentration of 100 mg/mL, respectively. Stock solutions were sonicated if necessary were solubility was an issue. All samples were then stored at 4 ^{o}C.

5.3.2 Cell culture

The human prostate cancer cell line, PC3; human breast cancer cell line, MCF7 and the African green monkey kidney cell line, Vero were cultured in DMEM (Dulbecco's Modified Eagle's medium) and 10% FBS (Fetal bovine serum). Suspensions of monolayer cultures of cells were seeded into 96 well microtiter plates at a density of 6000 cells/well using a volume of 100 µl in each well. The microtiter plates with cells were incubated at 37 ^{o}C, 5% CO_2, and 100% relative humidity for 24 hours prior to addition of test compounds to allow for cell attachment.

5.3.3 *In vitro* anticancer assay and IC_{50} determination

Anticancer activity was studied using a high content screening approach, and nuclear morphological changes were visualized by Hoechst 33342 staining after cells were treated with 50 and 200 µg/mL of each extract. One hundred microliter aliquots of the diluted extract in the fresh medium were used to treat cells. Cells were incubated at 37 ^{o}C in a humidified 5% CO_2 incubator for 48 hours. Melphalan was used as a positive control. Treatment medium was aspirated from all wells and replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL in phosphate-buffered saline) and incubated for 10 minutes at room temperature. Thereafter, cells were stained with propidium iodide (PI) at 100 µg/mL to enumerate the proportion of dead cells within the population. Cells were imaged immediately after addition of PI using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices). DNA distribution was analyzed using propidium iodide (PI), a double-stranded DNA-binding dye. Cells first need to be fixed and permeabilized to allow the entry of PI stain into the cell (Krishan, 1975). For the *in vitro* IC_{50} determination, cells were treated with increasing concentrations of each extract prepared from serial drug dilutions, 6.25 – 300 µg/mL. Concentration to inhibit 50 percent of the cell population was determined and only those obtained IC_{50} value concentrations were used for cell cycle analysis.

5.3.4 *In vitro* cell-cycle analysis

Cell cycle analysis was performed by propidium iodide (PI) based measurements of the DNA content of the cells by flow cytometry. One hundred microliters aliquots of the diluted compound in the fresh medium was used to treat cells. Cell lines were incubated at 37 ^{o}C in a humidified 5% CO_2 for 48 hours. Melphalan was used as a positive control. Treatment medium was removed from all wells and replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL) and Annexin V-FITC and then incubated for 10 minutes at room temperature. The plates were then inspected under an inverted microscope to guarantee growth and images were then acquired on the ImageXpress Micro XLS Widefield microscope (Molecular Devices). Thereafter, cells were stained with propidium iodide (PI) at 100 µg/mL to enumerate the proportion of dead cells within the population. Cells were imaged immediately after addition of PI using the ImageXpress Microscope.

5.3.5 Image quantification and analysis

Quantification of live and dead cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices). Acquired images were analyzed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module and the Cell Cycle Application Module. Acquired data were transferred to Microsoft Excel spreadsheet and data was analyzed and processed using GraphPad Prism version 4 for IC_{50} determination and Microsoft Excel for cell cycle analysis.

5.3.6 Statistical analysis

All experiments were performed in triplicated and data represent the mean ± standard deviation (SD). The statistical significance was measured by one-way ANOVA and p-values less than 0.05 were considered significant. GraphPad Prism version 4 was used as well as the MetaXpress software.

5.4 RESULTS

The results, as shown in **Figure 5.2-4**, indicated that methanolic extracts of *A. laricinus* had the anticancer activity against breast cancer (MCF-7) cells, with very little effect on non-cancerous (Vero) cells. Dichloromethane extracts of *S. asperulus* showed a dose-dependent cytotoxic effect against prostate (PC3) cells (**Figure 5.3**) and against breast (MCF-7) cells (**Figure 5.4**), with little cytotoxic effect against non-cancerous kidney (Vero) cells (**Figure 5.2**). The rest of the extracts from *S. asperulus* and *A. laricinus* did not show any concentration-dependent cytotoxicity in any of the tested cell lines (PC3, MCF-7, and Vero).

Furthermore, from this chapter, results (**Figure 5.6** and **Figure 5.8**) showed that *Senecio asperulus* dichloromethane extract arrested a significant number of PC3 cells at the early stage of mitosis and MCF-7 cells at a synthesis phase. Several MCF-7 cells arrested at the synthesis phase by *Senecio asperulus* dichloromethane extract was two-fold when compared with the positive control.

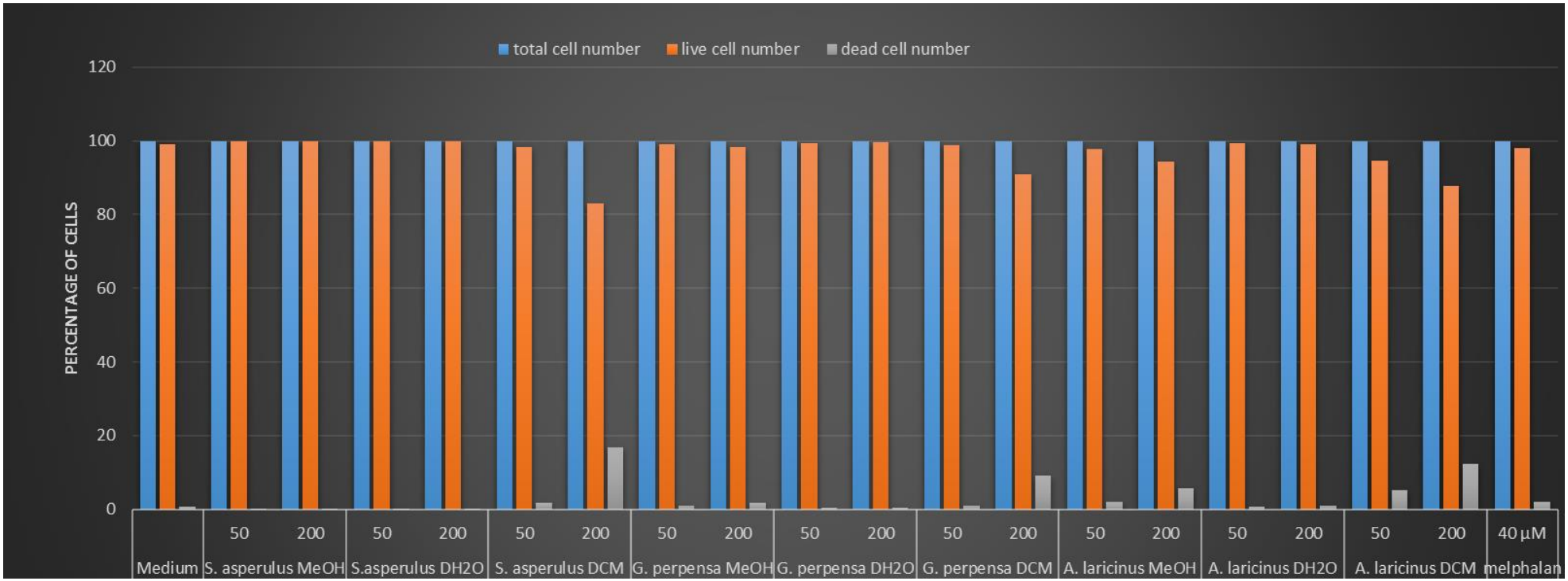


Figure 5.2. Cytotoxicity effect of *Senecio asperulus* and *Asparagus laricinus* extracts on the normal kidney (Vero).

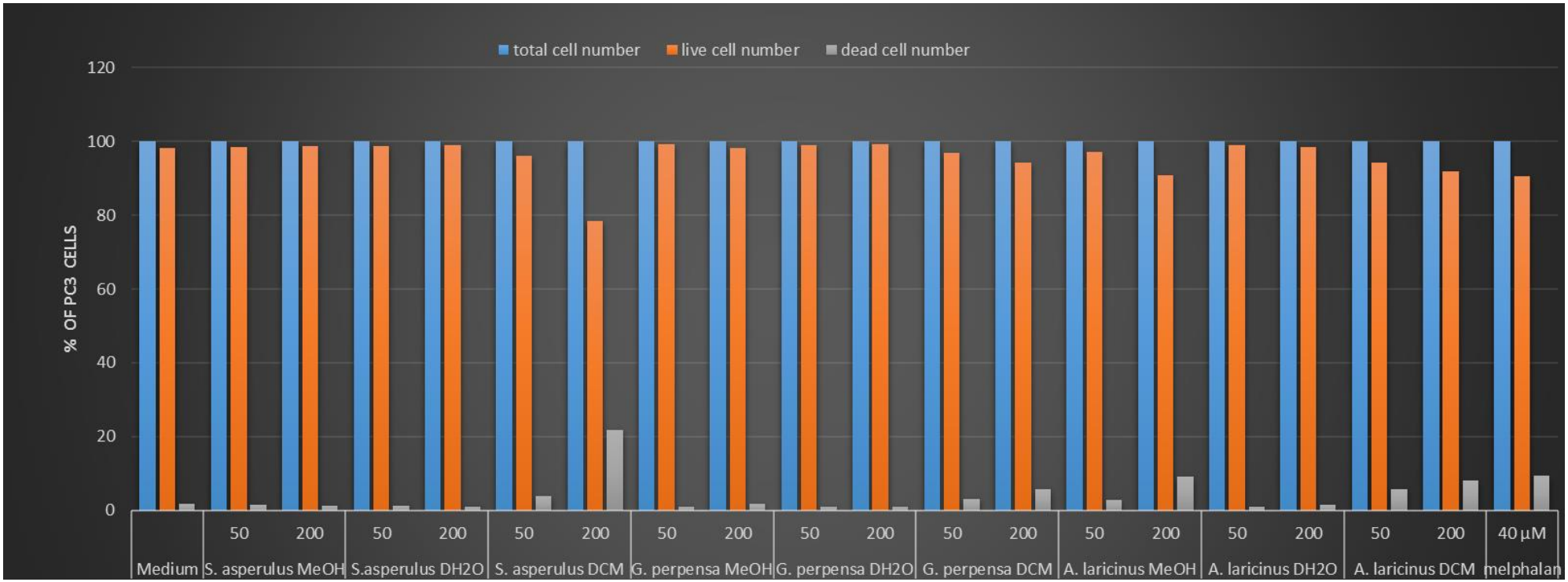


Figure 5.3. Cytotoxicity effect of *Senecio asperulus* and *Asparagus laricinus* extracts on prostate cancer cell line (PC3).

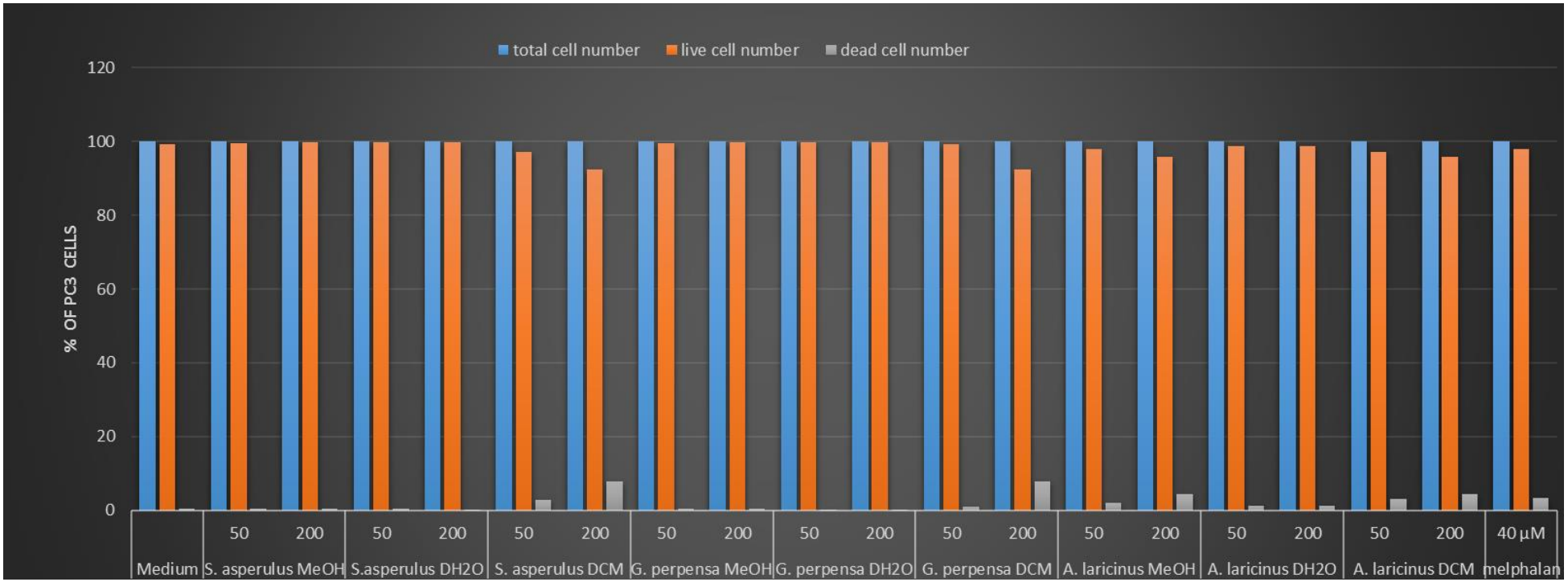


Figure 5.4. Cytotoxicity effect of *Senecio asperulus* and *Asparagus laricinus* extracts on breast cancer cell line (MCF-7).

From the anticancer analysis findings, two plant extracts (*Senecio asperulus* dichloromethane and *Asparagus laricinus* methanol extracts), showed to be less cytotoxic to non-cancerous cells and more cytotoxic to cancer cells analyzed, when compared to controls. Thus, their respective concentrations required to reduce 50% (IC_{50}) of prostate and or breast cell viability were determined and illustrated in **Figure 5.5. A and B**.

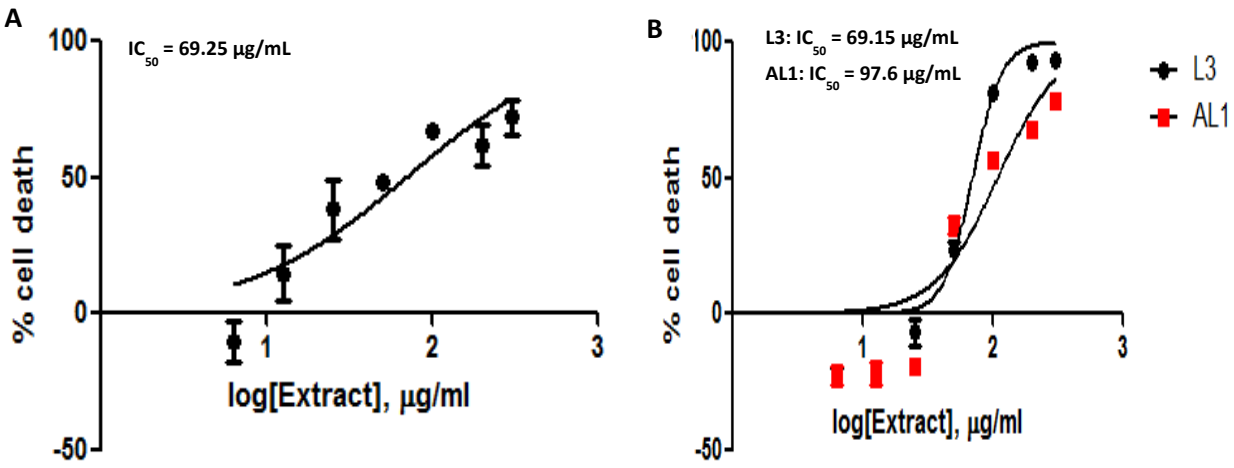


Figure 5.5. **A**: IC_{50} value of *Senecio asperulus* dichloromethane (L3) on prostate cancer cell lines. **B:** IC_{50} values of *Asparagus laricinus* methanol (AL1) and *Senecio asperulus* dichloromethane (L3) extracts on breast cancer cell lines.

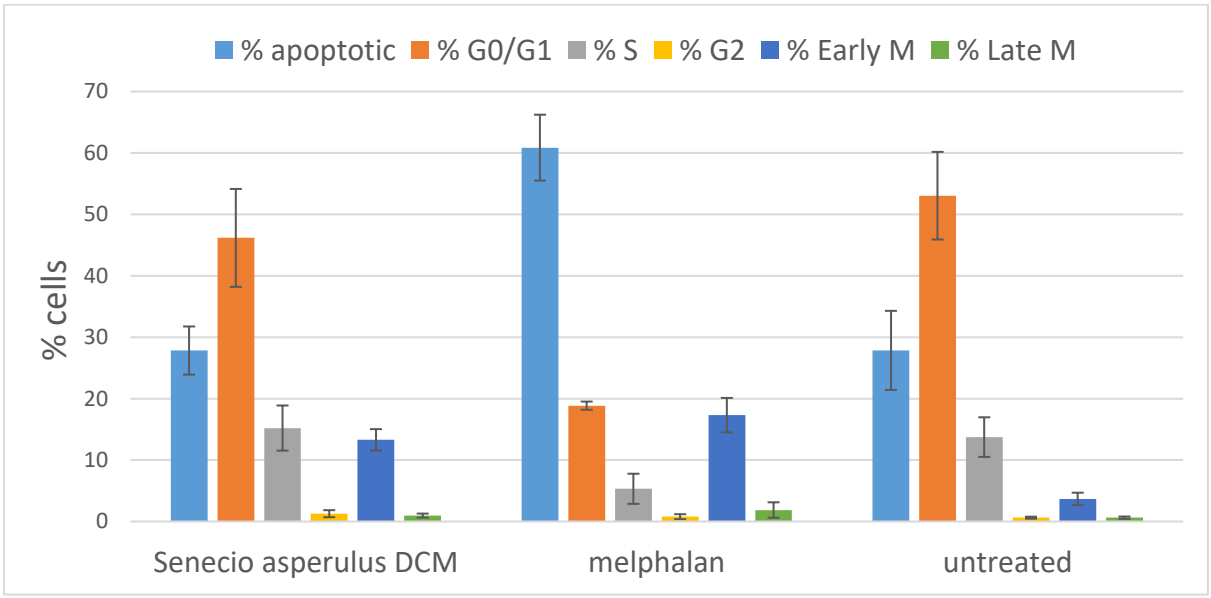


Figure 5.6. Distribution of PC3 cells in the different phases of the cell cycle when treated with *Senecio asperulus* dichloromethane extracts. Melphalan (40 µM) was used as a positive control. Error bars indicate standard deviation of quadruplicate values. G0/G1: Gap 0/Gap1 phase; S: synthesis phase; M: mitotic phase.

It is evident from the results in **Figure 5.5. B**, that the IC_{50} value of *Senecio asperulus* dichloromethane (L3) extract in both PC3 and MCF-7 cell lines was two-fold higher compared to those of *Asparagus laricinus* methanol (AL1) on MCF-7 cells. Thus, further studies to investigate whether the cytotoxic effects were due to apoptosis or necrosis were undertaken with both extracts *Senecio asperulus* and *Asparagus laricinus*, at their IC_{50} concentrations.

Furthermore, to evaluate the mechanism of action of these plant extracts on cancer cells, cell cycle analysis was also performed. At the end of the analysis, deoxyribonucleic acid (DNA) contents in different cell cycle phases were determined using Hoechst 33342, Annexin V-FITC and PI multiplex staining technique (**Figure 5.7**). The results were presented in the form of percentages as showed in **Figure 5.6** and **5.8**.

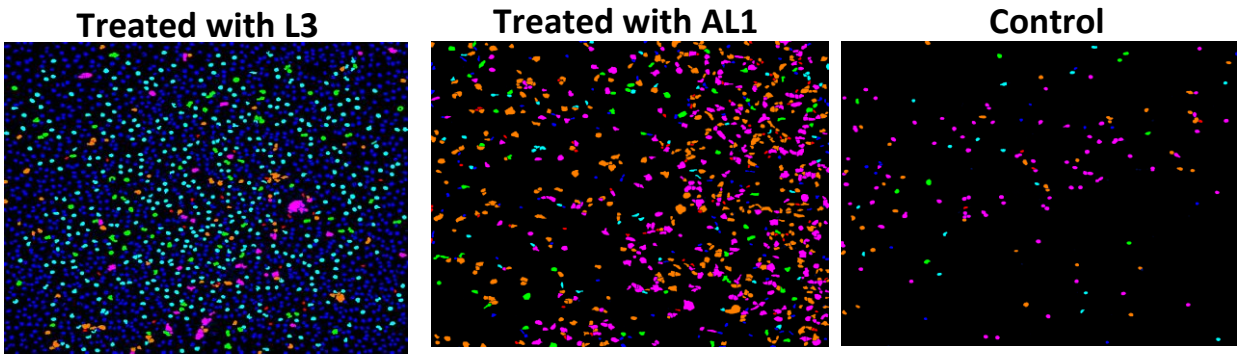


Figure 5.7. Staining uptake changes observed after Hoechst 33342, Annexin V-FITC and PI staining of MCF-7 cells treated with *Senecio asperulus* dichloromethane (L3), *Asparagus laricinus* methanol (AL1) extract and melphalan at determined IC_{50} concentrations.

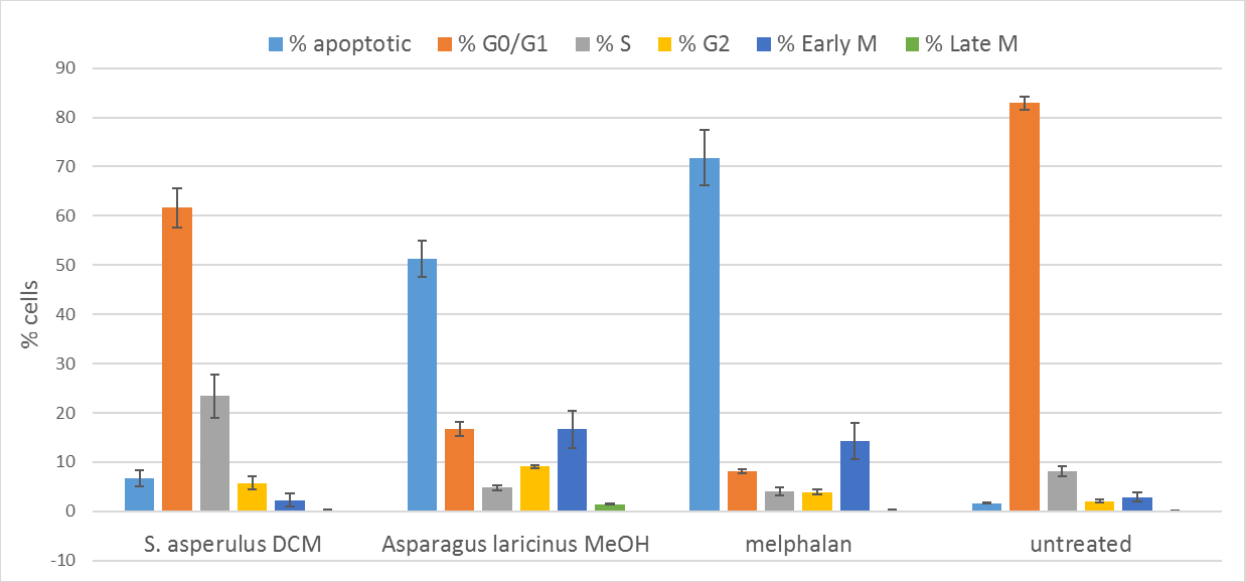


Figure 5.8. Distribution of MCF7 cells in the different phases of the cell cycle when treated with *Senecio asperulus* dichloromethane and *Asparagus laricinus* methanol extract. Melphalan (40 µM) was used as a positive control. Error bars indicate standard deviation of quadruplicate values. G0/G1: Gap 0/Gap1 phase; S: synthesis phase; M: mitotic phase.

5.5 DISCUSSION

The current study investigated the cytotoxicity activity of indigenous medicinal plants, *Senecio asperulus,* and *Asparagus laricinus*, against human breast (MCF-7) and prostate (PC3) cancer cells. The non-cancerous African green monkey kidney cell line (Vero) was used as a control as well as melphalan that was used as a positive control. The results showed decreased cell viability of MCF-7 and PC3 cancer cells in a dose-dependent manner (**Figure 5.2-5.4**) when treated with *Senecio asperulus* dichloromethane and *Asparagus laricinus* methanol extracts for a period of 48 hours. The dose-dependent cytotoxicity effect of the methanol extract of *A. laricinus*, dichloromethane extract of *S. asperulus* and dichloromethane extract of *Gunnera perpensa* indicated selectivity for cancer (MCF-3 and PC3) as they had little effect on Vero cells. The rest of the unmentioned extracts from all three-plant species were either not cytotoxic or were cytotoxic to all cell lines (both cancerous and non-cancerous). The cytotoxic extract to all cell lines was *A. laricinus* dichloromethane extract, and inactive ones included the methanolic and aqueous extracts of *S. asperulus* and *G. perpensa,* as well the aqueous extracts of *A. laricinus*.

An interesting anticancer agent should have more cytotoxic effect on cancer cell lines and less effect on non-cancerous cell lines. Thus, the observed selectivity is encouraging for potential drug development from *A. laricinus* methanolic extract as targeted cancer therapy. According to Ayoub et al (2014), cytotoxic agents should be effective at concentrations of up to 100 µg/mL. Since both extracts were active below 100 µg/mL, they can be considered as suitable candidates for chemotherapeutic drugs. *A. laricinus* DCM extract was cytotoxic on prostate, breast and Vero cells, thus due to lack of selectivity (killed both cancer and non-cancer cells), this extract was not investigated further for its anticancer mechanism of action. *Gunnera perpensa* DCM extract also showed very less cytotoxicity on Vero cells with moderate activity on prostate and breast cancer cells, the same selective way as *S. asperulus* DCM and *A. laricinus* methanol extracts. However, due to financial limitations, only the latter two mentioned extracts were selected to be studied further as not much research has been done on those two medicinal plants. While some Z-venusol compound with anticancer properties has been isolated and investigated from the South African *Gunnera perpensa* as reported by Khan et al (2004). Mathibe et al (2015), reported on the *in vitro* anticancer activity of Z-venusol in breast cancer cells and has demonstrated the mechanism of action of this compound to be through the initiation of apoptotic cell death.

In an attempt to propose a mechanism of action associated with the cytotoxicity observed from the anticancer results reported in this chapter, the *Asparagus laricinus* methanol and *Senecio asperulus* dichloromethane extracts were studied further to determine whether cell death was due to apoptosis or necrosis. It is worth mentioning that cell viability can decrease due to necrosis, autophagy or apoptosis. Necrosis is caused by factors external to the cell or tissue, such as infection, toxins, or trauma. Autophagy is triggered by nutrients undernourishment or withdrawal of other essential factors needed for cells to survive. Autophagy leads to the degradation of cytoplasmic materials, and this becomes an evident feature on the cell morphology analysis. Although autophagy is not inherently complicated, recent progress has confirmed that it plays a wide variety of pathophysiological and physiological roles which are sometimes multifaceted (Yoshimoto et al. 2004). Apoptosis on the other hand, can be defined as a highly ordered programmed cell death process of unhealthy cells, where

a cell dies as part of its normal program of development, or due to lack of growth factors, or when the immune system instructs it to die. However, cancer cells typically escape apoptosis by disabling the apoptotic pathways, thus continue to proliferate and even become resistant to the chemotherapeutic drug (Ferreira et al., 2004; Luqmani, 2005). Cell death due to apoptosis can be observed with the morphological changes in the cell shape such as: nuclear condensation, cell body shrinkage, cytoplasm fragmentation, plasma membrane blebs, and externalization of phosphatidylserine. Such changes suggest that cells have committed to a specific mode of cell death resulting in apoptosis (Selvan et al., 2018). Furthermore, it is known that drugs with apoptosis-inducing properties may reduce potential drug resistance.

For this study, apoptosis analysis was done using Hoechst 33342 nuclear staining assay. The apoptotic induced cell death by *Senecio asperulus* dichloromethane and *Asparagus laricinus* methanol extracts were investigated on MCF-7 and PC3 cells stained with Hoechst 33342, Annexin V-FITC and further stained with PI stain. As illustrated in **Figure 5.9,** Hoechst dye stains the nuclei of the live cells blue, while Annexin V-FITC dye stains the membrane of the apoptotic cells which have lost their mitochondrial membrane potential, green. This Annexin V-FITC dye binds to phosphatidylserine (PS) which the cell externalizes after committing to apoptosis so that macrophages can recognize that cell and remove it from the system. During cell death due to necrosis, cell membrane leaks, thus PI stain enters the cell while PS is also still inside the cell and intact. PI stains the dead nuclei of the cell red or pink.

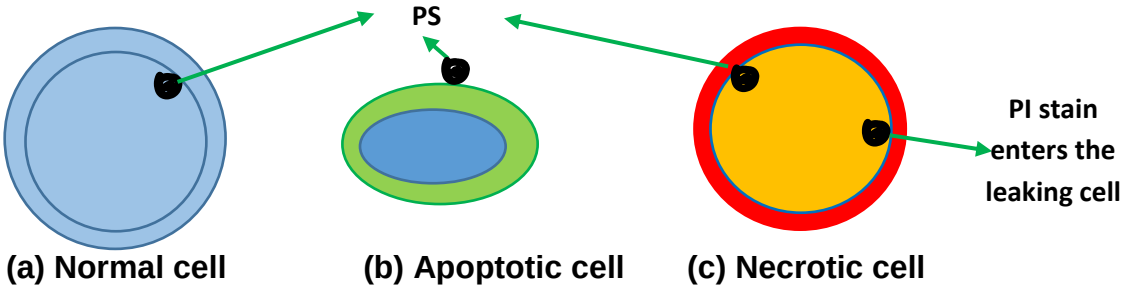


Figure 5.9. Staining uptake changes observed after Hoechst 33342, Annexin V-FITC and PI staining.

Acquired images were then analyzed using the MetaXpress software (**Figure 5.7**). The percentage of obtained morphological changes and also Annexin V-FITC stain uptake observations indicated that *Asparagus laricinus* methanol extract exhibits

significant activity in destroying MCF-7 cells by apoptosis when compared with controls (**Figure 5.8**). However, *Senecio asperulus* dichloromethane extract didn't show much evidence of apoptosis on PC3 cell line when compared to controls (**Figure 5.6**), even though this extract showed more cytotoxic activity on this cancer cell line.

Some cells die through programmed pathway while others die through obstruction accompanied by cell-cycle mechanisms (Dirsch et al., 2004; Kumar et al., 2018). Cell cycle analysis is performed to determine the state of DNA in response to treatment of the cell with a specific compound or extract. The distribution of DNA content is important as it leads to the identification of targets or pathways to target for the treatment of cancer and tumors (Planchais et al., 2000). During cell development, cells need to progress through every phase of the cell cycle to assure a full copy of DNA for a new daughter cell. There exist checkpoints which regulate the progression of cells through the cycle and cause cell cycle arrest if DNA damage or DNA stress has occurred. Faults in the G2-M arrest checkpoint allows a damaged cell to enter mitosis before repair and undergo apoptosis (DiPaola, 2002). Thus, efforts to promote the latter outcome may increase the cytotoxicity of chemotherapy, however, other studies also propose enhanced cytotoxicity to be associated with high cell-cycle arrest (Shapiro et al., 2001; Alimbetov et al., 2018). Cell cycle arrest is defined as a high proportion of cells found in the same cycle event at a specific time. Cell cycle arrest is maintained until DNA repair is complete (Nojima, 2004).

At the G2 phase, as cell approaches mitosis phase, cyclin-dependent protein kinases (Cdk1)/nuclear cyclin B level (B1) complex is essential for entry into and progression through mitosis (Wolf et al., 2007). Thus, activation of this Cdk1/B1 complex plays a key regulatory role in cell proliferation, while down-regulation of this complex expression induced G2/M phase arrest (Chang et al., 2003). However, continued and inappropriate overexpression of Cdk1/ cyclin B1 plays an opposite role by mediating pro-apoptotic signaling in response to mitotic arrest and causes non-specific cell death (Eichhorn et al., 2014). An increase in the percentage of MCF-7 cells at G2 phase as well as at the early mitosis phase when treated with *Asparagus laricinus* methanol extract suggests that the cytotoxicity mechanism was also stimulated by active cyclin B1/CDK1 complex in these cells. However, this can only be confirmed through a study of the regulation of apoptosis-related proteins in MCF-7 cancer cells by *Asparagus*

laricinus methanol extract. This will be performed in the future studies of this plant species.

5.6 CONCLUSION

This chapter was intended to investigate the cytotoxicity of three medicinal plants, *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* on prostate and breast cancer cell lines, and to further elucidate the mechanism of action of the active extract/s on the cell cycle of these cancer cell lines. *Senecio asperulus* dichloromethane extract was cytotoxic against PC3 and MCF-7 cancer cell lines, and *Asparagus laricinus* methanol extract showed cytotoxicity on MCF-7 cells only in a dose-dependent manner. Moreover, both extracts had little cytotoxicity against non-cancerous Vero cells. Their selectivity for cancer cells, their low IC_{50} as well as the cell cycle analysis prompted further investigation. The dichloromethane extract of *Senecio asperulus* appeared to arrest cells in the G2 as well as in the early mitotic (M) phase of the cell cycle on PC3 cells, while on the MCF7 cells, cell arrests were more in the S phase. These findings show that cytotoxicity of this extract against PC3 and MCF-7 cancer cell lines was not due to apoptosis, but rather due to cell arrest at the G2 and early mitosis phase. The methanol extract of *Asparagus laricinus* revealed cell arrest in the early M phase of the cell cycle but also shows a high percentage of MCF-7 apoptotic cells. Therefore, this extract selectively induced MCF-7 cell death through apoptosis at an acceptable growth inhibition concentration dose and, therefore, can be considered as a suitable candidate for future chemotherapeutic drugs.

5.7 REFERENCES

Akarolo-Anthony, S.N., Ogundiran, T.O., Adebamowo, C.A. 2010. Emerging breast cancer epidemic: evidence from Africa. Breast Cancer Research 12(4), S8.

Alimbetov, D., Askarova, S., Umbayev, B., Davis, T., Kipling, D. 2018. Pharmacological Targeting of Cell Cycle, Apoptotic and Cell Adhesion Signaling Pathways Implicated in Chemoresistance of Cancer Cells. International Journal of Molecular Science 19, 1690; DOI:10.3390/ijms19061690

Ayoub, I.M., El-Shazly, M., Lu, M.C., Singaba A.N.B. 2014. Antimicrobial and cytotoxic activities of the crude extracts of Dietes bicolor leaves, flowers and rhizomes. South African Journal of Botany 95, 97-101.

Brookes, K. B., and M. F. Dutton. 2007. “Bioactive Components of the Uteroactive Medicinal Plant, Gunnera Perpensa (or Ugobo): Research in Action.” South African Journal of Science 103 (5 & 6): 187–189.

Brummitt, R.K. 1992. Vascular plant families and genera. Royal Botanical Gardens, Kew 8, 14-18.

Chang, D.C., Xu, N., Luo, K.Q. 2003. Degradation of cyclin B is required for the onset of anaphase in Mammalian cells. Journal of Biological Chemistry 278, 37865–37873.

Cragg, G.M.; Newman, D.J.; Weiss, R.B. 1997. Coral reefs, forests, and thermal vents: the worldwide exploration of nature for novel antitumor agents. Semin Oncol. 24(2), 156-63.

DiPaola R.S. 2002. To Arrest or Not To G2-M Cell-Cycle Arrest. Clinical Cancer Research 8, 3311–3314.

Dirsch, V.M., Kirschke, S.O., Estermeier, M., Steffan, B., Vollmar, A.M. 2004. Apoptosis signaling triggered by the marine alkaloids ascididemin is routed via caspase-2 and JNK to mitochondria. Oncogene 23, 1586-1593.

Eichhorn, J.M., Alford, S.E., Sakurikar, N., Chambers, T.C. 2014. Molecular analysis of functional redundancy among anti-apoptotic Bcl-2 proteins and its role in cancer cell survival. Experimental Cell Research 322 (2), 415-424.

Ferreira, C.G., Epping, M., Kruyt, F.A., Giaccone, G. 2004. Apoptosis: target of cancer therapy. Clinical Cancer Research 8, 2024–2034.

Jemal, A., Bray, F., Forman, D., et al. 2012. Cancer burden in Africa and opportunities for prevention. Cancer 118, 4372–4384.

Khan, Fatima, Xolani K Peter, Rod M Mackenzie, Lynn Katsoulis, Ronette Gehring, Orde Q Munro, Fanie R van Heerden, and Siegfried E Drewes. 2004. “Venusol From Gunnera Perpensa: Structural and Activity Studies.” Phytochemistry. 65(8):1117–1121.

Kinghorn A.D. 2000. Plant Secondary Metabolites as Potential Anticancer Agents and Cancer Chemopreventives. Molecules. 5 285-88.

Kose, L.S., Moteetee, A., Van Vuuren, S. 2015. Ethnobotanical survey of medicinal plants used in the Maseru district of Lesotho. Journal of Ethnopharmacology 170, 184–200.

Krishan, A. 1975. Rapid flow cytofluorometric analysis of cell cycle by propidium iodide staining. Journal of Cell Biology 66, 188–193.

Kumar A, Sharma P, Gomar-Alba M, Shcheprova Z, Daulny A, Sanmartín T, Matucci I, Funaya C, Beato M, Mendoza M. 2018. Daughter-cell-specific modulation of nuclear pore complexes controls cell cycle entry during asymmetric division. Nat Cell Biol. 20(4):432-442.

Ho, L.H., Read, S.H., Dorstyn, L., Lambrusco, L., Kumar, S. 2008., Caspase-2 is required for cell death induced by cytoskeletal disruption. Oncogene 27, 3393-3404.

Lee JY, Kim EY, Jung KW, Shin A, Chan KK, Aoki D, et al. 2014. Trends in gynecologic cancer mortality in East Asian regions. Journal of Gynecologic Oncology 25, 174–82.

Luqmani, Y.A. 2005. Mechanisms of drug resistance in cancer chemotherapy. Medical Principles and Practice 14, 35–48.

Mammo F.K, Mohanlall V, Shode F.O. 2017. Gunnera perpensa L.: A multi-use ethnomedicinal plant species in South Africa. African Journal of Science, Technology, Innovation and Development. 9:1, 77-83.

Mashele, S.S., Kolesnikova, N. 2010. In vitro anticancer screening of Asparagus laricinus extracts. Pharmacologyonline 2, 246-252.

Mathibe L.J., Botha J., Naidoo S. 2016. Z-venusol, from Gunnera perpensa, induces apoptotic cell death in breast cancer cells in vitro. South African Journal of Botany 102:228–233.

Mugomeri, E., Chatanga, P., Hlapisi, S., Rahlao, L. 2014. Phytochemical characterization of selected herbal products in Lesotho. Lesotho Medical Association Journal. 12,38-47.

Nojima, H, 2004. G1 and S-phase checkpoints, chromosome instability, and cancer. Methods in Molecular Biology 280, 3–49.

Ogbole, O.O., Segun, P.A., Adeniji, A.J. 2017. In vitro cytotoxicity of medicinal plants from Nigeria ethnomedicine on Rhabdomyosarcoma cancer cell line and HPLC analysis of active extracts. BMC complementary and alternative medicine 17, 494.

Planchais, S., Glab, N., Inzé, D., Bergounioux, C. 2000. Chemical inhibitors: a tool for plant cell cycle studies. FEBS Letters 476, 78–83.

Porter, L.A., Donoghue, D.J. 2003. Cyclin B1 and CDK1: nuclear localization and upstream regulators. Progress in cell cycle research 5, 335–347.

Selvan, D.A., Mahendiran D, Kumar R.S., Rahiman AK. 2018. Garlic, green tea and turmeric extracts-mediated green synthesis of silver nanoparticles: Phytochemical, antioxidant and in vitro cytotoxicity studies. Journal of photochemistry and photobiology, B: Biology 180, 243-252.

Shapiro, G.I., Supko, J.G., Patterson, A., Lynch, C., Lucca, J., Zacarola, P.F., Muzikansky, A., Wright, J. J., Lynch, T. J., Jr., and Rollins, B. J. 2001. A Phase II trial of the cyclin-dependent kinase inhibitor flavopiridol in patients with previously untreated stage IV non-small cell lung cancer. Clinical Cancer Research 7, 1590–1599.

Sleath, P.R., Hendrickson, R.C., Kronhein, S.R., March, C.J., Black, R.A. 1990. Substrate specificity of the protease that processes human interleukin-1β. Journal of Biological Chemistry 265, 14526 – 14628.

Sylla, B.S., Wild, C.P. 2012. A million Africans a year dying from cancer by 2030: what can cancer research and control offer to the continent?. International Journal of Cancer 130, 245–250.

Van der Merwe, D., Swan, G.E, Botha, C.J. 2001. Use of ethnoveterinary medical plants in cattle by Setswana-speaking people in the Madikwe area of the North West Province of South Africa. Journal of the South African Veterinary Association 72(4), 189-196.

Wildlife Wholesale Nursery (WWN). Asparagus laricinus, 2018. (Accessed June 25, 2018). https://wildflowernursery.co.za/indigenous-plant-database/asparagus-laricinus/

Wolf, F., Sigl, R., Geley S. 2007. The end of the beginning': cdk1 thresholds and exit from mitosis. Cell Cycle 6, 1408–1411.

WHO Traditional Medicine Strategy 2014-2023. 2013. (Accessed June 20, 2018). http://www.searo.who.int/entity/health_situation_trends/who_trm_strategy_2014-2023.pdf?ua=1

World Health Organization, Regional office for Africa. 2017. (Accessed June 25, 2018). Cancer. https://www.afro.who.int/health-topics/cancer

Yoshimoto, K., Hanaoka, H., Sato, S., Kato, T., Tabata, S., Noda, T., Ohsumi, Y. 2004. Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. Plant Cell 16, 2967–2983.

CHAPTER 6

"Genotoxicity and anti-genotoxicity of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa*"

ABSTRACT

The use of traditional medicinal plant concoctions to cure or treat different kinds of diseases is common in African folk medicine. However, the effects of biologically active compounds from mostly used medicinal plants remains unknown. Thus, the aim of this chapter was to evaluate the genotoxicity/mutagenicity and anti-genotoxicity/anti-mutagenicity potential of *Asparagus laricinus* cladodes, *Senecio asperulus* and *Gunnera perpensa* roots extracts prepared using five different solvents. Neutral red uptake (NRU) assay, Alkaline comet assay, and Vitotox test were used to assess the toxicological profile of investigated medicinal plants on hepatic (C3A) cells and *Salmonella typhimurium* TA104 strains. Ethyl methane-sulfonate (EMS) and 4-nitroquinoline oxide (4NQO) were used as positive controls for the Comet and Vitotox assay, respectively. The results obtained from the Vitotox test for *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* correlated well with results obtained from NRU and Comet assays. Most tested extracts showed no *in vitro* toxicity and genotoxicity, except from the dichloromethane extracts of *Senecio asperulus* and *Gunnera perpensa*, as both were cytotoxic but not genotoxic after S9 metabolic activation. Both these extracts offer a step towards the further investigation of their possible cytotoxic effects on humans as they didn't show any significant ability to cause DNA damage on hepatic cells used. From the Vitotox test results, after *Salmonella typhimurium* strains were treated with a known mutagen, none of the extracts appeared to have antigenotoxic properties. Thus, this indicates that the anticancer activities of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* (**Chapter 5**), didn't result from the protection against genotoxicity. Moreover, the negative genotoxicity highlighted the safe use of these medicinal plants.

6.1 INTRODUCTION

Medicinal plants play an important role in our African communities' folk medicine as they are used as remedies for minor ailments to even major and serious ailments such as cancer, diabetes and even human immunodeficiency virus (Mustapha, 2014; Roy et al., 2017; Wu et al., 2001). Africa is blessed with rich fauna and flora. This abundance, especially in medicinal plants, contributes to the growing number of people using traditional medicinal plants. Other factors for the increased dependence on medicinal plants is that; they are easily accessible even at local markets, very affordable, and are widely believed to have fewer to no side effects (Ndhlala et al., 2011). Even pharmaceutical companies show increasing interest in plant-derived drugs mainly because of the current widespread belief that 'green medicine' is safe, with fewer side effects (Pavithra et al., 2010; Vaghasiya et al., 2011).

There are still challenging tasks for scientists working in drug discovery research. Some challenges include: to investigate the safety of herbal medicine, to distinguish favorable from adverse effects, and to ban poisonous plants or contaminations from herbal mixtures (Efferth and Kaina, 2011). However, these can be addressed by assessing the toxicity of plant-derived products to the tissue or organs of mammalian recipients. Over 50% of the drugs in clinical trials for anticancer activity were isolated directly or indirectly from natural sources (Cragg and Newman, 2000; Joshi et al., 2009). Most plants used as traditional medicine have *in vitro* mutagenic properties (Cardoso et al., 2006; Mohd-Fuat et al., 2007; Deciga-Campos et al., 2007), as a result, it is important to screen for their mutagenic potency. Therefore, plants with these properties must be considered potentially unsafe and further testing before their continued use is recommended. This chapter is aimed at investigating the mutagenic and anti-mutagenic properties of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa*.

6.2 LITERATURE REVIEW

Genotoxicity is when the chemical agent directly or indirectly damages the cells genetic information in the DNA, which may cause mutations or even lead to cancer when not repaired (Verschaeve, 2005), but not necessarily always. Direct DNA

damage leads to DNA structural changes, while indirect damage leads to the metabolic activation of metabolites formation or even affects chemicals acting directly on to the DNA (Gautam et al., 2016). Gene mutations can result from chromosomal abnormalities and re-arrangements through deletion, translocation, and inversion. Sometimes these mutations are corrected and reversed, but if not fixed, they pass on to the next generation of that cell during mitosis. Cells can protect themselves from a mutation that is genotoxic by DNA repair or programmed cell death process (apoptosis). Failure to commit to one of these two options leads to mutagenicity. The breakage of the DNA single and double-strand leads to unfixable chromosomal aberrations, gene and micronuclei damages which are unrepairable.

Mutagenicity is the ability of a chemical agent to cause mutations that result in cell death, and those agents are said to be genotoxic (Collins, 2004; Verschaeve, 2005). Therefore, all mutagens are genotoxic, but not all genotoxic agents are mutagenic. Moreover, mutations are known to be major contributors to carcinogenesis, thus mutagens are also most likely considered to be carcinogens (Makhuvele et al., 2018). However, not all mutations are caused by mutagens as they can also be spontaneous. Spontaneous mutations are the result of errors from spontaneous hydrolysis, replication, repair and recombination in natural biological processes and not from environmental mutagens (Griffiths et al., 2000).

Antimutagens are the agents that counteract the effects of mutagens by inactivating or preventing the Mutagen-DNA reaction or mutagenic compound transformation into mutagen. These can be natural or synthetic compounds functioning through certain mechanisms, such as, (i) directly interacting with mutagens, (ii) inhibiting the activation of a mutagens, (iii) blocking the mutagen binding to the target, and (iv) through generation of antioxidant mechanism (Gautam et al., 2016; Słoczyńska et al., 2014). Through these mechanisms, it is evident that antimutagens have the potential to eliminate or reduce the mutagenic effects of potentially harmful substances. Thus, the investigation of antimutagenic compounds presents new possibilities in anticancer drug discovery and this quest is currently expanding hastily in the field of cancer research (Ferguson and Philpot 2008; El-Sayed and Hussin 2013). To evaluate the mutagenicity and antimutagenicity of compounds in question, DNA damage in cells after being exposed to the test substance is investigated. For satisfactory evaluation

of the genotoxic potential of a compound, 3 endpoints need to be assessed which are: (1) gene mutation, (2) DNA damage, and (3) structural and numerical chromosome aberrations (Leandro et al., 2013; Słoczyńska et al., 2014). Therefore, many *in vitro* and *in vivo* toxicology tests methods including Vitotox © test, mammal cell micronucleus test, and Comet assay have been developed to assess the potential of substances to cause mutagenicity that may lead to cancer.

6.3 METHODOLOGY

6.3.1 C3A cell culture

Toxicology of the studied plant extracts was done using C3A hepatic cells. Cell suspensions of human C3A cells in Dulbecco's modified Eagle's culture medium supplemented with 10% fetal calf serum were seeded into each well of a 96-well microtiter plate such that the cell density was 40,000 cells/well. Plates were incubated overnight (24 hours) at 37 ^{o}C and 5% CO_2. Humidity was maintained using a water bath containing distilled water inside the incubator.

6.3.2 The neutral red uptake test

The NRU test was performed using the method by Repetto, 2008. After overnight incubation, the cells were then treated with dilutions of the extracts which were 4 µg/ml, 20 µg/ml, 50 µg/ml, and 100 µg/ml. Cells were further incubated for another 24 hours to allow extracts to work, then cells were washed in PBS after which 200 mL of a 0.625 mg/mL NR-solution was added. Cells were washed again with PBS after being incubated for 3 hours to remove excess dye. Two hundred milliliters of a 50:1 ethanol-acetic acid solution was then added, and cells could mix with this solution for 1,5 hours on the shaker, to remove the dye from the cells. The absorbances were measured with a microplate spectrophotometer at 540 nm wavelength against the blank. Results were expressed as a percentage of the OD determined from the average of the blank control culture read at 540 nm and set at 100%.

6.3.3 The comet assay

The protocol of Singh et al. (1988) was followed to evaluate the DNA damaging and protective effects of the two plant extracts. Microscope slides were pre-coated by spreading 300 µl of 1% normal melting point (NMP) agarose in water evenly over the slides and allowing the agarose to harden. C3A cells at a density of 200000 cells/ml were treated with different concentrations of the test sample in 24 well plates and incubated for 24 hours at 37 ^{o}C in a 5% carbon dioxide incubator. The plant extracts were tested at concentrations of 250, 100, 50 and 4 µg/ml. Ethyl methane-sulfonate (EMS) at 1 mM will be used as a positive control/mutagen. For mutagenicity testing, the cells will be exposed to plant extracts alone and for antimutagenicity testing, the cells were exposed to a combination of the plant extracts and 1 mM EMS. After incubation, cells were trypsinized and 10 µl of a 10 000-cell suspension which was added to 300 µl of 0.8% low melting point (LMP) agarose at 37 ^{o}C. The mixture was spread on the pre-coated slides and allowed to harden under a coverslip on ice. Once the agarose had been prepared, the coverslips were removed, and the microscope slides placed in lysis buffer overnight. Denaturation was conducted using the electrophoresis buffer at 17 ^{o}C for 40 minutes.

Electrophoresis was conducted using the same solution at 25V, the current was adjusted to 300 mA for 20 minutes. After electrophoresis, neutralization of the microscope slides was carried out in Tris buffer (pH 7.5) and slides were dried. The slides were then placed in ice-cold ethanol for 10 minutes and allowed to dry at room temperature. The gels were stained with 100 µl of 20 µg/ml ethidium bromide, left for 10 minutes and rinsed in distilled water. The slides were analyzed using a fluorescence microscope supplied with a camera. The tail length, % DNA in tail and tail moment were determined using the PC image-analysis program TriTek CometScoreTM. This program allows measurement of tail length, percentage DNA in tail and tail moment as parameters to measure DNA damage in the comet assay.

For mutagenicity testing, differences in parameters used to measure DNA damage (i.e. tail length, percentage DNA in tail and tail moment) were compared between sample concentration and solvent blank (negative control). For antimutagenicity

testing, the same parameters used for mutagenicity testing were used. In this case, the measurements in the test samples were compared to the positive control (EMS).

6.3.4 The VITOTOX® test

The VITOTOX® test was performed following the method described by Verschaeve (2005). *Salmonella typhimurium* bacteria which lacks the necessary oxidative enzyme systems for the metabolization of foreign compounds were used as they are capable of reacting with the DNA. The bacteria are, as for most other *in vitro* assay, treated with the test compound in the presence and absence of a post-mitochondrial supernatant ('S9'). Micro-organisms are incubated overnight, and then a dilution of the bacterial suspension is incubated for an hour on a rotative shaker. Multiwell plates are used so as to contain the solvent, different concentrations of the test compound, or the positive control for genotoxicity testing (4-Nitroquinoline 1-oxide with S9 or 4-NQO without S9).

Genotoxicity and toxicity measurements are performed using a microplate luminometer that enables online measurements of emitted light (e.g., every 5 minutes over a period of 4 hours). After completion of the measurements, the data are transferred into an Excel macro sheet and the signal to noise ratio (S/N), i.e., the light production of exposed bacteria divided by the light production of non-exposed bacteria, is calculated for each measurement. S/N is calculated for the *rec*N2-4 and *pr1* strain separately, as well as the ratio between the maximum S/N values of the *rec*N2-4 and *pr1* strains. All calculations occur automatically and were based on measurements between 60-240 minutes of incubation. Based on experimental grounds a compound was considered genotoxic when the following criteria were met:
○ max S/N (*rec*N2-4) / max S/N (*pr1*) (to be indicated as: *rec/*pr1) is greater than 1.5.
○ max S/N in *rec*N2-4 must show a good dose-effect relationship. Mann-Whitney was used for the statistical analysis and $p < 0.05$ was regarded as significant.

6.4 RESULTS

The vitotox assay, genotoxicity and cytotoxicity of the plant extracts are reported in **Figure 6.3-6.4**.

6.4.1 NRU test

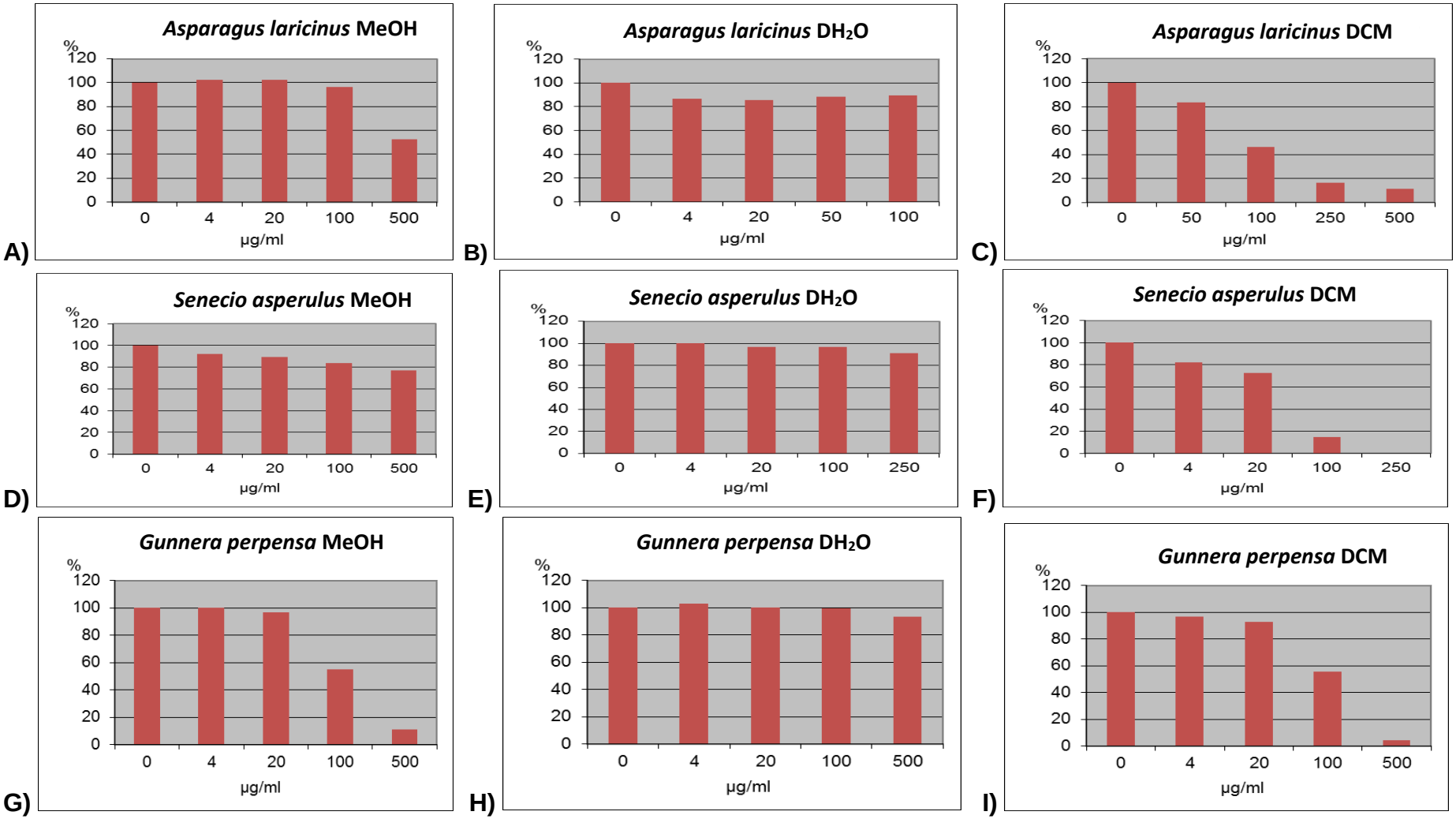


Figure 6.1: Neutral Red Uptake test results of *Asparagus laricinus, Senecio asperulus,* and *Gunnera perpensa* plant extracts.

6.4.2 Genotoxicity by comet assay

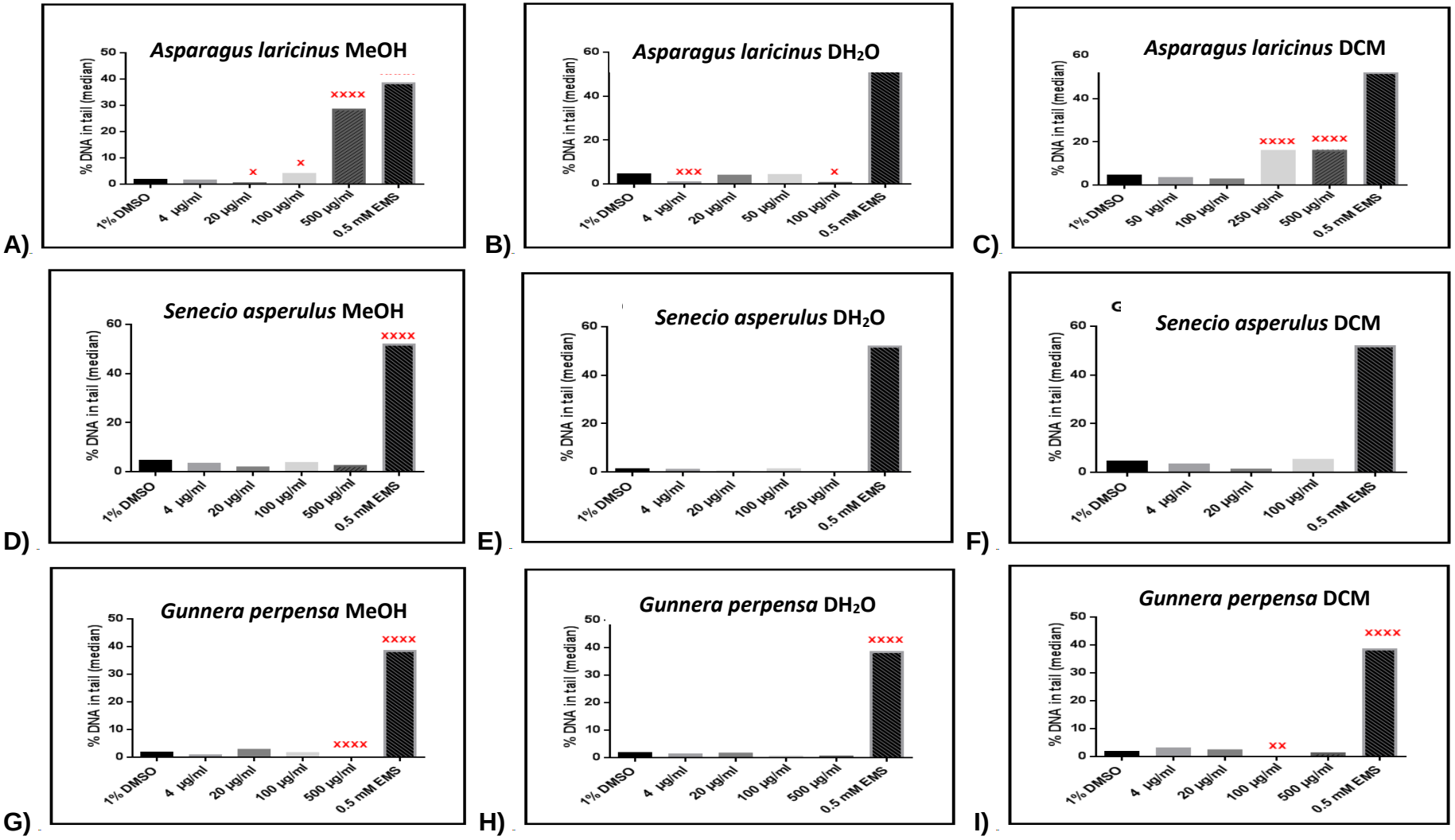


Figure 6.2: Comet test results of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* plant extracts. The statistically significant increases above background levels were indicated as **x** = $P < 0.05$; **xx** = $P < 0.01$, and **xxx** = $P < 0.001$.

6.4.3 Vitotox assay

For the antimutagenicity evaluation of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa,* the genotoxicity and cytotoxicity effects of these extracts with and without S9 were investigated and reported in **Figure 6.6 and 6.7**.

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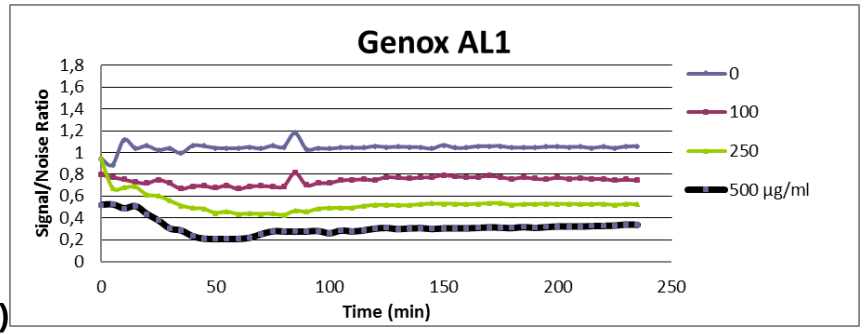

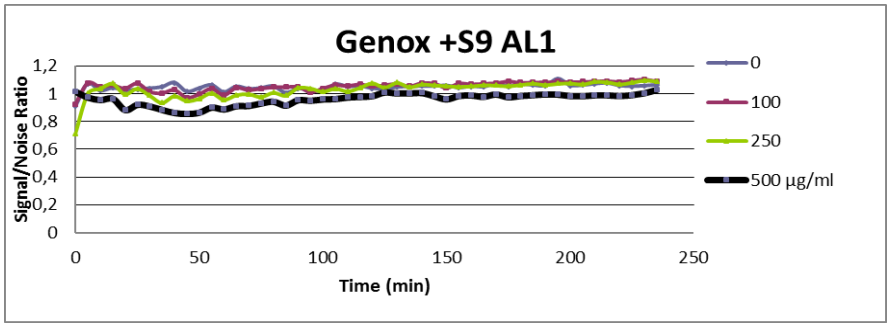

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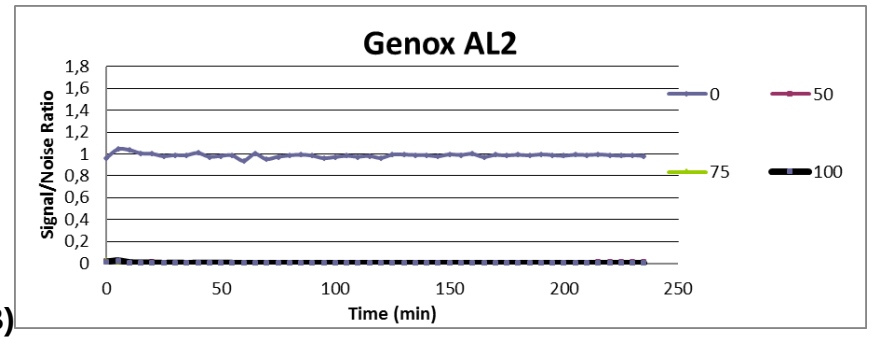

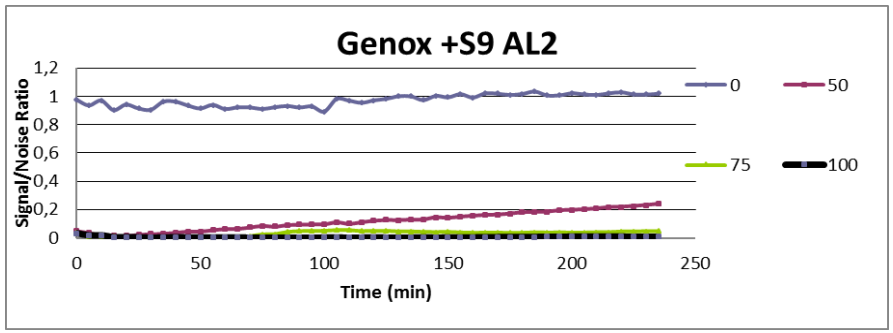

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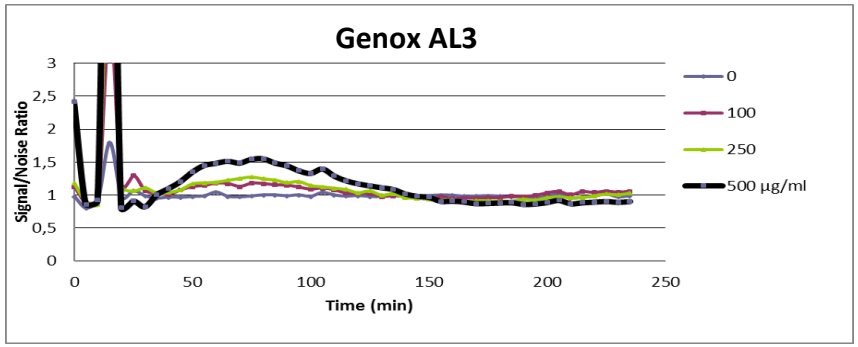

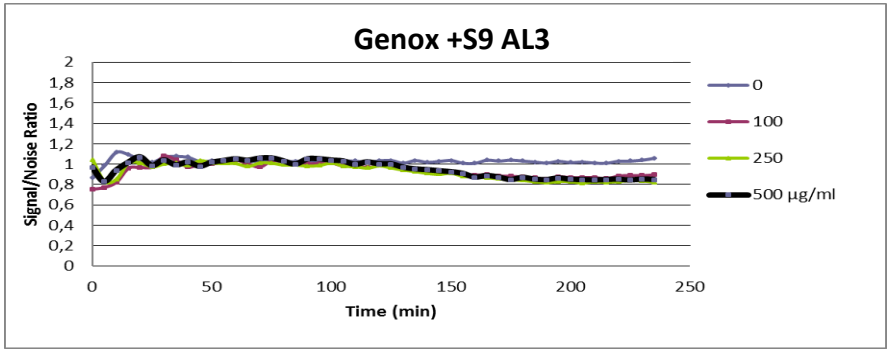

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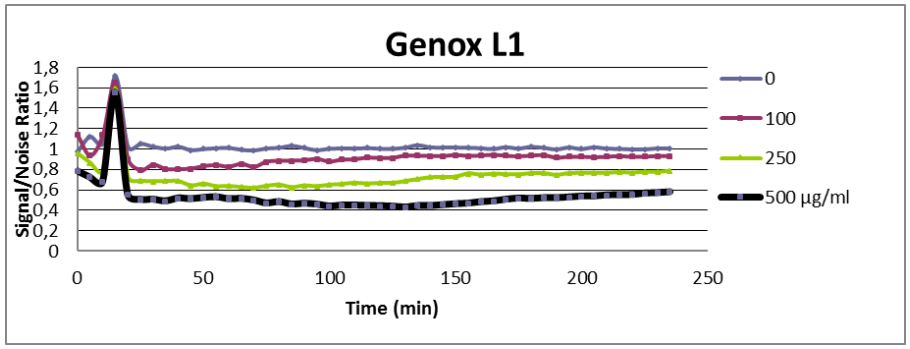

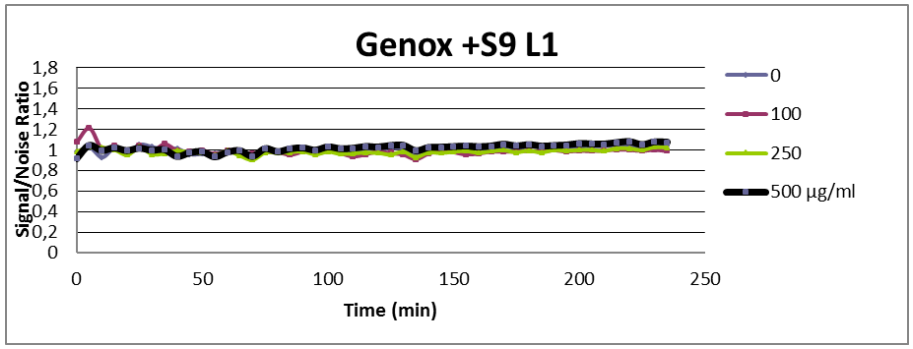

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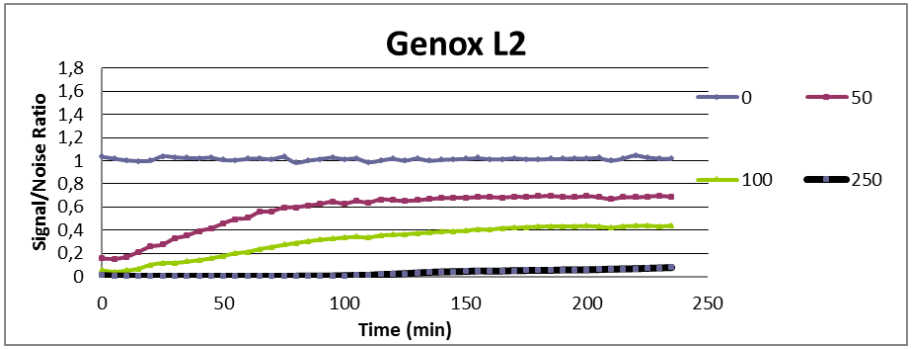

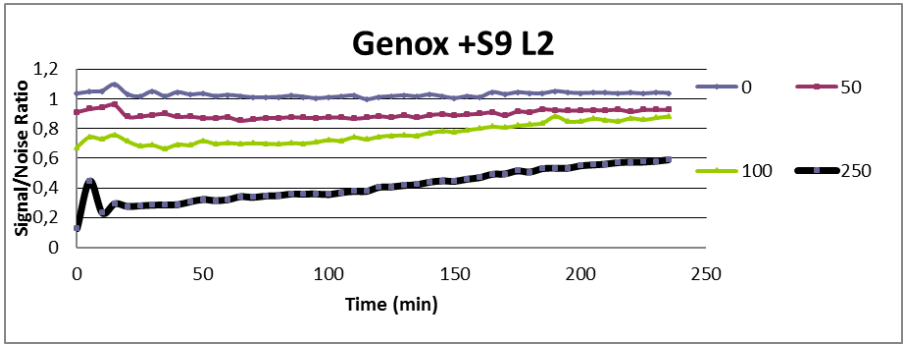

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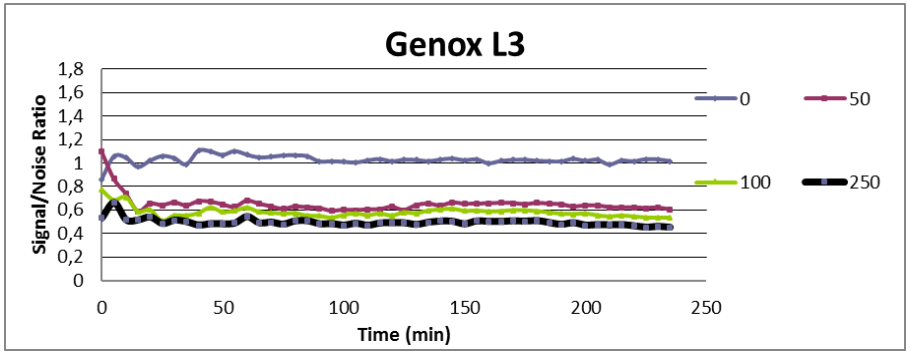

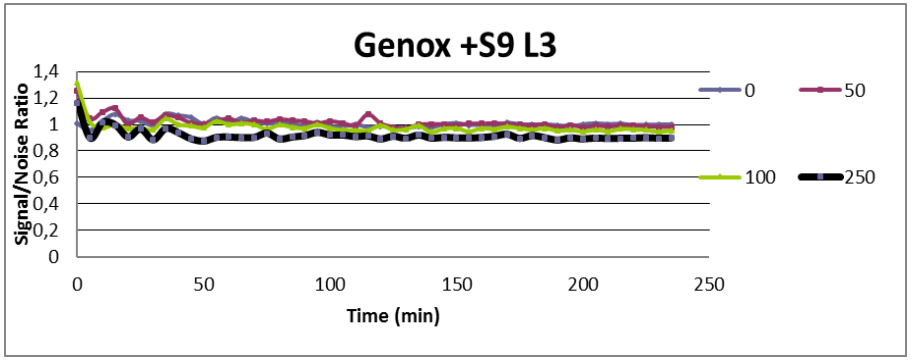

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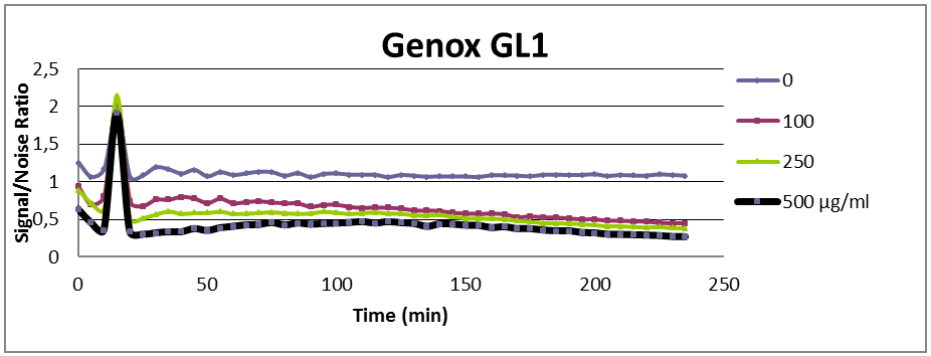

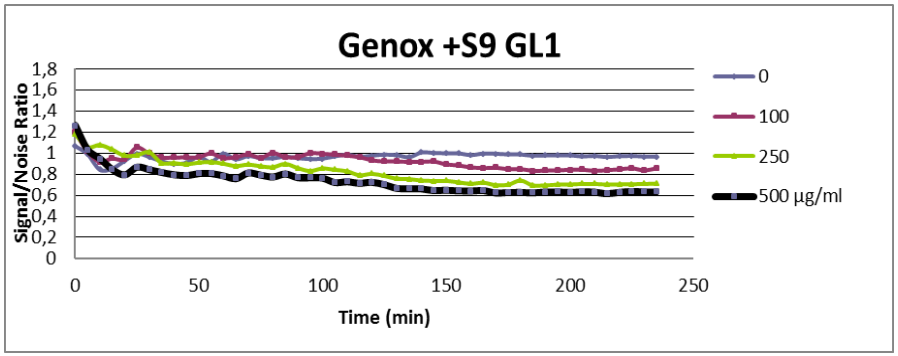

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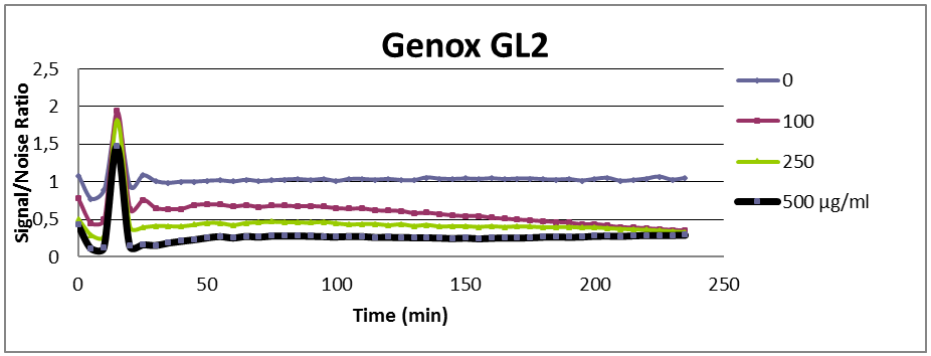

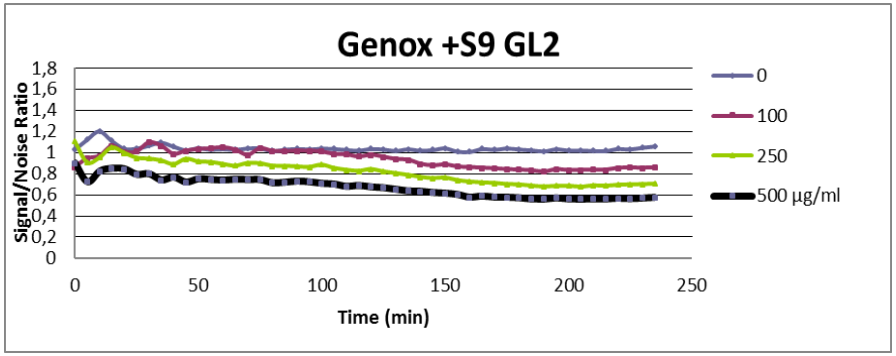

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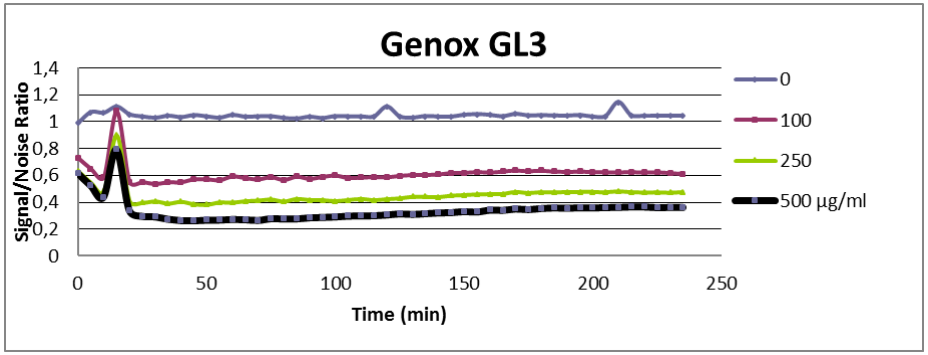

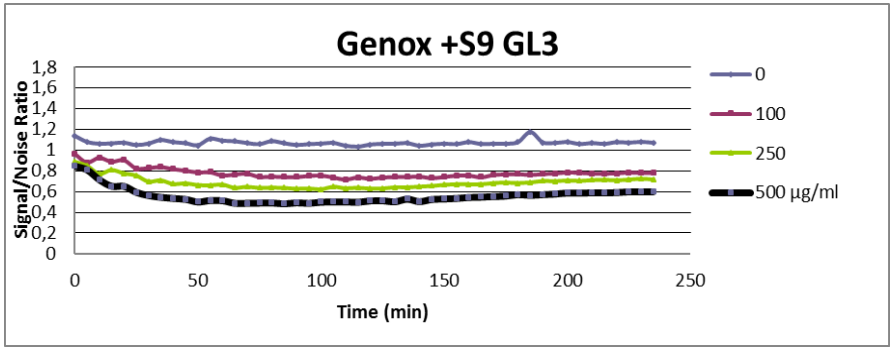

Figure 6.3: Genotoxicity results of *Asparagus laricinus* ***(AL)***, *Senecio asperulus* ***(L)*** and *Gunnera perpensa* ***(GL)*** plant extracts, with solvents used for extraction represented as **1** for Methanol, **2** for water and **3** for DCM.

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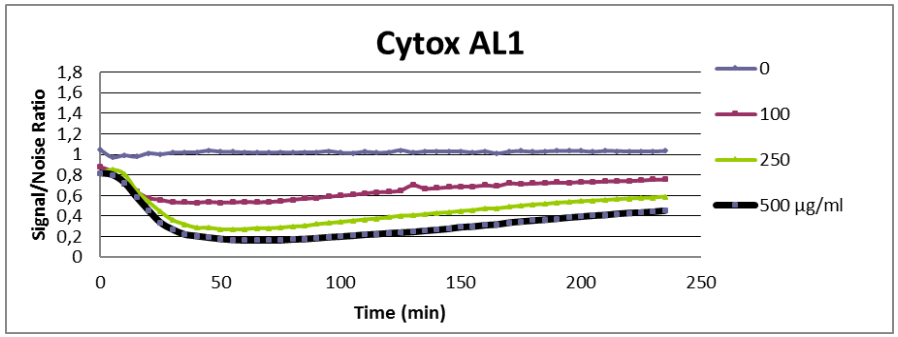

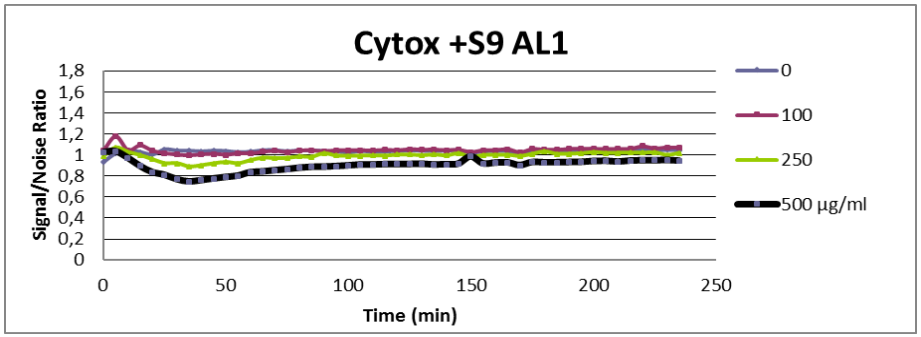

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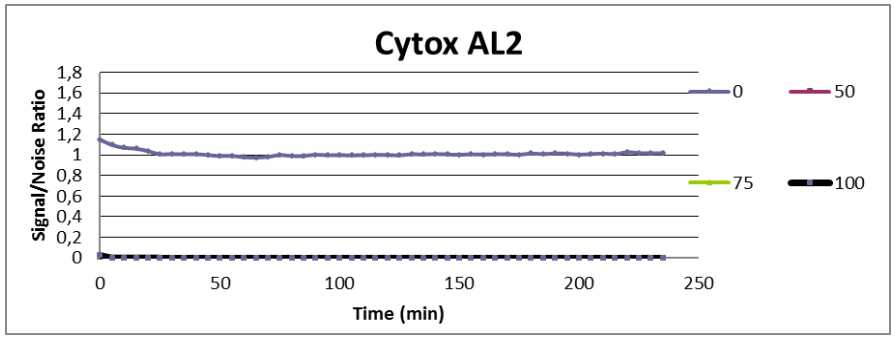

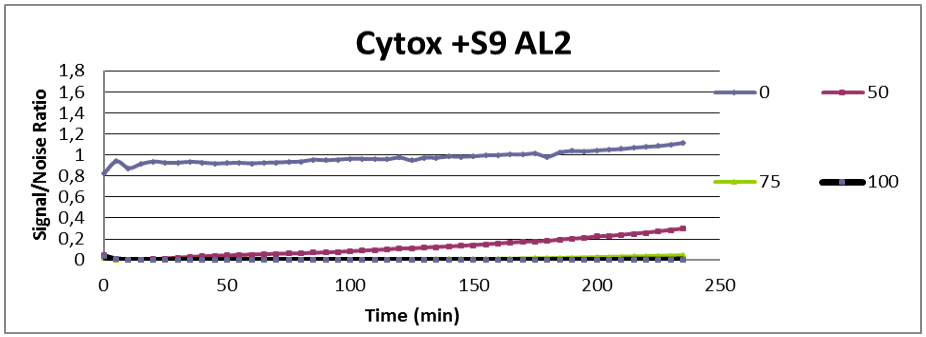

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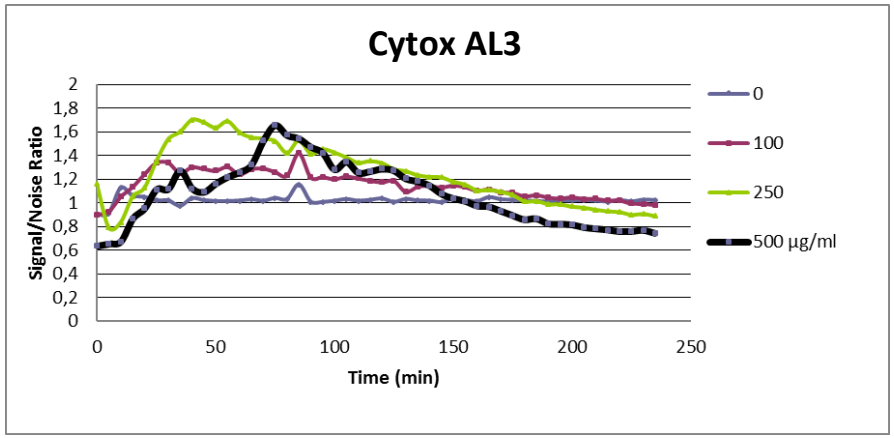

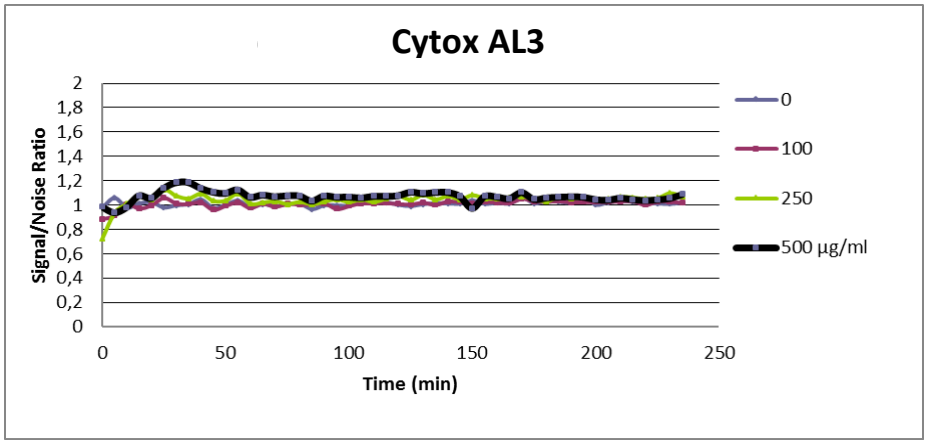

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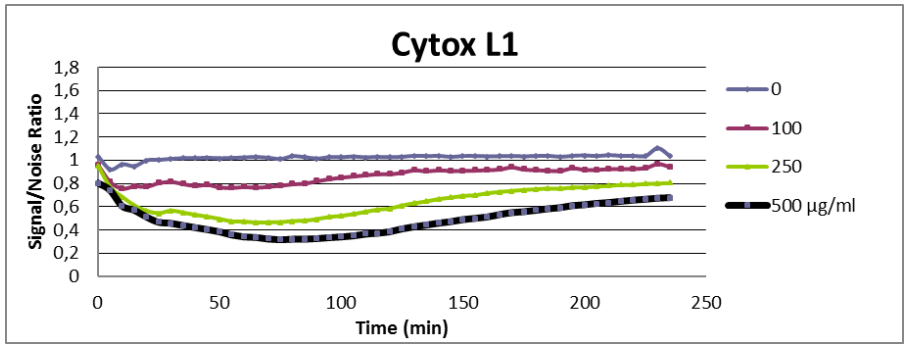

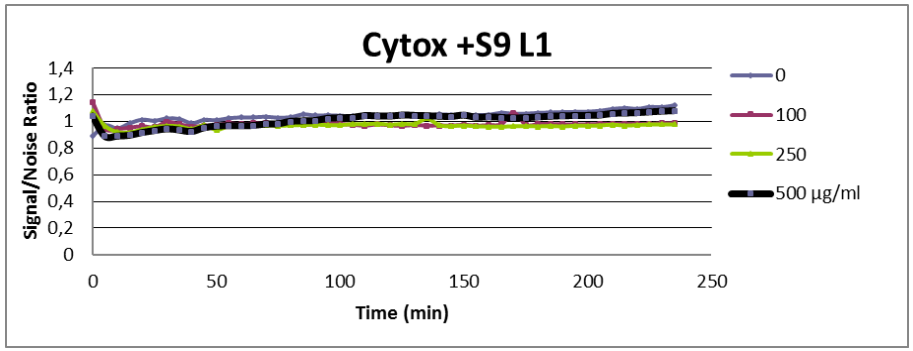

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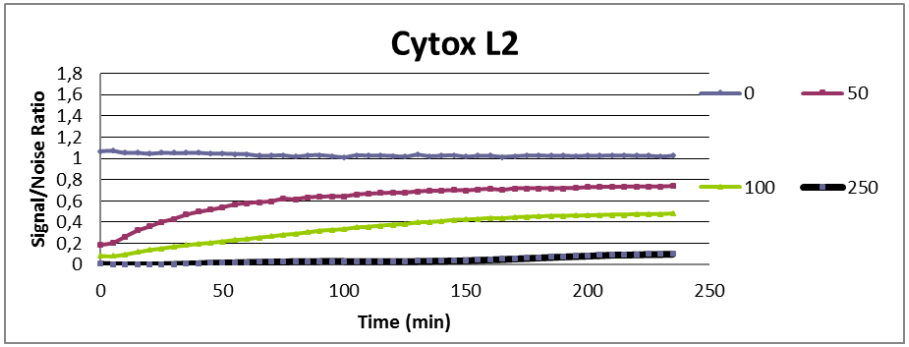

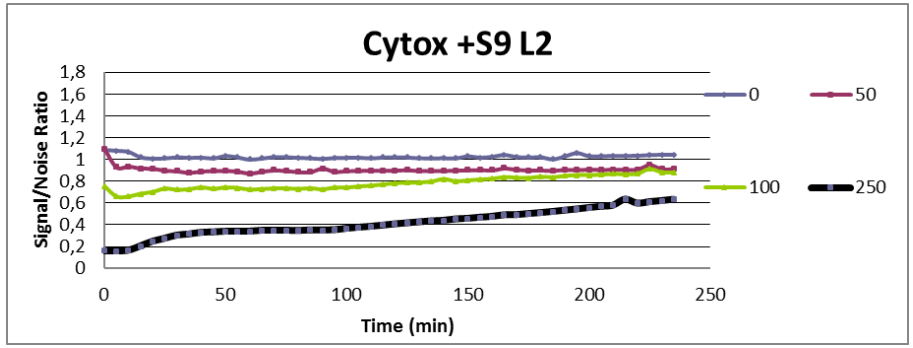

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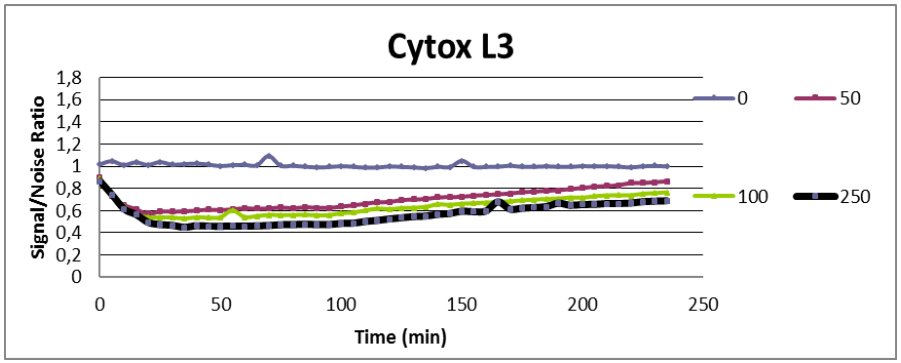

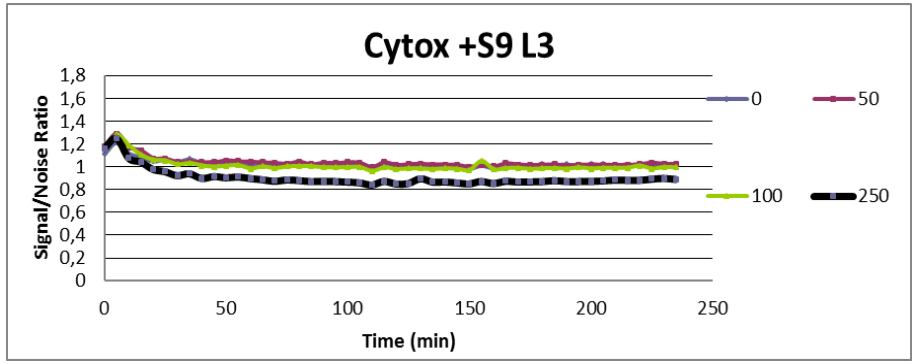

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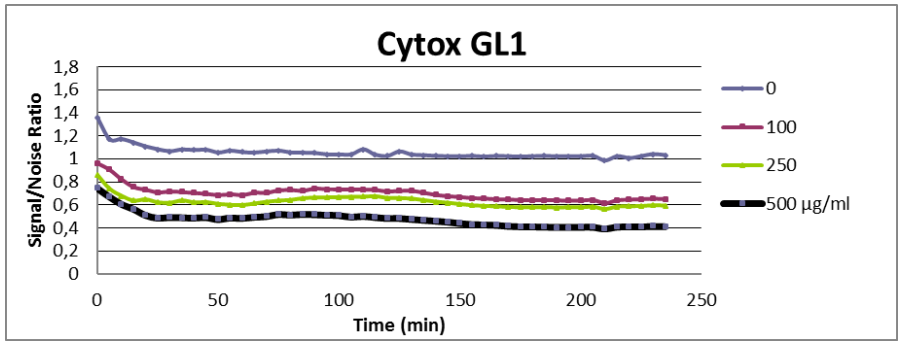

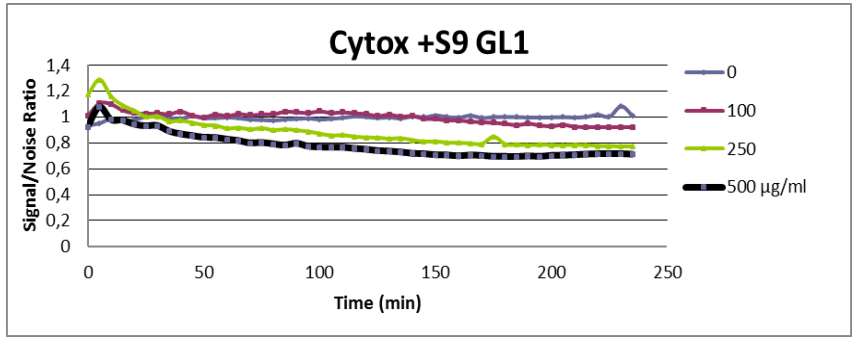

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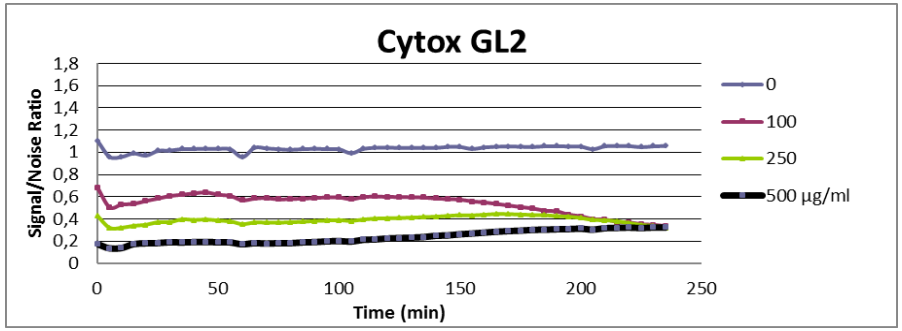

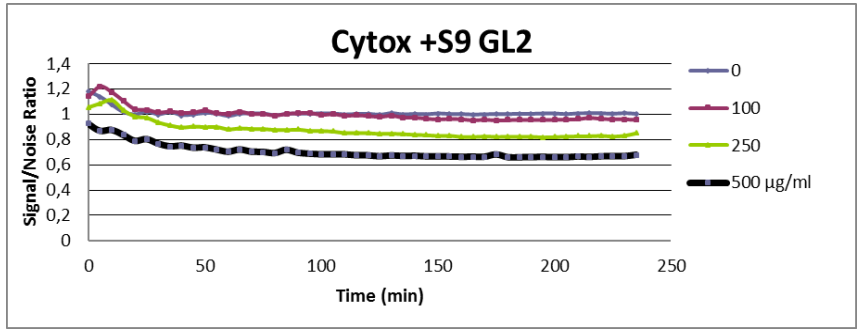

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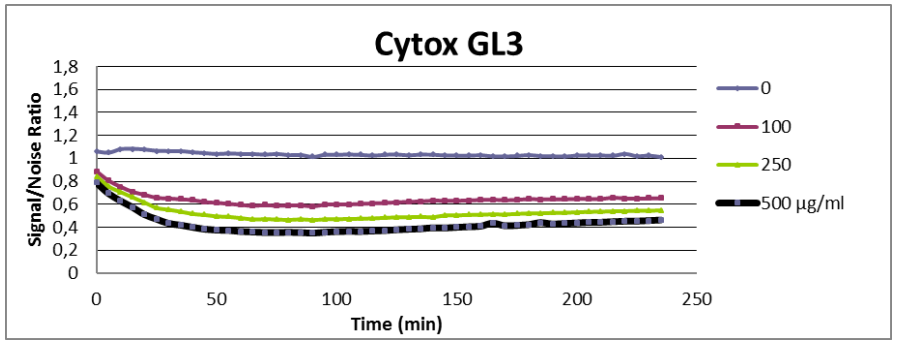

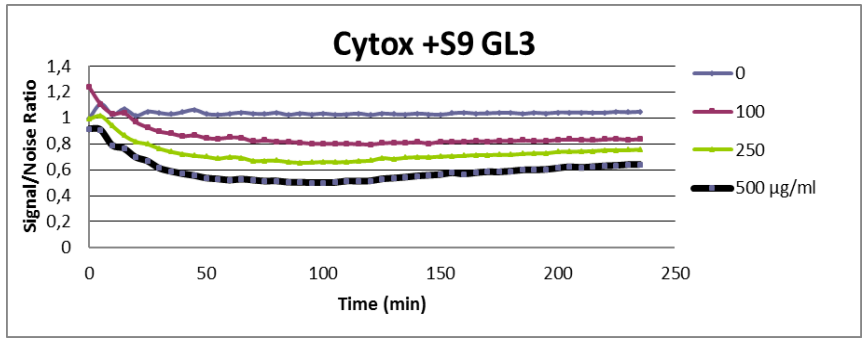

Figure 6.4: Cytoxicity results of *Asparagus laricinus* ***(AL)***, *Senecio asperulus* ***(L)*** and *Gunnera perpensa* ***(GL)*** plant extracts, with solvents used for extraction represented as **1** for Methanol, **2** for water and **3** for DCM.

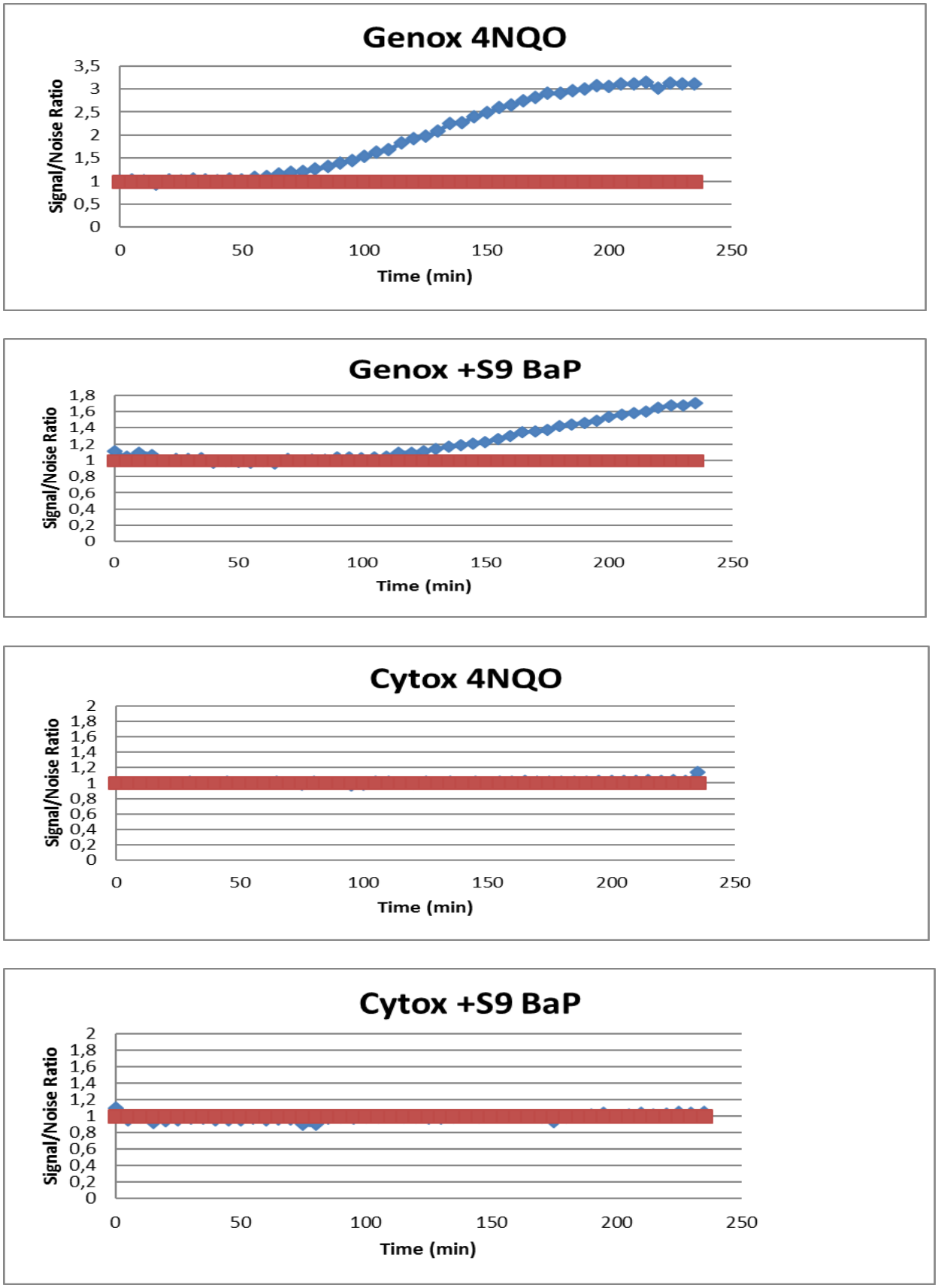

Figure 6.5: Genotoxicity and cytotoxicity results of controls.

6.5 DISCUSSION

For the continuous use of medicinal plants with a history of use for the treatment of various ailments and cancer to be recommended, their safety to mammal cells must be evaluated. As much as medicinal plants are believed to be safe by their users, research has proven that natural products, including medicinal plants, can be mutagenic (Cardoso et al., 2006; Mohd-Fuat et al., 2007; Deciga-Campos et al., 2007). Genotoxic compounds from medicinal plants can cause mutations that are mutagenic. However, plants with antimutagenic potential are considered as interesting sources for new therapeutic uses (Verschaeve et al., 2017). In this chapter we report on the mutagenicity and antimutagenicity of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* on hepatic (C3A) cells using two tests, the bacterial Vitotox test, and the alkaline comet assay.

The neutral red test is based on the ability to live cells to take up and bind the 3-amino-7dimethyl-2-methylphenazine hydrochloride (NR) dye. This dye is known to accumulate in the lysosomes of the viable cell after penetrating the cell membranes through nonionic diffusion (McGaw et al., 2014). Increased unabsorbed NR dye shows increased cell death. Viable cells can, therefore, be distinguished from dead or dying cells based on their NR uptake (NRU) and quantitative measurement of the number of viable cells can be determined. **Figure 6.1** reports on the cell viability of cells treated with different concentrations of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* plant extracts. Dichloromethane extracts of *Asparagus laricinus* and *Senecio asperulus* showed proliferation inhibition to C3A cells in a dose-dependent manner starting from a concentration of 50µg/ml, while *Gunnera perpensa* Methanol and DCM extracts showed cytotoxicity starting from the concentration of 100µg/ml. These findings were in consonance with cytotoxicity of the same plants on non-cancerous cells (Vero cells) as reported in **Chapter 5**.

The comet assay was performed only in the absence of S9 as cells used, C3A cells, already had an ability to retain their metabolic activity. Concentrations used in this test were guided by results obtained from the NRU test as well as the solubility of extracts, for example, some *Senecio asperulus* extracts maximum concentrations were lower than other extracts (**Figure 6.1, E** and **F**). Moreover, too toxic concentrations will

influence the percentages of DNA fragments in the tail of the formed comets. Thus, extracts that reduced the viability of cells at higher concentrations had their DNA damage properties assessed at lower concentrations. Furthermore, the comet assay was performed in the dark in order to prevent light-induced DNA damage (Azqueta and Collins, 2013). Deviations that were statistically significant for *Asparagus laricinus* were found at as low as 4 µg/ml for aqueous extracts, 20µg/ml for methanolic extracts and 250 µg/ml DCM extracts, and concentrations thereafter showed very increased DNA damage due to the observed percentage of the tail (**Figure 6.2**). Nonetheless, these effects were low when compared to DNA damage caused by the well-known mutagen, Ethyl methanesulfonate (EMS). *Senecio asperulus* extracts didn't show any formation of comets that were statistically significant as well as *Gunnera perpensa* aqueous extracts. However, *Gunnera perpensa* organic extracts did show DNA damage that was statistically significant at the highest tested concentration of 500 µg/ml for methanol extracts, and at 100 µg/ml for DCM extracts. For the latter, there was however no significant DNA damage increase as concentrations increased.

Vitotox test is based on the induction of SOS function transcription through the insertion of an operon-less "lux" gene next to recN resulting in light production when the bacterial DNA is damaged. The infusion of luxCDABE on the multicopy plasmid to recN promoter allows genotoxicity to be detected, while its fusion to pr1 promoter allows cytotoxicity to be detected. According to Verschaeve (2005), when recN-luxCDABE fusion occurs, the function of the samples' genotoxicity at sublethal levels will be to increase light production, while this light production is decreased as a function of the samples' toxicity due to the infusion of Pr1-luxCDABE. The vitotox assay, genotoxicity and cytotoxicity of the plant extracts were assayed simultaneously so as to identify false-positive results caused by non-specific light production induced by other mechanisms besides the genotoxic effect (Verschaeve et al., 1999).

The genotoxic strain with luciferase operon (TA104 recN2-4) and cytox strain expressing the lux operon (TA104 pr1) was used with and without metabolic activation by the S9 enzyme. S9 fractions have enzymes which play a major role in the activities of phase I and II metabolisms (Greim and Snyder, 2008). The genotoxicity of the plant extract was indicated by the light production after the genotoxic extract has activated the recN promoter in TA104 recN2-4 strain and the non-specific light production when

the compound activates the pr1 in TA104 pr1 strain was indicative of the cytotoxicity of the plant extract. The positive control used, 4-Nitroquinoline 1-oxide, is known to have mutagenic and carcinogenic effects. For the validity of the test, 4-NQO showed genotoxicity with S/N that was greater than 1.5 and no cytotoxicity with S/N of between 0.8 and 1 (**Figure 6.5**).

Asparagus laricinus methanol and *Senecio asperulus* aqueous extracts were neither genotoxic nor cytotoxic with or without S9. Their signal to noise ratio in response to Genox strain was less than 1.5 and to Cytox strain were below 0.8 in a dose-dependent manner. *Asparagus laricinus* water and *Gunnera perpensa* DCM extracts were found not to be genotoxic or cytotoxic with or without S9. Dichloromethane extracts of *Asparagus laricinus* was found to be cytotoxic as well as genotoxic with or without S9 activation, this was in line with NRU test and comet assay results. *Senecio asperulus* methanol extract was genotoxic and cytotoxic before the presence of S9, however, this extract remained cytotoxic but was not genotoxic when S9 was present. Dichloromethane extracts of *Senecio asperulus* were not genotoxic or cytotoxic without S9, but its toxicity was reversed by the presence of S9 as this extract became cytotoxic. This supports the cytotoxicity observed from the NRU test.

Moreover, both methanol and water extract of *Gunnera perpensa* were genotoxic but couldn't produce a signal to noise ratio that was above 0.8 for the Cytox strain. Thus, they were considered to be genotoxic and not cytotoxic. However, these extracts were not genotoxic but cytotoxic when S9 was present. This shows that the presence of S9 reversed the mutagenicity or even blocked the induced mutagenic activity of these extracts, thus making them genotoxic agents that are not mutagenic. According to Ndlala et al. (2011), *Gunnera perpensa* has an ability to inhibit the enzyme, thus this could be the reason why this plant was not genotoxic even after metabolism activation by the S9 fractions. The isolation of pure compounds as well the re-analysis of those compounds will assist in highlighting unknown mutagens from these plants. From our results, *Gunnera perpensa* extracts genotoxicity was reduced by the presence of S9 enzyme (from both Comet and Vitotox assays), this means the safety of this plant is modified when this plant is metabolized by the liver cells, however, this can only be confirmed after *in vivo* work has been is carried out on this plant.

To assess the antimutagenic effects of our selected medicinal plants, extracts were now tested with the presence of 4NQO, with and without the presence of S9 using the same concentrations and conditions as for the genotoxicity test. 4-nitroquinoline-N-oxide (4-NQO) is a base substitution agent that causes direct DNA damage by acting at G residues leading to the induction of GC to AT transitions (Fronza et al., 1992). From our results, all extracts tested did not significantly decrease or improve the genotoxicity of 4NQO, thus were considered as not having antigenotoxic activities. This implies that the observed anticancer activities of these plants, as reported in **Chapter 5,** are not due to genotoxicity protection from the plant, but rather through other mechanisms such as apoptosis as shown in **Chapter 5** for *Asparagus laricinus*.

6.6 CONCLUSION

Asparagus laricinus, Senecio asperulus and *Gunnera perpensa* have been used as traditional medicines for the treatment of several diseases including cancer. Numerous ethnobotanical studies have reported *Gunnera perpensa* to be one of the main ingredients for a concoction used by pregnant women for the maintenance of female reproductive health during pregnancy and the postpartum period. However, the safety of this plant has not been investigated using the comet and vitotox assays, more especially using liver cells. The presence of the S9 enzyme shows the manner in which the plant will be metabolized by the liver and this was outlined in this chapter. *Asparagus laricinus* could not cause DNA damage or be cytotoxic to liver cells, besides its dichloromethane extract that was mutagenic due to its genotoxic and cytotoxic properties revealed from all three tests performed. *Senecio asperulus* can be considered as a safe medicinal plant as it showed no mutagenicity or cytotoxicity. Moreover, *Gunnera perpensa* was found to be genotoxic and not cytotoxic, however, its genotoxic and cytotoxic effects are reversed when the S9 enzyme is present. This shows that during the metabolism of this plant by the S9, its genotoxicity properties are lost. Thus, the plant doesn't cause any DNA damage after it has been metabolized but rather becomes cytotoxic. Therefore, this plant is still questionable and shouldn't be used further until its cytotoxic mechanism is well understood. All tested extracts, unfortunately, didn't show any antimutagenic effects, thus can't be used to reverse DNA damage caused by mutagens.

6.7 REFERENCES

Arpita Roy, Shruti A, Navneeta B. 2017. A Review on Medicinal Plants against Cancer. Journal of Plant Sciences and Agricultural Research. 2(1):1-5.

Azqueta A, Collins A.R. 2013. The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Arch Toxicol. 87:949-968.

Canivenc-Lavier M, Vernevauta M, Totis M, Siess M, Magdalou J, Suscheteta M. 1996. Comparative effects of flavonoids and model inducers on drug-metabolizing enzymes in rat liver. Toxicology. 11(41):2297-2301.

Cardoso C.R, De Syllos Colus I.M, Bernardi C.C, Sannomiya M, Vilegas W, Varanda E.A. 2006. Mutagenic activity promoted by amentoflavone and methanol extract of Byrsonima crassa Niedenzu. Toxicology 225:55-63.

Collins A.R. 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol. 26(3):249-61.

Cragg G.M, Newman D.J. 2000. Antineoplastic agents from natural sources: Achievements and future directions. Expert Opinion on Investigational Drugs. 9:1-15.

Deciga-Campos M, Rivero-Cruz I, Arriaga-Alba M, Castaneda-Corral G, Angeles-Lopez G.E, Navarrete A, Mata R. 2007. Acute toxicity and mutagenic activity of Mexican plants used in traditional medicine. Journal of Ethnopharmacology. 110:334-342.

Efferth T, Kaina B. 2011. Toxicities by herbal medicines with emphasis to traditional Chinese medicine. Current Drug Metabolism. 12(10):989-996.

El-Sayed W.M, Hussin W.A. 2013. Antimutagenic and antioxidant activity of novel 4-substituted phenyl-2,2'-bichalcophenes and aza-analogs. Drug Des Devel Ther. 7:73-81.

Ferguson L.R, Philpott M. 2008. Nutrition and mutagenesis. *Annu Rev Nutr. 28:313-29.*

Fronza G, Campomenosi P, Iannone R, Abbondandolo A. 1992. The 4-nitroquinoline 1-oxide mutational spectrum in single-stranded DNA is characterized by guanine to pyrimidine transversions. Nucleic Acids Res. 20:1283-1287.

Gautam S, Saxena S, Kumar S. 2016. Fruits and Vegetables as Dietary Sources of Antimutagens. J Food Chem Nanotechnol. 2(3):97-114.

Greim H, Snyder R. 2008. Toxicity of Selected Chemicals. Toxicology and Risk Assessment: A Comprehensive Introduction. (Ed.), ISBN: 978-0-470-86893-5, John Wiley & Sons. pp. 513-534.

Griffiths A.J.F, Miller J.H, Suzuki D.T, Lewontin R.C, Gelbart W.M. 2000. An Introduction to Genetic Analysis, 7th edition. New York: W. H. Freeman. ISBN-10: 0-7167-3520-2.

Joshi B, Lekhak S, Sharma A. 2009. Antibacterial Property of Different medicinal plants: Ocimum sanctum, Cinnamomumzeylanicum, Xanthoxylumarmatum and Origanum Majorana. Kathmandu University of Sciences, Engineering and Technology. 5:143-150.

Leandro L.F, Munari C.C, Sato V.L.F.L, Alves J.M, Oliveira P.F, Mastrocola D.F.P, Cunha W.R. 2013. Assessment of the genotoxicity and antigenotoxicity of (+)-usnic acid in V79 cells and Swiss mice by the micronucleus and comet assays. Mutat. Res. 753:101-06.

Makhuvele R, Foubert K, Apers S, Pieters L, Verschaeve L, Elgorashi E. 2018. Antimutagenic constituents from Monanthotaxis caffra (Sond.) Verdc. Journal of Pharmacy and Pharmacology. 70(7):976-984.

McGaw L.J, Elgorashi E.E, Eloff J.N. 2014. Cytotoxicity of African Medicinal Plants Against Normal Animal and Human Cells. Toxicological survey of African medicinal plants. 8:181-233.

Mohd-Fuat A.R, Kofi E.A, Allan G.G.C. 2007. Mutagenic and cytotoxic properties of three herbal plants from Southeast Asia. Tropical biomedicine. 24(2):49-59.

Moon S.J, Kottgen M, Jiao Y, Xu H, Montell C. 2006. A taste receptor required for the caffeine response in vivo. Curr. Biol. 16(18):1812-1817

Mustapha A.A. 2014. Medicinal plants with possible anti- HIV activities: A Review International Journal of Medicinal Plants. Photon. 106:439-453.

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

Repetto G, Del Peso A, Zurita J.L. 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Prot. 3:1125–31.

Shih S.C, Sloper-Mould K.E, Hicke L. 2000. Monoubiquitin carries a novel internalization signal that is appended to activated receptors. EMBO J. 19(2):187-98

Singh N.P, McCoy M.T, Tice R.R, Schneider E.L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 175:184-191.

Słoczyńska K, Powroźnik B, Pękala E, Waszkielewicz A.M. 2014. Antimutagenic compounds and their possible mechanisms of action. J Appl Genet. 55(2):273-285.

Tice R.R. 2000. "Single Cell Gel/Comet Assay: Guidelines for in vitro and in vivo Genetic Toxicology Testing". Environmental and Molecular Mutagenesis. 35(3):206-21.

Vaghasiya Y, Dave R, Chanda. S. 2011. Phytochemical Analysis of Some Medicinal Plants from Western Region of India. Res.J. Med.Plant, 5: 567-576.

Verschaeve L, Van Gompel L, Thilemans L, Regniers P, Vanparys D, van der Lelie L. 1999. VITOTOX® bacterial genotoxicity and toxicity test for the rapid screening of chemicals. Environ. Mol. Mutagen. 33:240-248.

Verschaeve L. 2005. The Vitotox genotoxicity test. Recent Res. Devel. Applied Microbiol. Biotechnol. 2:1-17.

Verschaeve L, Edziri H, Anthonissen R, Boujnah D, Skhiri F, Chehab H, Aouni M, Mastouri M. 2017. In vitro Toxicity and Genotoxic Activity of Aqueous Leaf Extracts from Four Varieties of Olea Europea (L). Pharmacogn Mag. 13(1):S63–S68.

Williams C.A, Grayer R.J. 2004. Anthocyanins and Other Flavonoids. Natural Product Reports. 21(4):539-73.

Wu J.A, Attele A.S, Zhang L, Yuan C.S. 2001. Anti-HIV activity of medicinal herbs: usage and potential development. Am J Chin Med. 29(1):69-81.

CHAPTER 7

"Characterization and identification of active compounds from *Asparagus laricinus* and *Senecio asperulus*"

ABSTRACT

Nature has been a good supply of various medicinal agents for thousands of years and more than 50% of modern drugs have been isolated from natural sources such as plants. Both *Asparagus laricinus* and *Senecio asperulus* have been used in traditional remedies by Southern Africans for years. *Asparagus laricinus* and *Senecio asperulus* are traditionally used to treat inflammation-related disorders and cancer. The aim of this chapter is to determine the chemical composition of *Asparagus laricinus* and *Senecio asperulus* crude extracts. To describe a distinctive feature of these medicinal plants, Liquid chromatography-mass spectrometry (LC-MS) analysis was performed. The LCMS results of these plant extracts showed the presence of twelve known compounds: *Luteolin 7-O-rutinoside, Apigenin arabinoside-glucoside, Apigenin galactoside-arabinoside, 3-Methoxynobiletin, Apigenin 7-O-apiosyl-glucoside, Quercetin 3-O-rhamnosyl-galactoside, Rutin (Quercetin 3-O-rhamnosyl-glucoside), Kaempferol 3-O-galactoside 7-O-rhamnoside, Ecdysterone, Isorhamnetin 3-O-glucoside 7-O-rhamnoside, Syringaresinol, 20-hydroxyecdysone* and twenty eight unknown compounds from *Asparagus laricinus Burch.*. Moreover, *Senecio asperulus DC.* revealed the presence of two known compounds, *chlorogenic acid isomer, dicaffeoyl quinic acid* and nineteen unknown compounds. The abundance of compounds characterized and identified from these plants correlated with the reported total phenolic content results discovered in Chapter 3 of this study, as it was demonstrated that *Asparagus laricinus* had more phenols than *Senecio asperulus*.

7.1 INTRODUCTION

Plants have limitless abilities to produce chemical substances, mainly secondary metabolites, of which numerous compounds have been isolated from them (Rafael, 2008). Chemical analysis of *Asparagus laricinus Burch.* showed the presence of saponins, steroids, tannins, terpenoids and flavonoids and *Senecio asperulus DC.* showed the presence of flavonoids, tannins, terpenoids, and steroids as reported in Chapter 3. The derivatives of such chemical compounds are found in most plants; thus, their existence was expected. Alkaloids, flavonoids, terpenoids, tannins, phenolics and many others are very important for both humans and plants (Rafael, 2008). Phenolics are natural compounds that function as a defense mechanism used by plants against predators (Velderrain-Rodríguez et al., 2014). Phenols also contribute to plant reproduction and in plant-plant interference as they are colorful attractants for birds and insects, thus helping seed dispersal and pollination (Harborne, 1994). Phenolic acids and flavonoids have also been reported to have various pharmacological activities when used by humans, these include them having antioxidative, antimicrobial and anticarcinogenic effects (Noratto et al., 2010; Pierson et al., 2015; Puupponen-PimiaÈ et al., 2001; Wang et al., 2015)

It is estimated that about 61% of the 877 small molecule new chemical entities introduced as drugs worldwide in the past 30 years were developed from natural products (Satyajit and Lutfun 2007). An estimation of about 75% of anticancer drug candidates discovered so far are natural products or structural analogs of natural products. Approximately 60% of all drugs in clinical trials for a multiplicity of cancers are of natural origin. In the past, the identification of compounds was labor-intensive, time-consuming and required too much sample. However, modern drug discovery approaches apply full automation and robotics where hundreds of molecules can be screened, using several assays within a short time and very small amounts of compounds. A number of techniques are used for the identification and analysis of plants chemical constituents such as liquid chromatography-mass spectrometry detector (LC-MS) detector and liquid chromatography–nuclear magnetic resonance spectroscopy. The aim of this study was to determine the chemical composition of *Asparagus laricinus Burch* and *Senecio asperulus DC* crude extracts extracted with methanol, water, dichloromethane, methanol: dichloromethane and hexane.

7.2 LITERATURE REVIEW

There are two classes of compounds from plants, namely primary and secondary metabolites. Primary metabolites are required for the nourishment of the plant, while secondary metabolites are not a requirement for the plant's survival (Harborne and Baxter, 1999). Secondary metabolites are mostly compounds that are active for treatment and prevention of diseases in humans and animals and are thus termed bioactive compounds. Bioactive compounds are generally unique to individual plant species and are species-specific, but some may be found in several or many plant species of a genus, in several related genera, or even families (Kinghorn *et al.*, 2003). Asparagus species are known as medicinal plants that possess a variety of biological properties (Saxema and Chaurasia, 2001). To show that this genus is rich in compounds of medicinal value, previous investigations of *Asparagus* species revealed the presence of 8-methoxy-5,6,4'-trihydroxyisoflavone-7-O-β-D-glucopyranoside, the novel compound from *Asparagus racemosus* and *Oligofurostanosides (curillins G and H)* and *spirostanosides* from *Asparagus curillins*, to mention a few (Negi et al., 2010).

Senecio species are known to be rich with essential oils with epoxyfuranoeremophilane and (E)-β-farnesene and as their main compound (Kahriman et al., 2011). According to Zdero et al. (1989), *Senecio asperulus* (the part not mentioned) from South Africa was shown to contain pyrrolizidine alkaloids, furocremophilones, and some diterpenes. *Asparagus laricinus* cladodes and *Senecio asperulus* roots extracts have never been explored with the aim of characterizing these plant species. Thus, the compounds they have, have not been identified before. Considering, that a single plant may contain up to thousands of phytoconstituents, due to dissimilar compounds being present in different parts of the plant, possibilities of making new discoveries become self-evident. Our phytochemical investigation of *Asparagus laricinus* and *Senecio asperulus* has resulted in the identification of phytochemicals that these two medicinal plants have using LC-MS instrument which combined both Mass spectrometry and High-pressure liquid chromatography as shown in **Figure 7.1**.

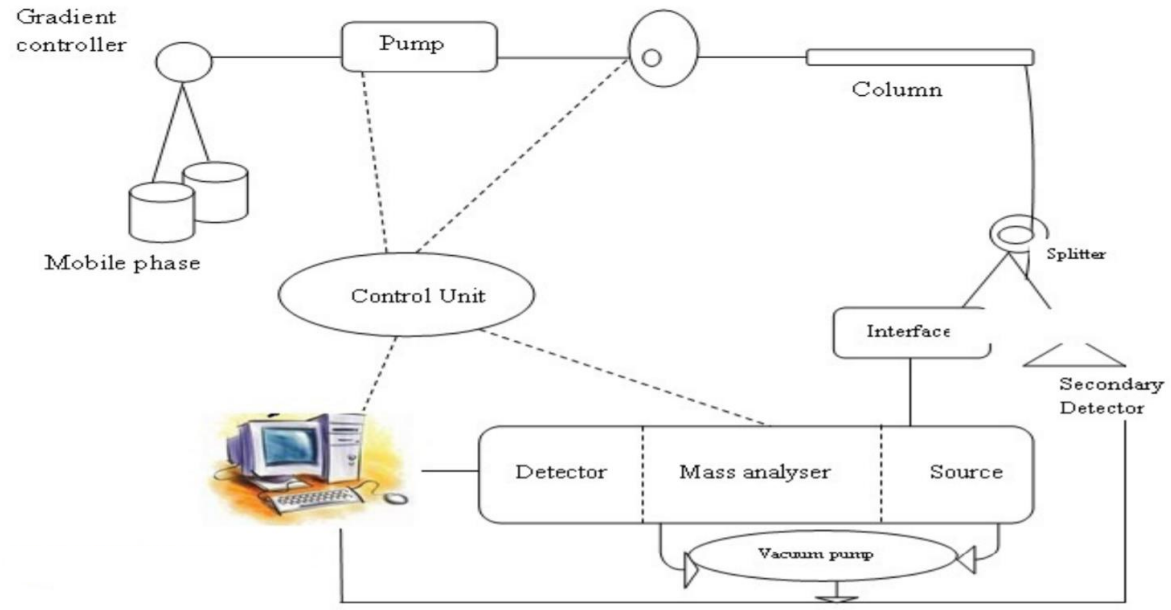

Figure 7.1: Illustration of a systematic LC-MS instrument (Parasuraman et al., 2014).

Liquid chromatography (LC) involves mass transfer of a sample through a polar mobile phase and non-polar stationary phase, while Mass spectrometry (MS) ionizes atoms so that they can be separated according to their molecular masses and charges (mass to charge ratio) (Korfmacher, 2005; Parasuraman et al., 2014). Liquid chromatography-Mass spectrometry (LC-MS) is a chromatographic technique used to separate a mixture of compounds with the purpose of characterizing and identifying the specific components of the mixture. Therefore, LC-MS combines techniques between MS and High-pressure liquid chromatography (HPLC), but not MS and LC. HPLC is an advanced type of Liquid chromatography where solvents travel under high pressures rather than through the force of gravity as with the normal LC. Furthermore, the application of both techniques simultaneously reduces experimental error and improves accuracy (Lim 2002).

Mass spectrometry in LC-MS helps to determine the elemental composition and structural elucidation of a sample (Pitt, 2009). With LC-MS, compounds are separated based on their physical and chemical properties, then the components within each peak are detected by ionization and identified based on their mass spectrum. The other major advantage of LC-MS includes sensitivity, specificity, and precision as analysis are performed at the molecular level (Parasuraman et al., 2014). Moreover,

this instrument only requires small quantities of the samples and is very convenient for low or non-volatile organic compounds, which cannot be handled with other chromatographic techniques, such as gas chromatography.

7.3 METHODOLOGY

7.3.1 Identification of active compounds with LC-MS

LC-MS/MS analysis for the identification of active compounds from *Senecio asperulus* and *Asparagus laricinus* crude extracts was carried out using the Waters Synapt G2, ESI positive/negative, Cone Voltage 15 V, lock mass: leucine encephalin instrument. This is an Agilent 1100 LC system consisting of degasser, binary pump, autosampler and column heater. The column outlet was coupled to an Agilent MSD Ion Trap XCT mass spectrometer equipped with an ESI ion source. Data acquisition and mass spectrometric evaluation were carried out on a computer with an Acquity binary solvent manager instrument system. For the chromatographic separation, Waters BEH C18, 2.1 x 100 mm column was used. The column was held at 95% solvent A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in acetonitrile) for 1 minute, followed by an 11 min step gradient from 5% B to 100% B, then it was kept for 4 min with 100% B; finally, the elution was achieved with a linear gradient from 100% B to 5% B for 2 minutes.

The flow rate was 0.4 ml/min and the injection volume was 0.01 ml. The following parameters were used throughout all MS experiments: for electrospray ionization with negative ion polarity the capillary voltage was set to 3 kV, the drying temperature to 350 ^{o}C and cone voltage of 15 V, the maximum nebulizer pressure to 15000 psi and the seal wash was 5 min. The total run time was 15 minutes, the scan speed was 26 000 m/z/s (ultra-scan mode) and the lock mass was Leucine enkephalin. The phenolics were identified using a combination of HPLC with diode array detection and liquid chromatography with atmospheric pressure chemical ionization mass spectrometry (ESI-LC/MS/MS) based on their ultraviolet spectra, mass spectra and by comparison of the spectra with those of available authentic standards.

7.4 RESULTS

Compounds present in *Asparagus laricinus* and *Senecio asperulus* were shown by peaks generated by the LCMS instrument used, with molecular masses on the y-axis and retention time on the x-axis (Figure 7.2-7.4).

The analysis of the elemental composition is an informative technique used in mass spectrometry to identify unambiguous compounds. The obtained retention time and molecular mass of each peak observed (Figure 7.3-7.6) was used to identify the molecular formula of each compound as shown in Figure 7.2.

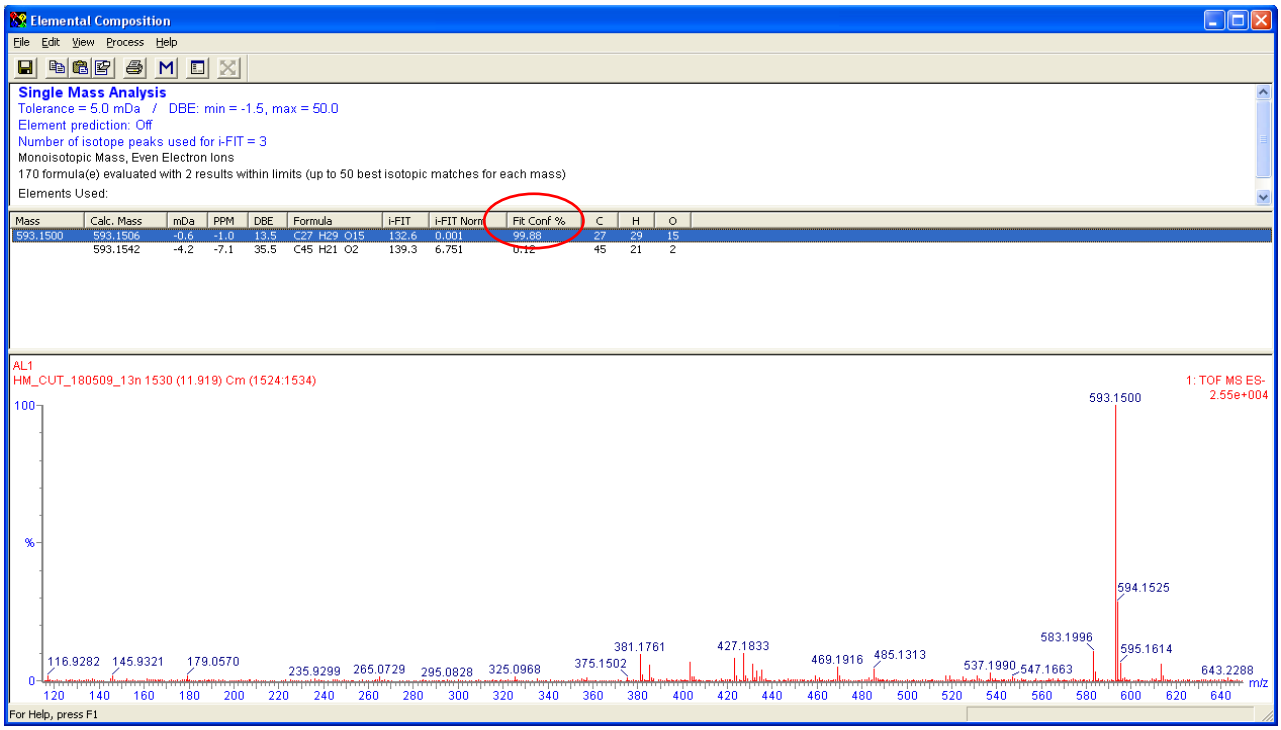

Figure 7.2: System used to identify compounds from the chromatogram.

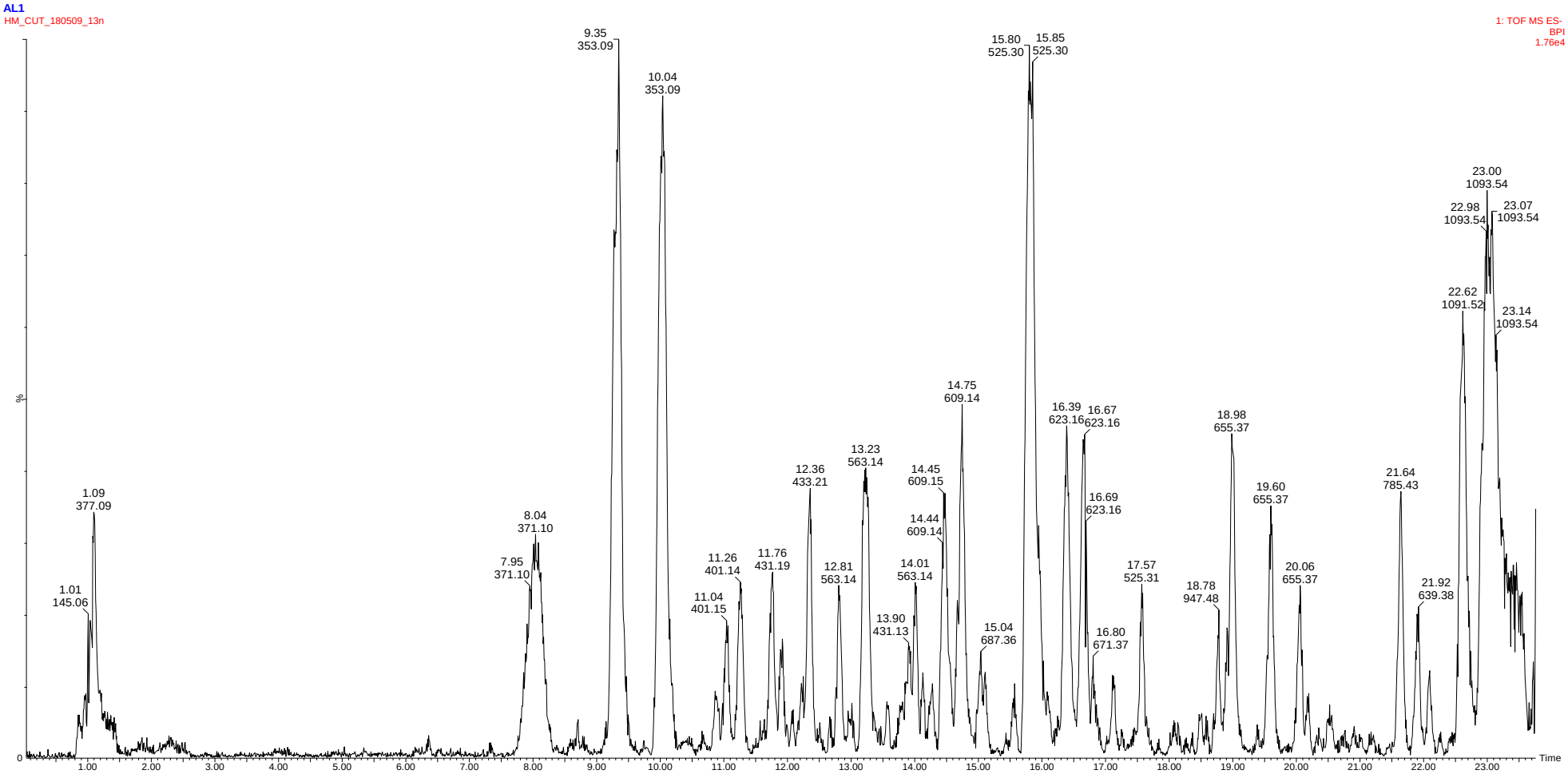

Figure 7.3: LC/MS chromatogram of *Asparagus laricinus* methanol extract (ESI negative). Nineteen phenolic components were identified.

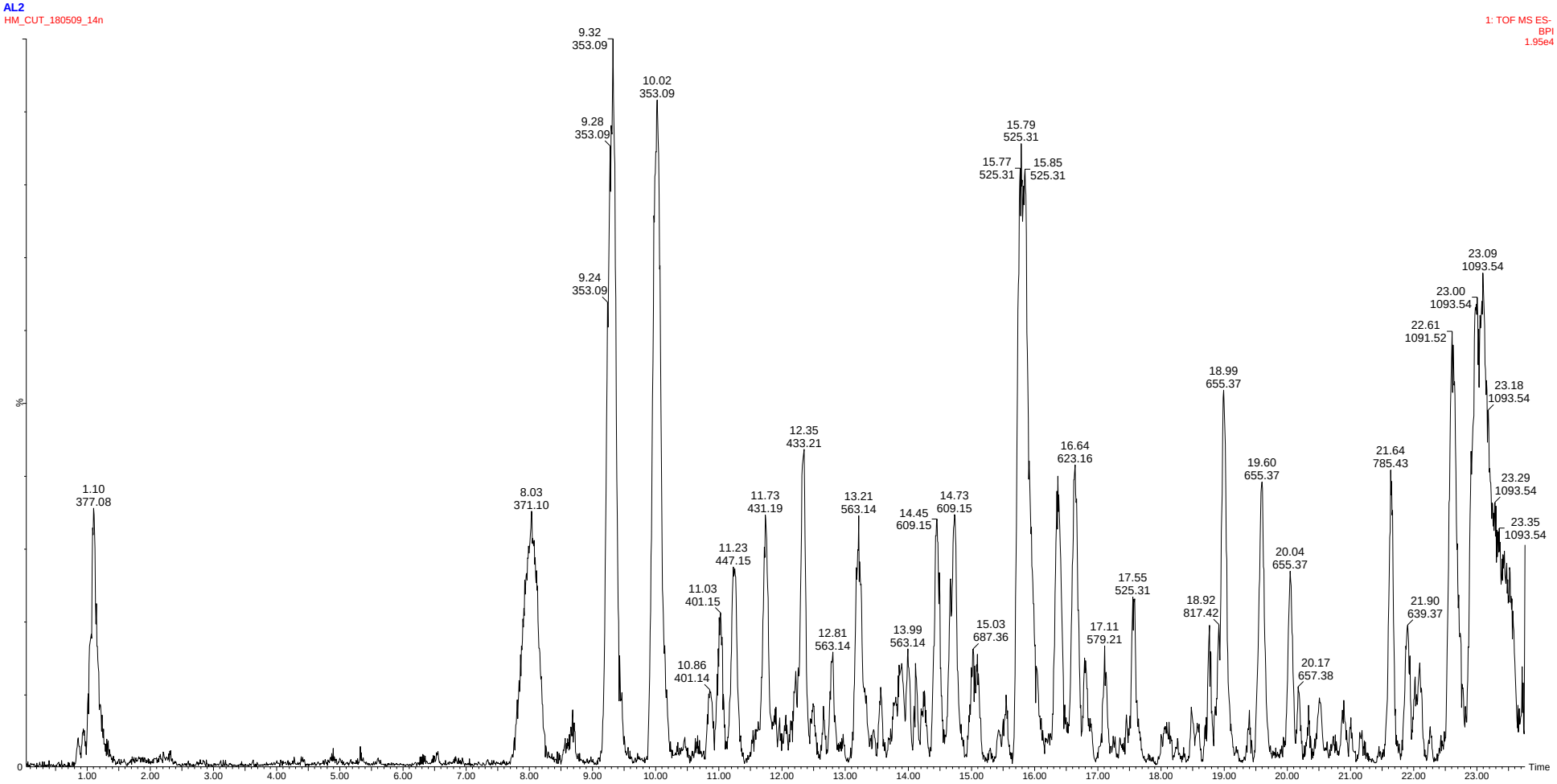

Figure 7.4: LC/MS chromatogram of *Asparagus laricinus* aqueous extracts (ESI negative). Twenty phenolic components were identified.

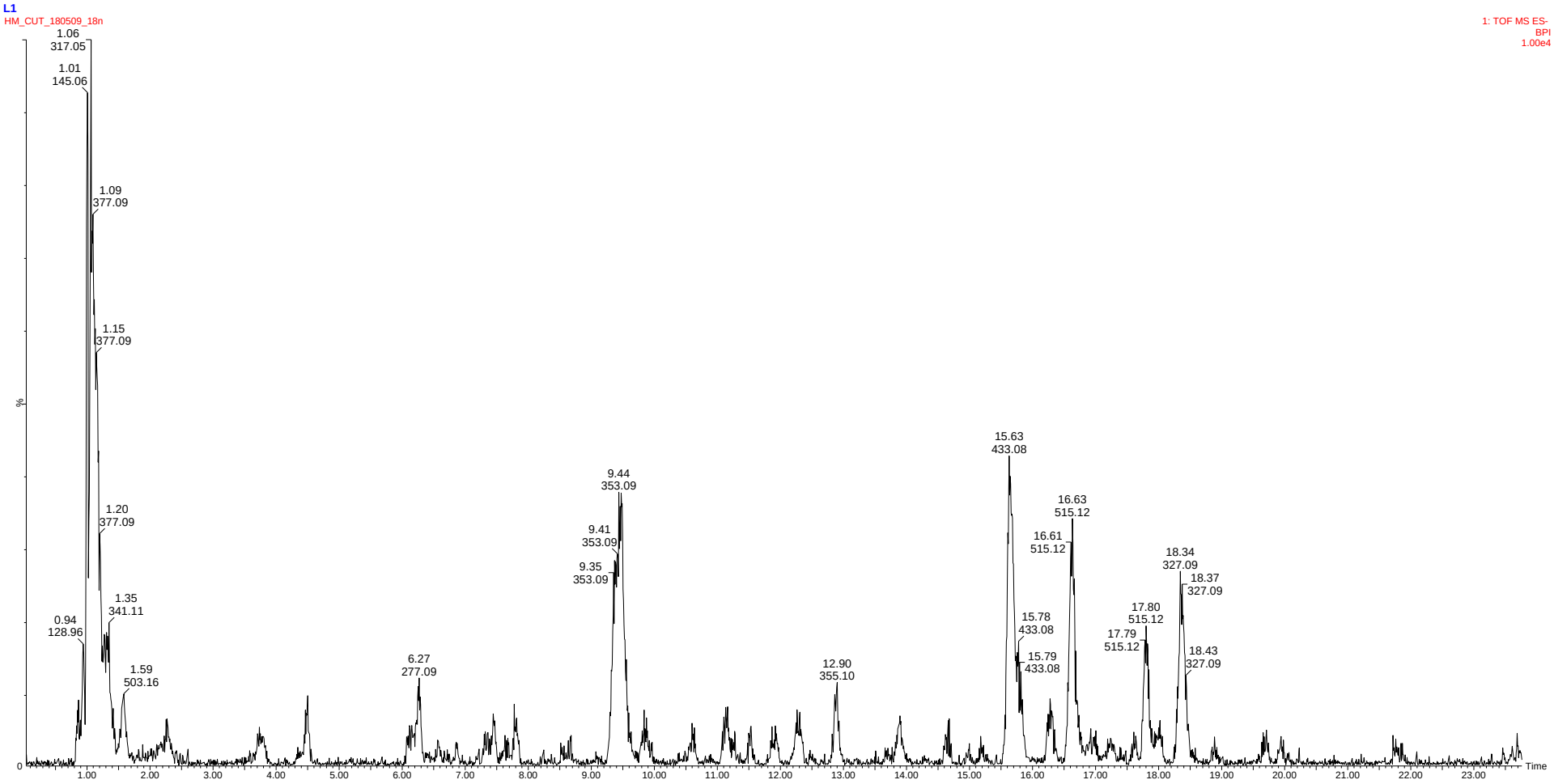

Figure 7.5: LC/MS chromatogram of *Senecio asperulus* methanol extracts (ESI negative). Thirteen phenolic components were identified.

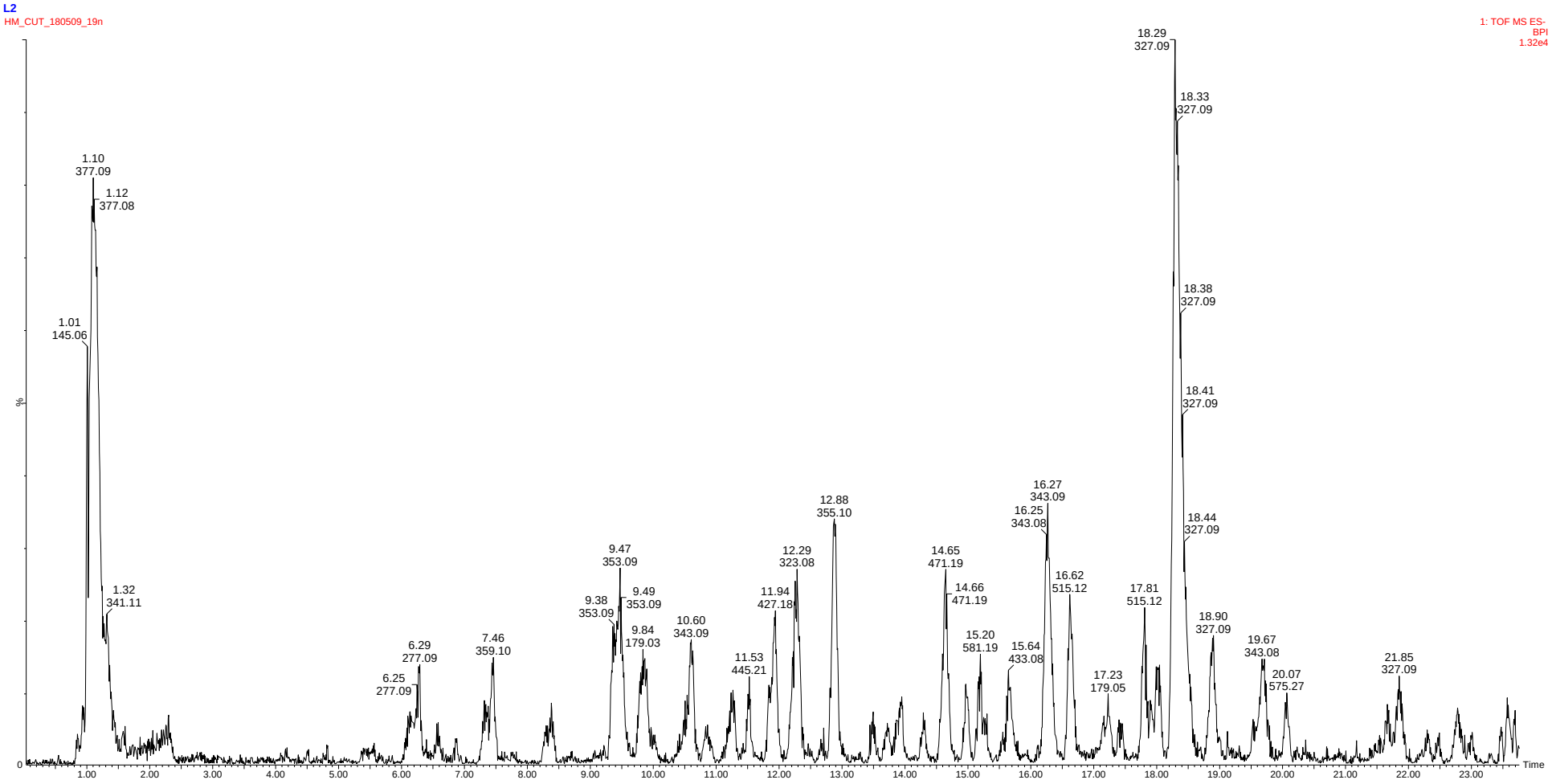

Figure 7.6: LC/MS chromatogram of *Senecio asperulus* aqueous extracts (ESI negative). Twenty-four phenolic components were identified.

Table 7.1: Summary of most abundant compounds present in *Asparagus laricinus Burch.* cladodes from both ESI negative and positive mode. **(m/z) = Molecular mass, Rt = retention time, ID= identification.**

Rt	m/z [M-H]-	Formula [M-H]-	Fragments	UV max	ID	Phytochemical class
8,04	371,1	C16H19O10	281 251 197 167	289	**Unknown**	Not known
9,35	353,08	C16H17O9	233 205,05	294	**Unknown**	Not known
10	353,08	C16H17O9	263 233 205 161	296	**Unknown**	Not known
10,89	755,2	C33H39O20	593 401 284 193 161	263	**Unknown**	Not known
11,26	401,14	C18H25O10	269 161 113 101	288	**Unknown**	Not known
11,8	519,17	C22H31O14	459 431 385	341	**Unknown**	Not known
11,92	593,2	C27H29O15	473 383 353 297	270 332	Luteolin 7-O-rutinoside	Flavonoids
12,36	433,2	C20H33O10	399 285 225 145 119	310	**Unknown**	Not known
12,67	563,14	C26H27O14	383 353 297 175	330	Apigenin arabinoside-glucoside	Flavonoids
13,21	563,14	C26H27O14	473 443 383 353 297	333, 271	Apigenin galactoside-arabinoside	Flavonoids
13,8	431,13	C22H23O9	339 295 267 241 226	279	3-Methoxynobiletin	

13,99	563,14	C26H27O14	473 443 383 353 297	332	Apigenin 7-O-apiosyl-glucoside	Flavonoids
14,1	787,27	C28H51O25	579 417 361 181	271	**Unknown**	Not known
14,24	461,14	C23H25O10	417 269 271 256	280	**Unknown**	Not known
14,47	609,14	C27H29O16	300 271 245 203 151	348	Quercetin 3-O-rhamnosyl-galactoside	Flavonoids
14,7	609,14	C27H29O16	300 271 245 203 151	348	Rutin (Quercetin 3-O-rhamnosyl-glucoside)	Flavonoids
15,03	641,35	C33H53O12	609 479 447 429 225	320	**Unknown**	Not known
15,54	593,15	C27H29O15	285 255 227 193	264	Kaempferol 3-O-galactoside 7-O-rhamnoside	Flavonoids
15,8	479,3	C27H43O7	479 159	247	Ecdysterone	Steroids
16,4	623,16	C28H31O16	314 299 271	351	Isorhamnetin 3-O-glucoside 7-O-rhamnoside	Flavonoids
16,6	623,16	C28H31O16	315 300 255	352	**Unknown**	Not known
17,12	417,15	C22H25O8	771 579 477 417 314 000	347	Syringaresinol	Polyphenols
17,57	479,3	C27H43O7	319 285 165 159 119	280	20-hydroxyecdysone	Steroids

Table 7.2: Summary of most abundant compounds present in *Senecio asperulus DC.* roots from both ESI negative and positive mode. **(m/z) = Molecular mass, Rt = retention time, ID= identification.**

RT	m/z [M-H]-	Formula [M-H]-	Fragments	UV max	ID
9,45	353,088	C16H17O9	191	325	chlorogenic acid isomer
11	353,0876	C16H17O9	191	305	chlorogenic acid isomer
15,6	433,0771	C20H17O11	271,179,135,253	330	**Unknown**
16,6	515,1187	C25H23O12	135,179,191,353	326	dicaffeoyl quinic acid
17,8	515,1198	C25H23O12	173,179,191,135,353	326	dicaffeoyl quinic acid
18,4	327,088	C18H15O6	97	280	**Unknown**

7.5 DISCUSSION

The crucial factor for conclusive accomplishment of isolating bioactive plant constituents depends on the selection of the correct plant and the relevant part of the plant, which contains the active compounds. This is because secondary metabolites are expressed in various combinations and concentrations in different parts of the plant, for example, the roots of the plants may contain fewer phenols than leaves. Consequently, certain secondary metabolites in specific organs and variation in bioactivity are often encountered in different parts of the same plant (O'Neill and Lewis, 1993). In this study, the LC-MS technique has been used to investigate the phenolic content of *Asparagus laricinus Burch.* and *Senecio asperulus DC*. plant extracts.

Concisely, for *Asparagus laricinus Burch.* LC-MS analysis in **Figure 7.3**, the first two components with the greatest peak area to be eluted were in the hydrophobic region as they had longer retention time (9.35 min and 10.04 min). Those with short retention time were more in the hydrophilic region, and those peaks were seen at 1.01 min and 8.04 min. However, peaks that were too flat with no UV absorbance such as those between 2.00 -7.00 min in **Figure 7.3**, were unlikely to contain polyphenolic groups, hence their m/z was not picked up by the LC-MS. The deprotonated molecule mass (m/z) measured by the MS in ESI negative and positive mode was used to determine the molecular formula of the compounds extracted. Fragmentation of the compounds

by the mass spectrum was also considered to obtain the molecular chemical formula of the compounds that formed significant peaks, and these were reported in **Table 7.1** and **7.2**.

The relative retention times and mass spectra of each extract components were compared with those of authentic samples (standards) and identified with the Elemental Composition database library programme. As shown in **Figure 7.3**, LC-MS analysis of the *Asparagus laricinus Burch.* methanol extract resulted in the identification of nineteen compounds, with the first identified compound of molecular mass 593,2 and 11,92 retention time as it had more than 90% similarity with the standard mass spectra in the library as shown in **Figure 7.2**. The LC-MS analysis of *Senecio asperulus DC.* extract resulted in the identification of two compounds, also with more than 90% similarity with the standard mass spectra in the library. Twenty-three compounds were found to be the most abundant compounds in the cladodes of *Asparagus laricinus Burch*, with twelve known and eleven unknown compounds. Four compounds were identified while two compounds were unknown from the total of six compounds that were found to be most abundant in *Senecio asperulus DC.*

A total of 41 compounds with 23 regarded as most abundant from *Asparagus laricinus* cladodes and 25 compounds with only six considered as abundant from *Senecio asperulus* roots were revealed from both negative and positive ESI mode*. Senecio asperulus DC.* revealed the presence of two known compounds, *chlorogenic acid isomer,* and *dicaffeoylquinic acid* and nineteen unknown compounds. This is the first report of the presence of these compounds from *Senecio asperulus* collected from Lesotho. Both identified compounds, *chlorogenic acid isomer* and *dicaffeoylquinic acid* have been proven to have antioxidant potential due to their ability to scavenge free radicals (Ortal et al., 2009; Xu et al., 2012). Therefore, the presence of this molecule in *Senecio asperulus DC.* supports its activity against free radicals, as demonstrated in **Chapter 4** of this study.

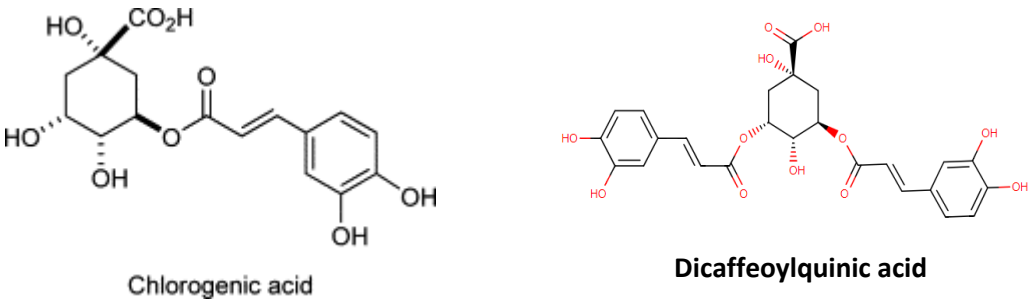

Figure 7.7: Structure of compounds identified from *Senecio asperulus DC.*

Fuku et al. (2013), isolated and identified three compounds using GC/MS; indole-3-carbinol, α-sitosterol, and ferulic acid from the *Asparagus laricinus* roots while we discovered a lot more from the cladodes of this plant. This support the distribution of secondary metabolites varies from different parts of the same plant, as the sensitivity of both LC/MS and GC/MS in metabolites detection is the same (Marquet, 2012; Prez et al., 2016). The following compounds were identified: Luteolin 7-O-rutinoside, Apigenin arabinoside-glucoside, Apigenin galactoside-arabinoside, 3-Methoxynobiletin, Apigenin 7-O-apiosyl-glucoside, Quercetin 3-O-rhamnosyl-galactoside, Rutin (Quercetin 3-O-rhamnosyl-glucoside), Kaempferol 3-O-galactoside 7-O-rhamnoside, Ecdysterone, Isorhamnetin 3-O-glucoside 7-O-rhamnoside, Syringaresinol, and 20-hydroxyecdysone from *Asparagus laricinus Burch.* There was no literature on the identification of these compounds from *Asparagus laricinus*, however, some of the identified flavonoids were reported to be present indeed in the Asparagaceae family. From the literature, steroidal saponins have been highlighted as a major bioactive constituent of the *Asparagus* family (Negi at al., 2010). Negi et al. (2010), further reported that; the primary chemical constituents of *Asparagus* species are tannins, flavonoids such as kaempferol, quercetin, and rutin, and the leaves contain diosgenin and quercetin-3-glucuronide.

Asparagus laricinus was found to have flavonoids in abundance. Flavonoids are classified into various classes (Singh et al., 2014), such as flavonols (quercetin), flavones (apigenin), flavanones (hesperetin, naringenin), flavonoid glycosides (astragalin, rutin), flavonolignans (silibinin), flavans (catechin, epicatechin) to name a few. Flavonoids are reported as antioxidant, antiproliferative, anti-tumor, anti-microbial, estrogenic, acetylcholinesterase, anti-inflammatory agents. They are also used to treat cancer, cardiovascular disease and neurodegenerative disorders

(Middleton and Chithan, 1993; Cushnie and Lamb, 2005). The presence of abundant flavonoids in this plant extract supported the anticancer, antioxidant, antimicrobial and anti-inflammatory activity yielded by *Asparagus laricinus* as reported in **Chapter 4** and **5**. Therefore, there was a correlation with the identified active compounds and pharmacological activities of this plant, and this was indeed expected. To further elucidate the action of each active compound present in *Asparagus laricinus* cladodes, each identified compound -even the unknown ones- must be isolated and analyzed separately to determine if the present phenols are functioning in synergy or they still function independently as this will allow for the pure active compounds to be used for new anticancer drugs development.

7.6 CONCLUSION

Traditional medicinal plants have been a good source of new medicinal agents for thousands of years, and more than 50% of modern drugs have been isolated from plants. The LC-MS analysis of *Asparagus laricinus Burch.* and *Senecio asperulus DC*. demonstrated the presence of respectively twenty-three and six compounds from these plant extracts of which only fourteen were identified and the rest are unknown thus still need to be investigated. The identified compounds have pharmacological properties that may be beneficial to human beings as chemopreventative agents. However, this can only occur after the isolation of these active compounds.

7.7 REFERENCES

Cushnie T.P.T, Lamb A.J. 2005. Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents. 26:343-356.

Fuku S, Al-Azzawi A.M, Madamombe-Manduna I.T, Mashele S. 2013. Phytochemistry and Free Radical Scavenging Activity of Asparagus laricinus. International Journal of Pharmacology. 9 (5):312-317.

Harborne J.B, Baxter H. 1999. The handbook of natural flavonoids. Vol 1& 2. Chichester: John Wiley & Sons. p. 122-13.

Harborne J.B. 1994. The flavonoids: Advances in research since 1986. London, UK: Chapman & Hall.

Kahriman N, Tosun G, Terzioğlu S, Karaoğlu S.A, Yayl N. 2011. Chemical Composition and Antimicrobial Activity of the Essential Oils from the Flower, Leaf, and Stem of Senecio pandurifolius. Rec. Nat. Prod. 5(2):82-91.

Kinghorn A.D, Farnsworth N.R, Soejarto D.D, Cordell G.A, Swanson S.M, Pezzuto J.M, Wani M.C, Wall M.E, Kroll N.H, Kramer R.A, Rose W.C, Vite G.D, Fiarchild C.R, Peterson R.W, Wild R. 2003. Novel strategies for the discovery of plant-derived anticancer agents. Pharmaceutical Biology. 41:53–67.

Korfmacher W.A. 2005. Foundation review: Principles and applications of LC-MS in new drug discovery. Drug Discov Today. 10(20):1357-67.

Lim C.K, Lord G. 2002. Current development in LC-MS for pharmaceutical analysis. Bio Pharm Bull. 25(5):547-57.

Marquet P. 2012. LC-MS vs. GC-MS, online extraction systems, advantages of technology for drug screening assays. Methods Mol Biol. 902:15-27.

Middleton E, Chithan K. 1993. The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer. In: J.B. Harborne (ed.), The flavonoids: Advances in research since 1986. London: Chapman and Hall. p. 145-166.

Negi J.S, Singh P, Joshi G.P, Rawat M.S, Bisht V.K. 2010. Chemical constituents of Asparagus. Pharmacogn Rev. 4(8):215–220.

Noratto G.D, Bertoldi M.C, Krenek K, Talcott S.T, Stringheta P.C, Mertens-Talcott S.U. 2010. Anticarcinogenic Effects of Polyphenolics from Mango (Mangifera indica) Varieties. j Agric Food Chem. 58(7):4104-12.

O'Neill M.J, Lewis J.A. 1993. The renaissance of plant research in the pharmaceutical industry. In: Kinghorn AD, Balandrin MF (eds). Human medicinal agents from plants. ACS Symposium Series 534. American Chemical Society, Washington, USA. p. 48-55.

Ortal D, Hugo E.G, Shlomo G. Margalit B. 2009. Antioxidant activity of 1,3-dicaffeoylquinic acid isolated from Inula viscosa. Food Research International. 42(9):1273-1280.

Parasuraman S, Anish R, Balamurugan S, Muralidharan S, Kumar KJ, Vijayan V. 2014. An Overview of Liquid Chromatography-Mass Spectroscopy Instrumentation. Pharmaceutical Methods. 5(2):47-55.

Pavithra PS, Janani VS, Charumathi KH, Indumathy R, Potala S, Verma RS. 2010. Antibacterial activity of plants used in Indian herbal medicine. Int J Green Pharm. 4:22- 8.

Pierson j-T, Curry M.C, Shaw P.N, Dietzgen R.G, Gidley M.J, Roberts-Thomson S.j, et al. 2015. Polyphenolic contents and the effects of methanol extracts from mango varieties on breast cancer cells. Food Sci Biotechnol. 24(1):265-71.

Pitt J.J. 2009. Principles and Applications of Liquid Chromatography-Mass Spectrometry in Clinical Biochemistry. Clin Biochem Rev. 30(1):19-34.

Perez E.R, Knapp J.A, Horn C.K, Stillman S.L, Evans J.E, Arfsten D.P. 2016. Comparison of LC–MS-MS and GC–MS Analysis of Benzodiazepine Compounds Included in the Drug Demand Reduction Urinalysis Program. Journal of Analytical Toxicology. 40:201-207.

Puupponen-PimiaÈ R, Nohynek L, Meier C, KaÈhkoÈnen M, Heinonen M, Hopia A and Oksman-Caldentey K.M. 2001. Antimicrobial properties of phenolic compounds from berries. Journal of Applied Microbiology. 90:494-507.

Rafael C.D, Magda N.L, Nadia R. 2008. Quantification of phenolic constituents and antioxidant activity of pterodon emarginatys vogel seeds. International Journal of Molecular Sciences. 9:606-614.

Satyajit D.S, Lutfun N. 2007. Chemistry for pharmacy students general, organic and natural product chemistry. The Atrium, Southern Gate, Chichester, West Sussex, England: John Wiley & Sons Ltd. p.55-57.

Saxena V.K, Chaurasia S. 2001. A new isoflavone from the roots of Asparagus racemosus. Fitoterapia. 72:307–9.

Singh M, Kaur M, Silakari O. 2014. Flavones: An important scaffold for medicinal chemistry. European Journal of Medicinal Chemistry. 84:206-239.

Velderrain-Rodríguez G, Palafox-Carlos H, Wall-Medrano A, Ayala-zavala j, Chen C.O, Robles-Sánchez M, et al. 2014. Phenolic compounds: their journey after intake. Food Funct. 5(2):189-97.

Wang W, Guo J, zhang J, Peng J, Liu T, Xin Z. 2015. Isolation, identification and antioxidant activity of bound phenolic compounds present in rice bran. Food Chem. 171:40-9.

Ward J.L., Harris, C., Lewis, J., Beale, M.H. 2003. Assessment of 1H NMR spectroscopy an multivariate analysis as a technique for metabolite fingerprinting of Arabidopsis thaliana. Phytochemistry. 62:949-957.

Wolfender J.L, Ndjoko K, Hostettmann K. 2003. Liquid chromatography with ultraviolet absorbance-mass spectrometric detection and with nuclear magnetic resonance spectroscopy: a powerful combination for the on-line structural investigation of plant metabolites. Journal of Chromatography A. 1000:437-455.

Xu J.G, Hu Q.P, Liu Y. 2012. Antioxidant and DNA-protective activities of chlorogenic acid isomers. J Agric Food Chem. 60(46):11625-30.

Zdero C, Bohlmann F, Liddell J. R. 1989. Seco-eremophilanes and other constituents from South African Senecio species. Phytochemistry. 28(12):3532-3534.

CHAPTER 8

"General conclusion"

ABSTRACT

The aim of this thesis was to evaluate the pharmacological properties of traditional medicinal plants used for the treatment of cancer by South African and Lesotho communities. To provide scientific evidence that can be used to translate ethnomedical reports on potential anticancer effects of *A. laricinus, G. perpensa* and *S. asperulus* agents or compounds into new, safe and effective anticancer treatment. For the achievement of this aim; *A. laricinus, G. perpensa* and *S. asperulus* plant extracts used for cancer treatment were screened for their phytochemical properties, antimicrobial, antioxidant, anti-inflammatory, antimutagenic and mutagenic activities. Furthermore, screened phytochemicals were identified with Liquid Chromatography-Mass Spectrometry (LC-MS/MS). Based on the experimental data collected from this research study, the following appropriate conclusions were drawn:

A. laricinus, G. perpensa, and *S. asperulus* plant extracts are potential antimicrobial, antioxidant, anti-inflammatory and anticancer agents. However, *A. laricinus, G. perpensa* and *S. asperulus* plant extracts did not have any antimutagenic and mutagenic effects. All three plants had phytochemicals with pharmacological properties. Moreover, *A. laricinus* and *S. asperulus* had newly identified active compounds from these species.

8.1 INTRODUCTION

Africa has rich plant biodiversity, with over 20 000 different explored species and plants are a great source of interest to the scientific community (Cherry, 2005). Moreover, more than 50% of the drugs in clinical trials were isolated from natural sources as they have unique chemical assortments that have resulted in them having diverse biological activities and drug-like properties (Cragg and Newman, 2000). In Lesotho and South Africa, there is an increase in the number of people using medicinal plants as their first line of the health care system, especially in rural areas. With this dependence on traditional medicinal plants, the effectiveness and safety of medicinal plants used must be evaluated. Benzo[*a*]pyrene, a compound isolated from Chinese medicinal plants with a history of uses for different diseases, was found to be mutagenic, thus causing permanent DNA damages to the host cells leading to cancer (Sakai et al., 1988). This emphasizes the fact that the safety of medicinal plants is more important as some compounds can cause more harm than good.

Cancer is one of the most prominent diseases in humans, with statistics of newly diagnosed and future estimated rates of prostate and breast cancer patients projecting each year. This disease is the foremost health concern globally, thus, eliciting increased research interest in search of new anticancer compounds. This is due to dissatisfactory results with current anticancer drugs due to their toxic side effects. However, hope still exists, as plants are rich sources of secondary metabolites that are known to possess unique therapeutic properties. Thus, plants remain a prime source of drugs for the treatment of cancer and can provide leads for the development of novel anticancer agents. Furthermore, pharmacological investigation of medicinal plants used traditionally is valuable as it will lead to the discovery of innovative active agents for current drug-resistant conditions and as potential chemotherapeutic agents. However, the union between the pharmacological activity and the safety assessment of medicinal plants cannot be divorced, thus the coherent approach in drug discovery requires the two to be investigated.

Chronic inflammation is the common link between carcinogenesis and bacterial infection caused by toxins, as well as carcinogenesis and DNA damage due to oxidative stress from reactive unstable oxidants (De Nunzio et al., 2011; Fang et al.,

2012). Besides environmental factors, normal metabolism processes in the human body cells also produce unstable oxygen species such as superoxide anion (O_2^-), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2) and when these are not stabilized, they cause damages to the cell DNA (Fang, 1991). Permanent genetical mutations may occur in cells where DNA is damaged and where the division of that damaged DNA occurs before it can be repaired. These cells with permanent mutations may begin to proliferate abnormally and rapidly thus result in carcinogenesis (Reddy *et al*., 2003).

The free radicals can oxidize biomolecules, i.e. nucleic acids, proteins, lipids, and DNA, thus leading to tissue damage and initiation of degenerative diseases. Oxidative damage plays a significantly pathological role in human diseases such as cancer, emphysema, cirrhosis, atherosclerosis and arthritis, etc. (Niki et al., 1994). Antioxidants are compounds that terminate the attack of reactive species such as free radicals, thus preventing aging and different diseases that are associated with oxidative damage inside the body system (Rice-Evans et al., 1996). The radical scavenging properties of antioxidants are known to eliminate or reduce the formation of oxidative stress by preventing the generation of free radicals. Almost all organisms are protected to some extent from free radical damage. Antioxidants have been contributing directly or indirectly to the prevention of pathogenesis and carcinogenesis (Ferrari and Torres, 2003).

An important positive development in the discovery of new anticancer agents from natural product sources research has been astounding (Kinghorn et al., 2003). In frequently reported studies, polyphenols have been suggested as responsible for potent biological activities of extracts prepared using methanol and ethanol (Michielin et al., 2009). Studies on the toxicological properties (using more than 1 point) of plant extracts are crucial to assure the safety of the extract and should be performed along with the determination of pharmacological properties of plant extracts, (Deciga-Campos et al., 2007). Thus, a rational approach to evaluate the anticancer properties of medicinal plants requires comprehensive knowledge on the efficacy and safety of the medicinal plants. This study was intended to evaluate the pharmacological activity of *Asparagus laricinus*, *Senecio asperulus* and *Gunnera perpensa* against cancer-

inducing factors, and in prostate and breast cancer cell lines, in order to determine the ability of these plant extracts as possible novel anticancer drugs.

8.2 SUMMARY OF RESULTS

Table 8.1: Summary of all experimental results obtained from this research project.

Analysis	*Asparagus laricinus Burch.*	*Senecio asperulus DC.*	*Gunnera perpensa L.*
Phytochemical screening	Saponins, Steroids, Tannins, Terpenoids, Flavonoids	Flavonoids, Tannins, Terpenoids, Steroids	Tannins, Flavonoids, Steroids, Terpenoids, Alkaloids, Phlobatannins
Antibacterial activity	Positive results	Positive results	Positive results
Antioxidant activity	Good	Good	Moderate
Anti-inflammatory activity	Weak	Weak	Inactive
Anticancer activity	Positive results	Positive results	Positive results
Mutagenic properties without S9	Not mutagenic	Not mutagenic	Mutagenic
Antimutagenicity properties	None	None	None
Characterization	14 compounds identified	2 compounds identified	Not performed

8.3 HOLISTIC DISCUSSION

Antioxidant supplements or dietary antioxidants may be sources of protection that the body needs to protect it against the damaging effects of free radicals. Various researchers have used the scavenging effect of a chemical on DPPH radical as a quick

and reliable parameter to assess the *in vitro* antioxidant activity. From the reaction of the extracts with DPPH radical, it was observed that the scavenging activity of all plants was dose-dependent (**Chapter 4**). The concentration of *Asparagus laricinus* required to scavenge 50% of DPPH radical present in the reaction mixture (EC_{50}) was higher compared to *Senecio asperulus* and *Gunnera perpensa,* but lower when compared to ascorbic acid. Thus, it was apparent that phenols, more especially flavonoids as they existed in abundance in *Asparagus laricinus* had strong antioxidant activities.

Disk diffusion is the most classic and old microbiology technique used for the determination of antimicrobial resistance globally. We can't evade the fact that there have been developments, and these include broth microdilution method using the microplate. This high throughput assay is very advantageous as many samples can be tested in one go. Nevertheless, the limitations of the broth microdilution method are primarily associated with the lack of or poor growth of many microorganisms (CLSI, 2009). Thus, the disk diffusion method is still the most widely used, even in diagnostic microbiology laboratories, due to its convenience, efficiency and reasonably affordable cost. The ability of *Asparagus laricinus*, *Senecio asperulus,* and *Gunnera perpensa* extract to inhibit the growth of selected bacterial strains was evaluated by using the disk diffusion method. The lowest concentration (MIC) of the extract required to inhibit the growth of micro-organisms tested was determined. The MIC value indicates the antimicrobial property of an extract. Extract with MIC < 1000 µg/ml is regarded as exhibiting activity (Seyoum et al*.,* 2006).

Asparagus laricinus showed to have more total phenols that other plant species as reported in **Chapter 3** and showed good antibacterial potential (**Chapter 4**), this shows that this plant has secondary metabolites that it uses to protect itself from microorganisms at its natural habitat. It is known that, in most instances, Gram-positive bacteria are more susceptible to plant extracts than Gram-negative ones due to the cell wall variations; but, this was not the case, as both Gram-positive and Gram-negative bacteria growth was inhibited by the aqueous and methanolic extracts of *Asparagus laricinus*. However, *S. saprophyticus* and *S. aureus,* which both belongs to the genus *Staphylococcus*, were resistant to the dichloromethane extracts of this plant species. It has been reported that *Staphylococcus* strains could develop resistance

and have waves of resistance as their virulence factors interfere with bacterial killing (Chambers and DeLeo, 2009; Pantosti et al., 2007). Thus, this could be the reason for this observed resistance to even high concentrations of *Asparagus laricinus* plant extracts.

Senecio asperulus aqueous extract showed to possess most secondary metabolites and was the most active plant extract as it managed to inhibit the growth of all strains tested against. Ngo et al., (2017) illustrated that organic solvents used for extraction do affect the levels of yielded phytochemicals contents from plants. The methanolic extracts of both *Senecio asperulus* and *Gunnera perpensa* failed to inhibit the growth of the Gram-positive and negative microorganisms selected for this study even at higher concentrations of 500 µg/ml, except for *S. faecalis*. However, both these plants showed the presence of most phytochemicals that have antimicrobial activities. Thus, this inactivity could be that present flavonoids which were extracted by methanol solvent were either in small quantities or not active against selected micro-organisms.

Gunnera perpensa dichloromethane extracts also inhibited most microorganisms and only one organism (*S. faecalis*) was resistant even at the highest tested concentration. Moreover, *Gunnera perpensa* water and methanol extracts were both inactive to all tested strains. Raafat & Sahl (2009), demonstrated that the *in vitro* antimicrobial activity is dependent upon various intrinsic and extrinsic factors, such as molecular weight, degree of deacetylation, viscosity, solvent, pH, test strains, temperature. Moreover, various types of protocols for the antimicrobial activity analysis may yield different results as the polarity of the natural compound can affect the diffusion of compounds onto the culture medium (Moreno et al., 2006), as far as the disk diffusion method is concerned. Compounds with less polarity diffused slower than the high polar ones, thus, this could be the reason there was no observed activity of *Gunnera perpensa* methanol extracts.

Studies have shown that dietary phytochemical antioxidants can remove free radicals and among them, phenolic compounds, such as flavonoids and catechin in edible plants, exhibit potent antioxidant activities (Decker, 1995; Fang 2002). The phytochemical screening results from this study revealed the presence of metabolites with pharmacological properties from *Asparagus laricinus*, *Senecio asperulus,* and

Gunnera perpensa. Recently, intensive research has been focused on developing tumor therapies from saponins, and *Asparagus laricinus* showed the presence of these secondary metabolites. Saponins exhibit potent anticancer activity in several human cancer cells through apoptosis-inducing pathways (Kaskiw et al*.,* 2009). The bioactivity of saponins from *Asparagus laricinus* was supported by the anticancer activity of this plant on breast cancer cells through apoptosis as demonstrated by the mechanism of action analysis. Moreover, further investigations on the regulation of apoptosis-related proteins will be performed in the future to strengthen this occurrence. Anti-proliferative results of *Asparagus laricinus* on non-cancerous Vero cells and liver cells variations that were observed between cytotoxicity assays (MTT assay and Vitotox test) used in **Chapter 5** and **Chapter 6**, ascribed to the fact that different endpoints were tested (genomics and proteomics).

Asparagus laricinus, *Senecio asperulus* and *Gunnera perpensa* extracts have been used as traditional medicines to treat a variety of diseases and their safety needs to be determined. For mutagenicity testing of these extracts, two-points were looked at, and those were the DNA damage with the comet assay and gene mutations with the Vitotox test. Hepatic (C3A) cells were used for the comet assay while the bacterial strains used were the *Salmonella typhimurium* TA104 strains with the presence and absence of metabolic activation for the Vitotox assay. The DNA damage caused by single- or double-strand breaks leads to the formation of DNA fragments scattering and forming a tail of the comet. Strain *rec*N2-4 gives an indication of genotoxicity as it has luciferase operon, while strain TA104 pr1 indicates cytotoxicity of the tested extract as it expresses the lux operon. Genotoxicity and cytotoxicity of the plant extracts were assayed simultaneously with the Vitotox test, so as to identify false-positive results caused by non-specific light production induced by other mechanisms besides the genotoxic effect (Verschaeve et al., 1999).

According to our vitotox results, *Asparagus laricinus* was safe, not genotoxic with or even without metabolic activation, and both comet and Vitotox findings supported this. Neither *Asparagus laricinus* (excluding DCM extract) nor *Senecio asperulus* (excluding methanol extract) was significantly mutagenic to the *Salmonella* tester strains. However, after the metabolic activation, *Asparagus laricinus* dichloromethane and *Senecio asperulus* water and methanol extracts were generating non-specific light

that was not dose-dependent, thus considered as cytotoxic. These results were in consonance with observations made from the neutral red uptake (NRU) test, as there was slight inhibition of C3A cells proliferation from these extracts. Another extract that showed clear genotoxicity and cytotoxicity was *Asparagus laricinus* dichloromethane extract. All three assays, NRU, Comet, and Vitotox showed that *Asparagus laricinus* dichloromethane was genotoxic and cytotoxic, these findings were also in agreement with anticancer screening results reported in **Chapter 5**, as this extract inhibited the proliferation of both cancer cells and non-cancerous cells.

Gunnera perpensa was considered as mutagenic. However, its mutagenic effects were masked, or blocked by the S9 enzyme. There was no DNA damage caused by this plant on liver cells from the comet assay and from the vitotox test. *Gunnera perpensa* was genotoxic without metabolic activation. However, this genotoxicity vanished when there was metabolic activation. *Gunnera perpensa* is therefore not harmful when consumed, this was concluded based on results observed from both toxicology tests performed. According to Verschaeve et al. (2017), plants with antimutagenic potential are considered as interesting sources for new therapeutic uses. However, none of the tested extracts reversed or reduced the mutagenic effects caused by a known mutagen, 4-Nitroquinoline 1-oxide (4-NQO).

The purpose of the identification of phenolic compound present in *Asparagus laricinus* and *Senecio asperulus* plant extracts was to characterize these medicinal plants, as *Gunnera perpensa* has been characterized before, and five compounds were identified from its methanolic extract: 3,3',4'-tri-O-methyl ellagic acid lactone, ellagic acid lactone, 1,1'-biphenyl-4,4'-diacetic acid, p-hydroxybenzaldehyde and Z-methyl lespedezate (Brookes and Dutton, 2007). Some of these compounds are known as antihaemorrhagic, antimutagenic and anticarcinogenic agents. Contrary to the presence of compounds reported by Brookes and Dutton (2007), none of the *Gunnera perpensa* extracts showed antimutagenic properties and only *Gunnera perpensa* dichloromethane extracts (**Chapter 5**) showed to have anticancer properties. However, this extract was not specific as it inhibited the growth of non-cancerous cells too. *Gunnera perpensa* was genotoxic *in vitro* without S9 activation, but not cytotoxic, this could mean that some of the compounds present in this plant are mutagens.

To avoid unnecessary isolation of compounds, the chemical screening process was enhanced so as to identify already known compounds swiftly (Wolfender et al., 2003). Moreover, targeted metabolites analytical approach increases the chances of discovering novel bioactive compounds and for this to be achieved, compounds from the whole plant part should be screened for abundant compounds before isolations are done. For the rationalized characterization of the active constituents, LC-MS spectrometry data could be used as a first pass screen to rapidly determine and characterize differences in the molecular composition of plant samples (Ward et al., 2003). To circumvent the process of repeating the isolation of already known compounds, LC-MS was used on crude extracts of our selected plants. LC-MS results confirmed the total phenolic content of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* plants, as we identified a correlation between the estimated total phenolic content and a number of compounds identified. *Asparagus laricinus* had twenty-three most abundant compounds of which only fourteen are known, while *Senecio asperulus* had six abundant of which only 2 were known. *Senecio asperulus* had less estimated total phenolic content when compared to *Asparagus laricinus,* which had a high estimated total phenolic content. As much as some compounds identified from these plants were known, they were novel to these plant species.

8.4 HOLISTIC CONCLUSION

From discussions unpacked in the Chapters of this study, *Asparagus laricinus* methanol extract was found to be one of the most promising niches for the development of targeted breast cancer drugs. This plant can be considered safe to be used in view of the evidence obtained from its non-cytotoxic effects on kidney (Vero) cells, non-mutagenicity and non-cytotoxicity effects on liver (C3A) cells. *Senecio asperulus* had an ability to induce breast and prostate cancer cell lines death through cell arrest at the synthesis phase and early mitotic (G2/M) phase G2, but not through apoptosis. This plant also didn't show signs of being mutagenic as it didn't cause DNA damage to the liver cells or the ability to cause mutation on the *Salmonella typhimurium* strains used. *Gunnera perpensa* had anticancer potential on breast and prostate cancer cells. This plant was however shown to be cytotoxic and mutagenic without S9 activation. Nonetheless, these mutagenic effects were not present with S9

activation, thus showing that this plant is safe to be used as it didn't cause any DNA damage when the metabolism. In summary, all tested plant extracts used to treat cancer by South African and Lesotho communities had pharmacological properties (antibacterial, antioxidant and anticancer activities, with *Senecio asperulus* having anti-inflammatory activity as well) and are safe to be used.

8.5 LIMITATIONS OF THE STUDY

- It was not possible to do this study using human subjects, as the test plants have not been used in animal models and clinical trials yet.
- It was not possible to test the plant extracts against a larger number of tumor cell lines and bacterial strains at this stage owing to limited resources and time.
- It was financially challenging to collect the same plant species from two different countries (Lesotho and South Africa), so as to compare the bioactivity and chemical variations as geographical positions do influence the composition of secondary metabolites in the plant.

8.6 RECOMMENDATIONS FOR FURTHER RESEARCH

This study was intended to contribute to the knowledge base of the anticancer therapeutic potential of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa*. It should be noted that plant material used in this study were chosen based on the ethnobotanical claims made by cancer-treating traditional healers in Lesotho as well as supported by recorded ethnobotanical studies. *Asparagus laricinus* was reported to be used for prostate cancer patients in Limpopo province in South Africa, *Senecio asperulus* was reported to be used for cancer treatment in Lesotho and *Gunnera perpensa* was reported to be used for cancer treatment in Lesotho and in the Eastern Cape province in South Africa. Moreover, a mixture of *Senecio asperulus* and *Gunnera perpensa* aqueous extracts have been avowed by a traditional herbalist in Lesotho as an effective concoction for the treatment of breast and prostate cancer. Since other compounds are extracted better with different solvents because of the

differences in polarity, extraction using a variety of solvents was performed to demonstrate a vast difference in their pharmacological activities as far as the experimental results are concerned. However, to account for possible geographical and chemotypic variations, plant material should be studied from several populations in comparison with each other; for example, effects of *Asparagus laricinus Burch* from Lesotho and South Africa, in the future. Based on the results observed from this study, the recommendations below were made:

i. The activity-guided fractionation, isolation, and identification of identified compounds are imperative, as these may lead to the development of novel treatments in the global struggle against cancer and cancer-related ailments.

ii. No single testing method can provide a comprehensive profile of the antioxidant and anti-inflammation capacity of a plant species, because of the complexity of oxidation-anti-oxidation and inflammation-induction processes. Therefore, different methods are to be used to determine the antioxidant potential and the regulation of other inflammation-related enzymes such as COX-2.

iii. It is recommended that a thorough geographical variation study be performed on each species to explain the chemical diversity between individuals within a population and between populations.

iv. Isolation and structural elucidation of unknown compounds should be studied further so that they can be named.

v. The study on the isolation and identification of the detected unknown compounds can be conducted, but now with the pure and targeted compounds using the guidance of compounds identified under phytochemical screening analysis.

vi. Isolated compounds to be tested on breast (MCF-7) cells again to determine if the activity of tested extracts with anticancer activities was due to a specific compound or several compounds working in synergy.

vii. Toxicity of each isolated compound should then be investigated.

viii. *In vivo* pharmacological investigation (targeting diseases that these plants have been traditionally used for) of active isolated compounds and crude extracts (as the activity might be synergical from all compounds in each plant).

ix. *In vivo* anticancer activity investigation of *Asparagus laricinus, Senecio asperulus* and *Gunnera perpensa* as some compounds behave differently *in vivo* when compared to *in vitro*.

x. Investigation of a mixture of *Senecio asperulus* and *Gunnera perpensa* as anticancer drugs for prostate and breast cancer as their activity might be due to synergistic effect when compounds from both plants extracts act together simultaneously.

xi. *In vivo* anticancer activity of the *Senecio asperulus* and *Gunnera perpensa* mixture as claimed by the traditional herbalist in Lesotho, using a mixture of both plants be investigated.

8.7 REFERENCES

Brookes K.B, Dutton M.F. 2007. “Bioactive Components of the Uteroactive Medicinal Plant, Gunnera Perpensa (or Ugobo): Research in Action.” South African Journal of Science. 103(5 & 6):187–189.

Chambers H.F, DeLeo F.R. 2009. Waves of Resistance: Staphylococcus aureus in the Antibiotic Era. Nat Rev Microbiol. 7(9):629–641.

Clinical and Laboratory Standards Institute (CLSI). 2009. Performance standards for antimicrobial disk susceptibility tests; approved standard-tenth edition M02-A10. National Committee for Clinical Laboratory Standards 29.

Cragg G.M, Newman D.J. 2000. Antineoplastic agents from natural sources: Achievements and future directions. Expert Opinion on Investigational Drugs. 9:1-15.

De Nunzio C, Kramer C, Marberger M, Montironi R, Nelson W, Schröder F, SciarraA, Tubaro A. 2011. The controversial relationship between benign prostatic hyperplasia and prostate cancer: The role of inflammation. European Urology. 60:106-117.

Deciga-Campos M, Rivero-Cruz I, Arriaga-Alba M, Castaneda-Corral G, Angeles-Lopez G.E, Navarrete A, Mata R. 2007. Acute toxicity and mutagenic activity of Mexican plants used in traditional medicine. Journal of Ethnopharmacology. 110:334-342.

Decker E.A. 1995. The role of phenolics, conjugated linoleic acid, carnosine and pyrrolquinolinequinone as nonessential dietary antioxidants. Nutrition Reviews. 53:49.

Fang YZ. 1991. Effect of ionizing radiation on superoxide dismutase in vitro and in vivo. In: Fang YZ (English edition). Advances in Free Radical Biology and Medicine. Vol 1:1. Beijing: Atomic Energy Press.

Fang YZ. 2002. Free radicals and nutrition. In: Fang YZ, Zheng RL (eds.) Theory and Application of Free Radical Biology. Beijing: Scientific Press. p. 647.

Fang Y, De Marco V.G, Nicholl MB. 2012. Resveratrol enhances radiation sensitivity in prostate cancer by inhibiting cell proliferation and promoting cell senescence and apoptosis. Cancer Science. 103:1090-1098.

Ferrari C.K.B, Torres E.A.F.S. 2003. Biochemical pharmacology of functional foods and prevention of chronic diseases of aging. Biomedicine & Pharmacotherapy. 57:251-260.

Hertog M.G.L, Hollman P.C.H, Van de Putte B. 1993. Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. Journal of Agricultural and Food Chemistry. 41:1242-1246.

Kang J. 2012. Principles and Applications of LC-MS/MS for the Quantitative Bioanalysis of Analytes inVarious Biological Samples, Tandem Mass Spectrometry - Applications and Principles. Dr. Jeevan Prasain (Ed.), ISBN: 978-953-51-0141-3, InTech. pp. 441-492.

Kaskiw M.J, Tassotto M.L, Mok M, Tokar S.L, Pycko R, Th'ng J, Jiang Z.H. 2009. Structural analogues of diosgenyl saponins: Synthesis and anticancer activity. Bioorganic & Medicinal Chemistry. 17(22):7670-9.

Kinghorn A.D, Farnsworth N.R, Soejarto D.D, Cordell G.A, Swanson S.M, Pezzuto J.M, Wani M.C, Wall M.E, Kroll N.H, Kramer R.A, Rose W.C, Vite G.D, Fiarchild C.R, Peterson R.W, Wild R. 2003. Novel strategies for the discovery of plant-derived anticancer agents. Pharmaceutical Biology. 41:53–67.

Michielin E.M.Z, Salvador A.A, Riehl C.A.S, Smania A, Smania E.F.A, Ferreira S.R.S. 2009. Chemical composition and antibacterial activity of Cordia verbenacea extracts obtained by different methods. Bioresource Technology. 100:6615-6623.

Moreno S, Scheyer T, Romano C.S, Vojnov A.A. 2006. Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radic. Res. 40:223–231.

Niki E, Shimaski H, Mino M. 1994. Antioxidant-free and biological defense. Gakkai Syuppn Center, Tokyo. p. 3-16.

Ngo T.V, Scarlett C.J, Bowyer M.C, Ngo P.D, Vuong Q.V. 2017. Impact of Different Extraction Solvents on Bioactive Compounds and Antioxidant Capacity from the Root of Salacia chinensis L. Journal of Food Quality. Article ID 9305047, 8 pages https://doi.org/10.1155/2017/9305047.

Pantosti A, Sanchini A, Monaco M. 2007. Mechanisms of antibiotic resistance in Staphylococcus aureus. Future Microbiol. 2(3):323-34.

Raafat D, Sahl, H.G. 2009. Chitosan and its antimicrobial potential – a critical literature survey. Microbial Biotechnology. 2:186-201.

Reddy L, Odhav B, Bhoola K.D. 2003. Natural products for cancer prevention: A global perspective. Pharmacology and therapeutics. 99:1-13.

Rice-Evans C.A, Miller N.J, Paganga G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Rad. Biol. Med. 20(7):933.

Sakai Y, Nagase H, Ose Y, Sato T, Kawai M, Mizuno M. 1988. Effects of medicinal plant extracts from Chinese herbal medicines on the mutagenic activity of benzo[a]pyrene. Mutation Research/Genetic Toxicology. 206(3):327-334.

Seyoum A, Asres K, El-Fiky F.K. 2006. Structure-radical scavenging activity relationships of flavonoids. Phytochemistry. 67:2058-2070.

Ward J.L, Harris C, Lewis J, Beale M.H. 2003. Assessment of 1H NMR spectroscopy an multivariate analysis as a technique for metabolite fingerprinting of Arabidopsis thaliana. Phytochemistry. 62:949 - 957.

Wolfender J.L, Ndjoko K, Hostettmann K. 2003. Liquid chromatography with ultraviolet absorbance-mass spectrometric detection and with nuclear magnetic resonance

spectroscopy: a powerful combination for the on-line structural investigation of plant metabolites. Journal of Chromatography A. 1000:437-455.

Yan L.L, Zhang Y.J, Gao W.Y, Man S.L, Wang Y. 2009. In vitro and in vivo anticancer activity of steroid saponins of Paris polyphylla var. yunnanensis. Experimental Oncology. 31(1):27-32.

Yen G, Wu S, Duh P. 1996. Extraction and identification of antioxidant components from the leaves of mulberry (Morus alba L.). Journal of Agricultural and Food Chemistry. 44:1687–90.

APPENDIX

(A) PLANT COLLECTION PERMIT (from South Africa)

OFFICIAL PERMIT

THE DEPARTMENT OF ECONOMIC DEVELOPMENT, TOURISM AND ENVIRONMENTAL AFFAIRS

destea
department of economic, small business development, tourism and environmental affairs
FREE STATE PROVINCE

98 ZASTRON STREET AGRICULTURAL BUILDING THIRD FLOOR BLOEMFONTEIN, 9300 FREE STATE SOUTH AFRICA	PERMIT OFFICE PRIVATE BAG X20601 BLOEMFONTEIN, 9300 TEL.: +27 (0) 51 400 9527/26 FAX: +27 (0) 51 400 9523

PERMIT HOLDER DETAILS

ID NUMBER	9012130226083		
NAME	Polo-Ma-Abiele Hildah Mlengwana		
PHYSICAL ADDRESS	02 Scott Park, Faure Avenue, Willows Bloemfontein	POSTAL ADDRESS	Moronnie Muthi Shop
MOBILE NUMBER:	051-507 3345		
FAX NUMBER	N/A		
EMAIL ADDRESS	pmlsoell@cut.ac.za		

THIS PERMIT IS ISSUED IN TERMS OF THE NATIONAL ENVIRONMENTAL MANAGEMENT: BIODIVERSITY ACT (10 OF 2004) (THREATENED OR PROTECTED SPECIES REGULATIONS) AND IN TERMS OF THE FREE STATE NATURE CONSERVATION ORDINANCE (8 OF 1969). AUTHORISATION IS HEREBY GRANTED TO THE HOLDER OF THIS PERMIT TO;

GENERAL PERMIT

To import the following plants: 5kg Gunnera perpensa, 5kg Senerio asperulus, 5kg Asparagus laricinus and 5kg Raphionacme hirsuta from Mr Motlalsi Nthlo, Dept of Agric Research Plant Genetic Resource Maseru, Lesotho to the above mentioned address.

Subject to the conditions at the back.

PERMIT HOLDER'S SIGNATURE	APPROVED BY THE MEC DEPARTMENT OF ECONOMIC, SMALL BUSINESS DEVELOPMENT, TOURISM AND ENVIRONMENTAL AFFAIRS	
EXPIRY DATE 31 July 2018 RETURN PERMIT AFTER EXPIRY	PERMIT NUMBER NC.553/2017	DATE ISSUED 31 May 2017

Department of Economic & Small Business Development Tourism & Environmental Affairs
2017 -06- 13
Private bag X 20601
Bloemfontein 9300
Free State Province

(B) PLANT COLLECTION PERMIT (from Lesotho)

MINISTRY OF TOURISM ENVIRONMENT AND CULTURE

Name of the Director of Environment: **Stanley Damane**. email:stanleydamane@hotmail.com

Project title:

'Evaluation of pharmacological properties of traditional medicinal plants used for the treatment of cancer by South African and Lesotho communities'

Purpose of Study:

plants are playing an important role as the source of effective anticancer agents in the developing countries communities, even though claims of their pharmaceutical uses have not been validated. The search for anticancer agents from plants sources is directed to the discovery and development of novel compounds that will target cancerous cells only and not be toxic to normal cells. The aim of the intended study will be on the pharmacological and molecular characterisation of plants (*Asparagus laricinus, Raphionacme hirsute, Senecio asperulus* and *Gunnera perpensa)*, endemic to Africa,with potential antimutagenic/ anticarcinogenic properties and to build scientific support for further development of these plants derived agents/ compounds into novel anticancer (combination) treatments.

Location:
Plant collection to be done at Mohale's Hoek and research analytical work to be conducted at Central University of Technology, Free State, South Africa and Antwerp University, Belgium

Specific conditions and restrictions:

ACCESS

Access to the research location(s) is limited to foot travel and horse.

HISTORICAL AND ARCHAEOLOGICAL PROTECTION / PRESERVATION

Any object of antiquity, historic, relic or other object of scientific or other interest uncovered or discovered on site shall be left in place, protected and immediately reported to the Authority.

FIELD EQUIPMENT PLOTS/TRANSECT

All equipment left on the site including plot markers must be specifically authorized in advance. Label all equipment with your name, date of installation, phone number and words "Research Study". If you are authorized to place equipment or plot markers, you will be required to GPS their location.

HAZARDOUS MATERIAL

Specific authorization must be obtained before using chemicals or hazardous materials on the site. For specific information regarding the transport, use, and disposal of chemicals or hazardous materials please contact the ***Department of Environment*** in the Ministry of Tourism Environment and Culture head office in Maseru.

LEAVE NO TRACE

The permit must make reasonable efforts to follow Leave No Trace outdoor ethics and principles during all field activities to minimize impacts on resources or experiences of other visitors. This includes proper collection and disposal of all litter, waste materials and other site altering materials brought on site by permittee or any permit participant.

FIRES

No fires are permitted in research area by any unauthorized body or individual.

COMMERCIAL FILMING

Your permit does not authorize the bearer or those that accompany them to conduct commercial filming activities.

OTHER DELIVERABLES

Copies of investigator field notes, maps, slides, photographs, charts/graphs and data (including GIS data and associated metadata) are required to be submitted to the authority (MTEC) at the completion of the study. In addition, two copies of all final reports, publications, and theses/dissertations produced as a result of the permitted project must also be provided to the authority. Additional deliverables, such as making oral presentation to the Park staff and/or the public may also be required.

CONFIDENTIALITY AGREEMENT

Investigators agree to keep confidentiality of any protected information that may develop or otherwise be acquired as part of this work. With regard to the protected information, investigators are considered agents of the Ministry of Tourism Environment and Culture (MTEC) and must not share or release information through any means except specifically authorized by the MTEC. Acquisition or development of any such information does not constitute a release to the public. Investigators are to understand that protected information must not be disclosed through any means including websites, maps, scientific articles, presentations and speeches. Lastly Investigators are to comply with general MTEC conditions, site-specific conditions, and project specific conditions if applicable. This includes review of any reports, presentations or summaries prior to publication. Signature of this research permit binds the investigator and his or her representative or collaborators to this condition.

SPECIMENS

Any specimens collected under this permit, any components of any specimens (including natural organism, enzymes, genetic material or seeds), and research results derived from collected specimens are to be used for scientific or educational purposes only and may not be used for commercial purposes unless the permittee has entered into a Cooperative Research and Development Agreement (CRADA) with Ministry of Tourism Environment and Culture or Department of Environment. The sale of collected specimens and or other transfer to third parties is prohibited. Breach of any of the terms of this permit will be grounds for revocation of this permit and denial of future permits. Further more if the permittee sells or otherwise transfers collected specimens, any components thereof, or any products or research results developed from such specimens or their component without CRADA, permittee will pay the Ministry of Tourism Environment and Culture (MTEC) a royalty rate of twenty percent (20%) of cross revenue from such sales. In addition to such royalty, the MTEC may seek other damages to which the Ministry may be entitled and injunctive relief against the permittee.

Recommended by Nature Conservation Division Staff (name and title)
DAVID MOHAPI - ENVIRONMENT OFFICER

Approved by Director of Environment
STANLEY M. DAMANE

Date Approved
23/4/2017

I agree to all conditions and restrictions of this permit as specified
(Not valid unless signed and dated by researcher)

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THIS PERMIT MUST BE CARRIED AT ALL TIMES WHILE CONDUCTING RESEARCH ACTIVITIES IN THE DESIGNATED SITES

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Cytotoxicity and cell cycle analysis of *Asparagus laricinus Burch.* and *Senecio asperulus DC.* on breast and prostate cancer cell lines

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ABSTRACT

Aims: Medicinal plants play an important role in our African communities for treatment and prevention of various diseases including cancer. This study was aimed on evaluating the cytotoxicity activities of *Asparagus laricinus Burch.* and *Senecio asperulus DC.*

Main methods: *In vitro* cytotoxicity screening was carried out using fluorescent cellular stains on human prostate cancer (PC3), human breast cancer (MCF-7) and the non-cancerous African green monkey kidney (Vero) cell lines. The cells were imaged with the ImageXpress Micro XLS Widefield fluorescent Microscope, and the acquired images were analysed using the MetaXpress software and the Multi-Wavelength cell scoring application module. Melphalan was used as a positive control in all experiments.

Key findings: *Asparagus laricinus* methanol and *Senecio asperulus DC.* dichloromethane extracts exhibited cytotoxicity activity against breast cancer cells with IC_{50} values of 97.6 μg/mL and 69.15 μg/mL, respectively. Cell cycle analysis suggested that *Asparagus laricinus* methanol extract induced cell death selectively through apoptosis observed from Annexin V-FITC and PI stain. Cell cycle analysis also showed that *Senecio asperulus DC.* dichloromethane extracts induced breast cancer cells death through cell arrest at the synthesis phase and G2 phase. *Senecio asperulus DC.* dichloromethane extracts further showed cytotoxicity activity against prostate cancer cells with IC_{50} values of 69.25 μg/mL due to cell arrest at the G2 and early mitotic (G2/M) phase.

Significance: We, therefore, propose that the methanol extract of *Asparagus laricinus* is a suitable aspirant for future breast cancer chemotherapeutic drug, due to its selective cytotoxicity on cancer cells and not on non-cancerous cells.

1. Introduction

Cancer is a serious public health problem and it continues to be the leading cause of mortality and morbidity worldwide (Lee et al., 2014; Ogbole et al., 2017). In the African region, the most common cancers are breast, cervical, liver and prostate cancer (World Health Organization, 2017). Prostate cancer is leading cancer in males: with nearly 1 in 5 men to be diagnosed with this illness during their lifetime and more than 4000 men being diagnosed with prostate cancer every year in South Africa (Sylla and Wild, 2012; Seigel et al., 2018). Breast cancer is also a growing health problem in sub-Saharan Africa (Akarolo-Anthony et al., 2010; Jemal et al., 2012; Sylla and Wild, 2012) and has now surpassed cervical cancer as the leading cause of death in many countries, with 94 378 new cases of breast cancer diagnosed annually (Akarolo-Anthony et al., 2010; Seigel et al., 2018). Additionally, the number of other types of cancer cases and death are estimated to increase over the next two decades (World Health Organization, 2017), despite current advancements in scientific knowledge. Several chemotherapeutic agents are available and in use for the management of cancer, nevertheless, the problem of indiscriminate toxicity and serious adverse events still exist (Ogbole et al., 2017). Thus, there is a significant increase in scientific and commercial interest in the continued discovery of novel anticancer agents from natural product sources.

African countries still depend greatly on traditional medicinal remedies for the treatment of different types of cancers as access to western medicine is limited (WHO, 2013). In Limpopo Province, South Africa, unexpected improvements have been observed on

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patients who had been terminally ill due to advanced prostate cancer and had used a decoction of *Asparagus laricinus Burch.* (Mashele and Kolesnikova, 2010). *Asparagus laricinus Burch.* (Asparagaceae) is a very hardy, evergreen, shrubby *Asparagus* with fine, feathery foliage and silvery, zigzag branchlets (Van der Merwe et al., 2001). It has myriads of tiny white, nectar-rich flowers that develop during spring and summer. These flowers are fragrant and attract insects and birds (Wildlife Wholesale Nursery, 2018). *A. laricinus Burch.* bears attractive red and blackberries that also attract birds. This species has alternate cladodes which grow in clusters of 4–6. *Asparagus laricinus Burch.* can grow up to 1.5 m. This plant species is used medicinally to treat tuberculosis, sores, red water, uterine infection, general alignments, umbilical cord inflammation, and serves as a diuretic (Van der Merwe et al., 2001). *A. laricinus Burch.* is native to Botswana and South Africa, Lesotho and Swaziland. *A. laricinus Burch.* is known as cluster-leaf asparagus in English, as Lesitwane in Setswana and Lerara tau in Sesotho (Van der Merwe et al., 2001; Mugomeri et al., 2014).

Senecio asperulus DC. belongs to the genus *Senecio* and is from the daisy family, Asteraceae, that includes ragworts and groundsels (Brummitt, 1992). *Senecio asperulus DC.* is known as Moferefere or Letapisa in Sesotho (Kose et al., 2015). *Senecio asperulus DC.* is a very aromatic plant with yellow flowers when young which turns to woolly flowers as the plant gets old and known to be highly toxic (Quattrocchi, 2016). In South Africa, leaves of this plant are medicinally used as an infusion mixture with other medicinal plants for the treatment of rheumatoid arthritis, sore throat, mouth ulcers, flu and cold (Quattrocchi, 2016). The plant is very common in the Eastern Cape province, KwaZulu-Natal, and Western Cape province of South Africa. In the kingdom of Lesotho, *S. asperulus DC.* is abundant and widely spread across the country. According to traditional medicinal plants practices in Mohale's Hoek, Lesotho, *Senecio asperulus DC.* is used on its own or in combination with other medicinal plants for the treatment of various ailments by laypeople (Kose et al., 2015; Mugomeri et al., 2014). Roots of *S. asperulus DC.* are used mostly in Lesotho for a treatment of various diseases such as treat herpes, syphilis, itching feet, arthritic joints, colds and flu, sore throat, sore joints, swollen gums, and used as a vasodilating agent, thus, improves circulation (Kose et al., 2015; Mugomeri et al., 2014).

Herbal medicine has always been one of the main components of healthcare systems for ages. However, most ethnobotanical claims have not yet been investigated scientifically. The investigation of traditionally used medicinal plants is valuable as a source of potential chemopreventative and chemotherapeutic agents since there are dissatisfactions with current anticancer treatment options due to their limitations. Plant-derived natural products provide an interesting source for screening and ultimately isolate novel potent molecules to combat a variety of ailments, including the modern disease "cancer". Thus, the cytotoxicity of both *Senecio asperulus DC.* and *Asparagus laricinus Burch.* was determined against breast cancer cell line (MCF-7), prostate cancer cell line (PC3) and non-malignant African green monkey kidney cell line (Vero).

2. Materials and methods

2.1. Plant material collection

Plant material was collected from Mohale's Hoek district, Lesotho and Free State Province, South Africa (SA). Plant identification was authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa by Dr Zietsman and Mr Nenugwi. Plant materials voucher numbers were PHM02 for *Senecio asperulus DC.* (from Lesotho) and MAS001 for *Asparagus laricinus Burch.* (from SA) and were delivered at National Botanical Gardens herbarium. Plant names were checked with http://www.theplantlist.org. The roots of *Senecio asperulus DC.* and cladodes *Asparagus laricinus Burch.* were washed, air dried at room temperature (25 °C) and then ground into fine powder using an electric blender.

2.2. Plant extraction methods

For extraction, maceration method was used as adapted from Azwanida (2015). Powdered plant materials were soaked separately in purified water, 100% methanol (MeOH), 1:1 (v/v) absolute methanol: dichloromethane (MeOH: DCM), 100% dichloromethane (DCM) and 100% Hexane for 72 hours with occasional stirring. The extracts were then filtered, and aqueous extracts were concentrated in a freeze dryer and organic solvents with a rocket evaporator.

2.3. Sample preparation

The plant extracts were reconstituted in dimethyl sulphoxide (DMSO) to give a final concentration of 100 mg/mL, respectively. Stock solutions were sonicated if necessary were solubility was an issue. All samples were then stored at 4 °C.

2.4. Cell culture

The human prostate cancer cell line, PC3; human breast cancer cell line, MCF7 and the African green monkey kidney cell line, Vero were cultured in DMEM (Dulbecco's Modified Eagle's medium) and 10% FBS (Fetal bovine serum). PC3 cell lines were purchased from Japanese Collection of Research Bioresources Cell Bank, while, MFC7 and Vero cells were purchased from ATCC Cell Biology collection. Suspensions of monolayer cultures of cells were seeded into 96 well microtiter plates at a density of 6000 cells/well using a volume of 100 µl in each well. The microtiter plates with cells were incubated at 37 °C, 5% CO_2, and 100% relative humidity for 24 hours prior to addition of test compounds to allow for cell attachment (Freshney, 2005).

2.5. In vitro cytotoxicity assay and IC_{50} determination

Cytotoxicity was studied using a high content screening approach, and nuclear morphological changes were visualized by Hoechst 33342 staining (Crowley et al., 2016), after cells were treated with 50 and 200 µg/mL of each extract respectively. One hundred microliter aliquots of the diluted extract in fresh medium were used to treat cells. Cells were incubated at 37 °C in a humidified 5% CO_2 incubator for 48 hours. Melphalan was used as a positive control. Treatment medium was aspirated from all wells and replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL in phosphate buffered saline) and incubated for 10 minutes at room temperature. Thereafter, cells were stained with propidium iodide (PI) at 100 µg/mL to enumerate the proportion of dead cells within the population. Cells were imaged immediately after addition of PI using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices). DNA distribution is analysed using propidium iodide (PI), a double-stranded DNA-binding dye. Cells first need to be fixed and permeabilized to allow the entry of PI stain into the cell (Krishan, 1975). For the *in vitro* IC_{50} determination, cells were treated with increasing concentrations of each extract prepared from serial drug dilutions, 6.25–300 µg/mL. Concentration to inhibit 50 percent of the cell population was determined and only those obtained IC_{50} value concentrations were used for cell cycle analysis.

2.6. In vitro cell-cycle analysis

Cell cycle analysis was performed by propidium iodide (PI) based measurements of the DNA content of the cells by flow cytometry using the method by Crowley et al. (2016) and Moore et al. (1997). One hundred microliters aliquots of the diluted compound in fresh medium was used to treat cells. Cell lines were incubated at 37 °C in a humidified 5% CO_2 for 48 hours. Melphalan was used as a positive control. Treatment medium was removed from all wells and replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL) and Annexin V-FITC and then incubated for 10 minutes at room temperature. The plates were then inspected under an inverted microscope to guarantee growth and images were then acquired on the ImageXpress Micro XLS Widefield microscope (Molecular Devices). Thereafter, cells were stained with propidium iodide (PI) at 100 µg/mL to enumerate the proportion of dead cells within the population. Cells were imaged immediately after addition of PI using the ImageXpress Microscope.

2.7. Image quantification and analysis

Quantification of live and dead cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices). Acquired images were analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module and the Cell Cycle Application Module. Acquired data was transferred to Microsoft Excel spreadsheet and data was analysed and processed using GraphPad Prism version 4 for IC_{50} determination and Microsoft Excel for cell cycle analysis.

2.8. Statistical analysis

All experiments were performed in triplicated and data represent the mean ± standard deviation (SD). The statistical significance was measured by one-way ANOVA and p-values less than 0.05 were considered significant.

3. Results

Aqueous, methanol, dichloromethane, hexane and methanol: dichloromethane extracts of *Senecio asperulus DC.* and *Asparagus laricinus Burch.* were screened at 2 concentrations (50 and 200 µg/mL) for cytotoxicity against prostate (PC3) and breast (MCF-7) cancer cells and against non-cancerous kidney (Vero) cells, respectively. Since the assay protocol used could provide quantitative data, the total number of cells were compared with the number of live and dead cells using a dual staining cytotoxicity assay. All activities were compared with both negative (medium only) and positive (melphalan) controls and only p-values < 0.005 were statistically significant. The results, as shown in Figs. 1, 2, and 3, indicated that methanolic extracts of *A. laricinus Burch.* had some cytotoxic effect against breast cancer (MCF-7) cells, with very little effect on non-cancerous (Vero) cells. The hexane extract of *S. asperulus DC.* as well as the methanol: dichloromethane extract of *A. laricinus Burch.* which were cytotoxic against all the cell lines used. While dichloromethane extracts of *S. asperulus DC.* showed a dose-dependent cytotoxic effect against prostate (PC3) cells (Fig. 2) and against breast (MCF-7) cells (Fig. 3), it had little cytotoxic effect against non-cancerous kidney (Vero) cells (Fig. 1). The rest of the extracts from *S. asperulus DC.* and *A. laricinus Burch.* did not show any concentration-dependent cytotoxicity in any of the tested cell lines (PC3, MCF-7, and Vero).

For all instances (Figs. 1, 2, 3, 4, 5, 6, and 7), the plant extracts are represented by the abbreviations in brackets. Thus, for *Senecio asperulus DC.* the extracts are methanol (L1); aqueous (L2); dichloromethane (L3); hexane (L4); and methanol: dichloromethane (L5). *Asparagus laricinus Burch.* extracts are methanol (AL1); aqueous (AL2); dichloromethane (AL3); hexane (AL4) and methanol: dichloromethane (AL5). Similarly, the controls are Medium only (MO) and melphalan (melph) at a concentration of 40 µg/mL.

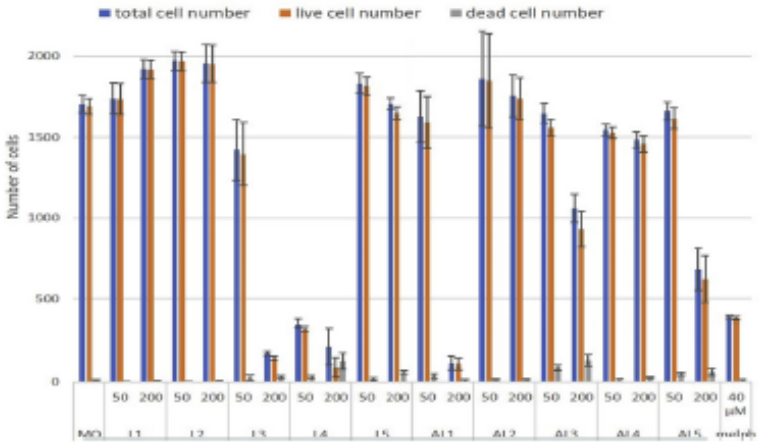

Fig. 1. Cytotoxicity effect of *Senecio asperulus DC.* and *Asparagus laricinus Burch.* extracts on normal kidney (Vero). Error bars indicate standard deviation of triplicate values.

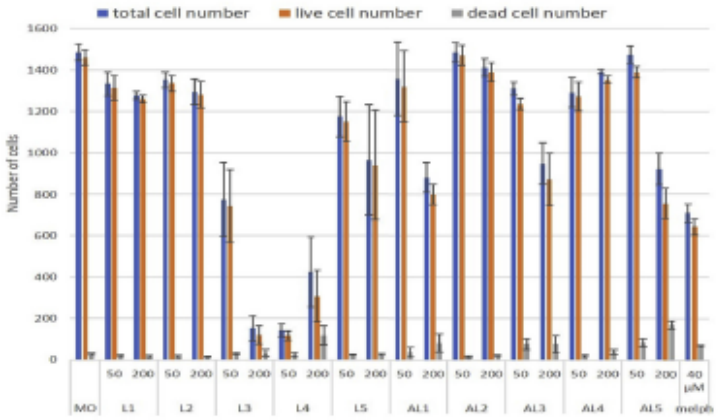

Fig. 2. Cytotoxicity effect of *Senecio asperulus DC.* and *Asparagus laricinus Burch.* extracts on the prostate cancer cell line (PC3). Error bars indicate standard deviation of triplicate values.

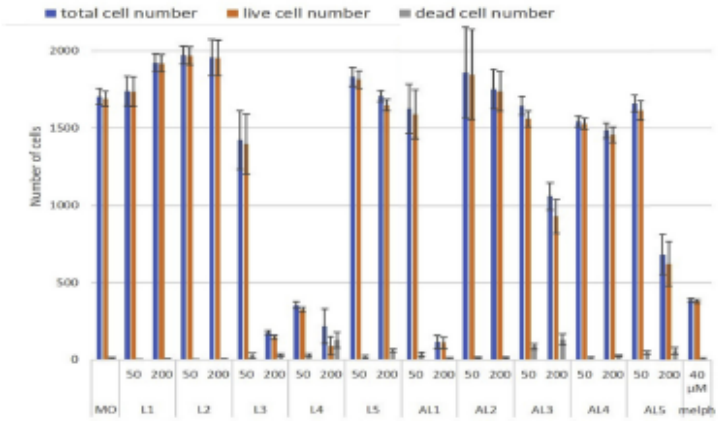

Fig. 3. Cytotoxicity effect of *Senecio asperulus DC.* and *Asparagus laricinus Burch.* extracts on breast cancer cell line (MCF-7). Error bars indicate standard deviation of triplicate values.

From the cytotoxicity screening findings, two plant extracts (*Senecio asperulus DC.* dichloromethane and *Asparagus laricinus Burch.* methanol extracts), showed these characteristics when compared to controls. Thus, their respective concentrations required to reduce 50% (IC_{50}) of prostate and or breast cell viability were determined and illustrated in Fig. 4A and B.

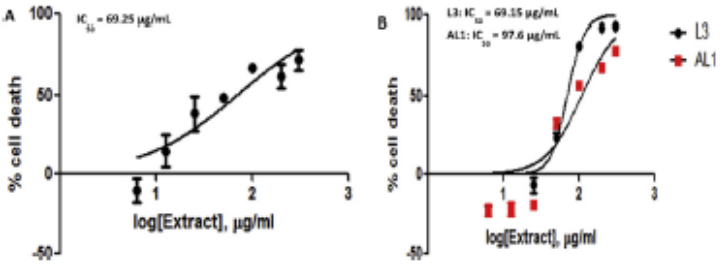

Fig. 4. IC_{50} values of *Senecio asperulus DC.* dichloromethane (L3) on prostate cancer cell lines (A). IC_{50} values of *Asparagus laricinus Burch.* methanol (AL1) and *Senecio asperulus DC.* dichloromethane (L3) extracts on breast cancer cell lines (B).

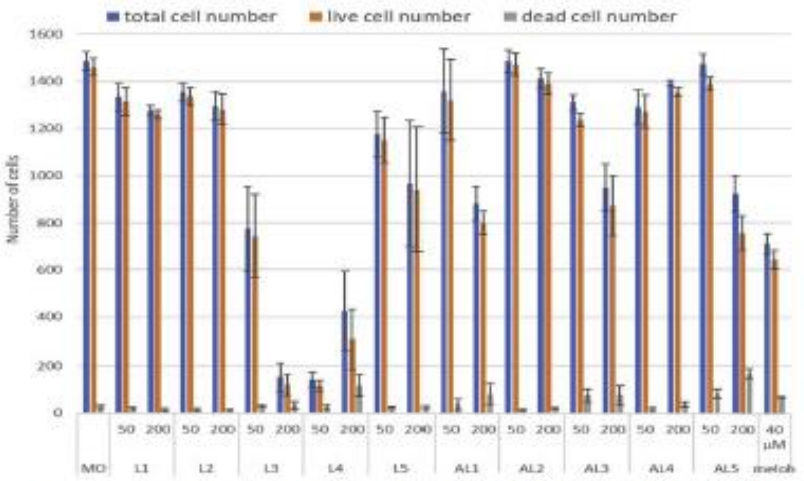

Fig. 2. Cytotoxicity effect of *Senecio asperulus DC.* and *Asparagus laricinus Burch.* extracts on the prostate cancer cell line (PC3). Error bars indicate standard deviation of triplicate values.

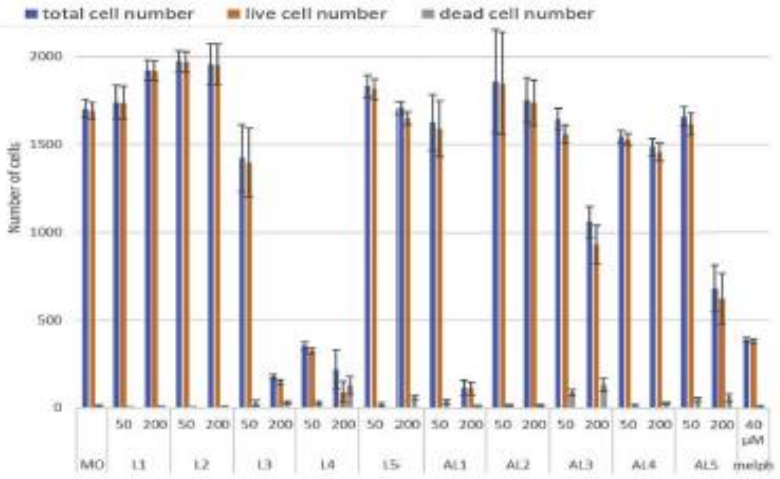

Fig. 3. Cytotoxicity effect of *Senecio asperulus DC.* and *Asparagus laricinus Burch.* extracts on breast cancer cell line (MCF-7). Error bars indicate standard deviation of triplicate values.

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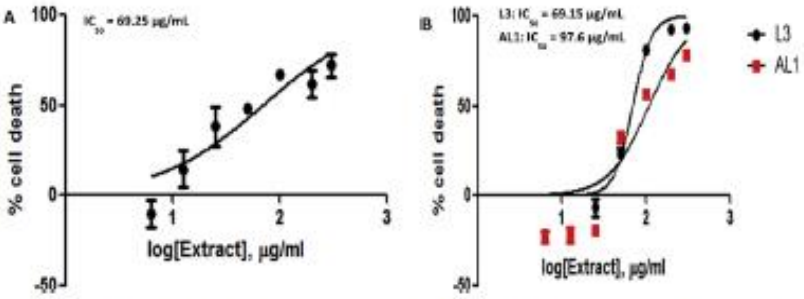

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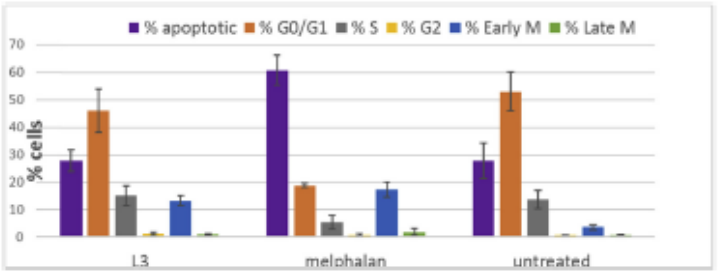


Fig. 5. Distribution of PC3 cells in the different phases of the cell cycle when treated with *Senecio asperulus DC.* dichloromethane (L3). Melphalan (40 μM) was used as a positive control. Error bars indicate the standard deviation of quadruplicate values. G0/G1: Gap 0/Gap1 phase; S: synthesis phase; M: mitotic phase.

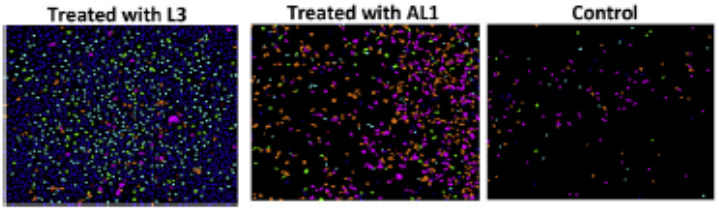


Fig. 6. Morphological changes observed after Hoechst 33342, Annexin V-FITC and PI staining of MCF-7 cells treated with L3, AL1, and melphalan at determined IC_{50} concentrations.

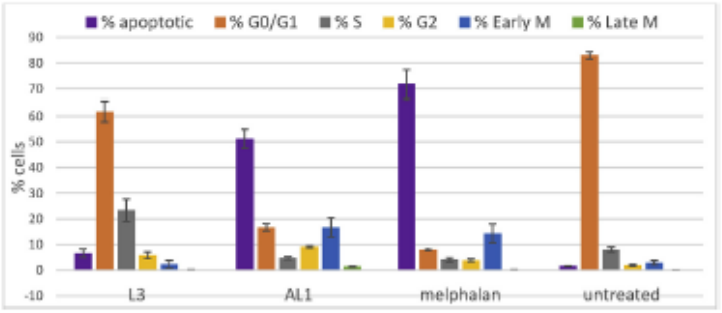
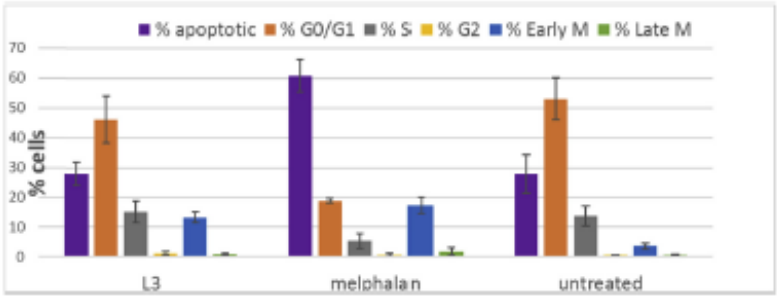


Fig. 7. Distribution of MCF7 cells in the different phases of the cell cycle when treated with *Senecio asperulus DC.* dichloromethane (L3) and *Asparagus laricinus Burch.* methanol (AL1). Melphalan (40 μM) was used as a positive control. Error bars indicate the standard deviation of quadruplicate values. G0/G1: Gap 0/Gap1 phase; S: synthesis phase; M: mitotic phase.

It is evident from the results in Fig. 4B, the IC_{50} value of *Senecio asperulus DC.* dichloromethane (L3) extract in both PC3 and MCF-7 cell lines was two-fold higher compared to those of *Asparagus laricinus Burch.* methanol (AL1) on MCF-7 cells. Thus, further studies to investigate whether the cytotoxic effects were due to apoptosis or necrosis were undertaken with both extracts L3 and AL1, at their IC_{50} concentrations. Furthermore, to evaluate the mechanism of action of these plant extracts on cancer cells, cell cycle analysis was also performed. At the end of the analysis, deoxyribonucleic acid (DNA) contents in different cell cycle phases were determined using Hoechst 33342, Annexin V-FITC and PI multiplex staining technique (Fig. 6). The results were presented in the form of percentages as showed in Figs. 5 and 7.

4. Discussion

The current study investigated the cytotoxicity activity of indigenous medicinal plants, *Senecio asperulus DC.* and *Asparagus laricinus Burch.*, against human breast (MCF-7) and prostate (PC3) cancer cells. The non-cancerous African green monkey kidney cell line (Vero) was used as a control as well as melphalan that was used as a positive control. The results showed decreased cell viability of MCF-7 and PC3 cancer cells in a dose-dependent manner (Figs. 1, 2, and 3) when treated with *Senecio asperulus DC.* dichloromethane and *Asparagus laricinus Burch.* methanol extracts for a period of 48 hours. The dose-dependent cytotoxicity effect of the methanol extract of *A. laricinus Burch.* and the dichloromethane extract of *S. asperulus DC.* indicates selectivity for cancer (MCF-3 and PC3) as they had little effect on Vero cells. An interesting anticancer agent should have a more cytotoxic effect on cancer cell lines and less effect on non-cancerous cell lines. Thus, the observed preliminary selectivity is encouraging for further research to fully elucidate the anticancer actions of *A. laricinus Burch.* methanolic extract as potential targeted breast cancer therapy for future drug development studies. According to Ayoub et al. (2014), cytotoxic agents should be effective at concentrations of up to 100 μg/mL. Since both extracts were active below



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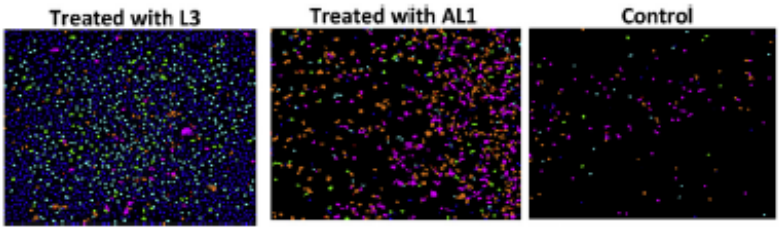

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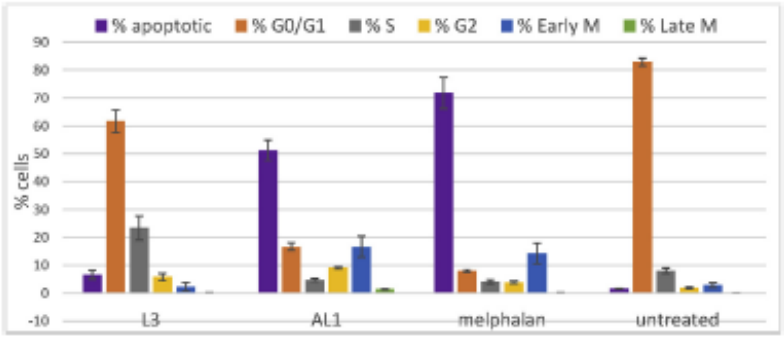

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100 μg/mL, they can be considered as suitable candidates for chemotherapeutic drugs. In an attempt to propose a mechanism of action associated with the cytotoxicity observed, these two plant extracts were studied further to determine whether cell death was due to apoptosis or necrosis.

It is worth mentioning that cell viability can decrease due to necrosis, autophagy or apoptosis. Necrosis is caused by factors external to the cell or tissue, such as infection, toxins, or trauma. Autophagy is triggered by nutrients undernourishment or withdrawal of other essential factors needed for cells to survive. Autophagy leads to the degradation of cytoplasmic materials, and this becomes an evident feature of cell morphology analysis. Although autophagy is not inherently complicated, recent progress has confirmed that it plays a wide variety of pathophysiological and physiological roles which are sometimes multifaceted (Yoshimoto et al., 2004). Apoptosis on the other side can be defined as a highly ordered programmed cell death process of unhealthy cells, where a cell dies as part of its normal programme of development, or due to lack of growth factors, or when the immune system instructs it to die. However, cancer cells typically escape apoptosis by disabling the apoptotic pathways, thus continue to proliferate and even become resistant to the chemotherapeutic drug (Ferreira et al., 2004; Luqmani, 2005). Cell death due to apoptosis can be observed with the morphological changes in the cell shape such as; nuclear condensation, cell body shrinkage, cytoplasm fragmentation, plasma membrane blebs, and externalization of phosphatidylserine. Such changes suggest that cells have committed to a specific mode of cell death resulting in apoptosis (Selvan et al., 2018). Furthermore, it is known that drugs with apoptosis-inducing properties may reduce potential drug resistance.

For this study, apoptosis analysis was done using Hoechst 33342 nuclear staining assay. The apoptosis effect of *Senecio asperulus DC.* dichloromethane and *Asparagus laricinus Burch.* methanol extracts were investigated on MCF-7 and PC3 cells stained with Hoechst 33342, Annexin V-FITC and further stained with PI stain (Crowley et al., 2016). The latter stain is generally for dead cells which have lost their cell integrity, as it intercalates into the DNA strands of the cell and staining its red color when observed under a fluorescent microscope. Therefore, dead cells are identified by this red/fluorescent propidium iodide staining after cells have been treated with test agents. Annexin V-FITC is very selective for cells death due to early apoptosis as it targets and binds to externalized phosphatidylserine (apoptotic cell surface marker) of the plasma membrane. During early stages of apoptosis, phospholipids on the plasma membrane translocate from the inner to the outer leaflet, thus exposing the phosphatidylserine which is recognized by, and fluorescently labeled by PS-binding protein, annexin-V. This stains the cell membrane in green color. Moreover, Hoechst 33342 stains the nuclear region of the cell into a bright blue color. Acquired fluorescence images were then analysed using the MetaXpress software. The obtained morphological changes and also Annexin V-FITC and PI stain uptake observations indicated that *Asparagus laricinus Burch.* methanol extract exhibits significant activity in destroying MCF-7 cells by apoptosis when compared with controls (Figs. 6 and 7). However, *Senecio asperulus DC.* dichloromethane extract didn't show much evidence of apoptosis on PC3 cell line when compared to controls (Fig. 6), this was shown by less red-stained cells. Thus, cells that died with loss of cell membrane integrity, were fewer when compared to melphalan which is known of inducing cell death through apoptosis. This finding was no surprise as plants belonging to the *Senecio* family are known to be toxic (Quattrocchi, 2016), thus this explains its high cytotoxic properties on PC3 cell lines (Fig. 2).

Some cells die through programmed pathway while others die through obstruction accompanied by cell-cycle mechanisms (Dirsch et al., 2004). Cell cycle analysis is performed to determine the state of DNA in response to treatment of the cell with a specific compound or extract. The distribution of DNA content is important as it leads to the identification of targets or pathways to target for the treatment of cancer and tumors (Planchais et al., 2000). During cell development, cells need to progress through every phase of the cell cycle to assure a full copy of DNA for a new daughter cell. There exist checkpoints which regulate progression of cells through the cycle and cause cell cycle arrest if DNA damage or DNA stress has occurred. Faults in the G2-M arrest checkpoint allows a damaged cell to enter mitosis before repair and undergo apoptosis (DiPaola, 2002). Thus, efforts to promote the latter outcome may increase the cytotoxicity of chemotherapy, however, other studies also propose enhanced cytotoxicity to be associated with high cell-cycle arrest (Shapiro et al., 2001; Alimbetov et al., 2018). Cell cycle arrest is defined as a high proportion of cells found in the same cycle event at a specific time. Cell cycle arrest is maintained until DNA repair is complete (Nojima, 2004).

From our study, results (Fig. 6) showed that *Senecio asperulus DC.* dichloromethane extract arrested a significant number of PC3 cells at the early stage of mitosis and MCF-7 cells at a synthesis phase. Several MCF-7 cells arrested at the synthesis phase by *Senecio asperulus DC.* dichloromethane extract was two-fold when compared with the positive control (Figs. 5 and 7). At the G2 phase, as cell approaches mitosis phase, cyclin-dependent protein kinases (Cdk1)/nuclear cyclin B level (B1) complex is essential for entry into and progression through mitosis (Wolf et al., 2007). Thus, activation of this Cdk1/B1 complex plays a key regulatory role in cell proliferation, while down-regulation of this complex expression induced G2/M phase arrest (Chang et al., 2003, Porter and Donoghue, 2003). However, continued and inappropriate overexpression of Cdk1/cyclin B1 plays an opposite role by mediating pro-apoptotic signaling in response to the mitotic arrest and causes non-specific cell death (Eichhorn et al., 2014). An increase in the percentage of MCF-7 cells at G2 phase as well as at the early mitosis phase when treated with *Asparagus laricinus Burch.* methanol extract suggests that the cytotoxicity mechanism was also stimulated by active cyclin B1/CDK1 complex in these cells. However, this can only be confirmed through a study of the regulation of apoptosis-related proteins in MCF-7 cancer cells by *Asparagus laricinus Burch.* methanol extract.

5. Conclusion

This study was carried out to investigate the cytotoxicity of two medicinal plants, *Senecio asperulus DC.* and *Asparagus laricinus Burch.* on prostate and breast cancer cell lines, and to further elucidate the mechanism of action of the active extract/s on the cell cycle of these cancer cell lines. *Senecio asperulus DC.* dichloromethane extract was cytotoxic against PC3 and MCF-7 cancer cell lines, and *Asparagus laricinus Burch.* methanol extract showed cytotoxicity on MCF-7 cells only in a dose-dependent manner. Moreover, both extracts had little cytotoxicity against non-cancerous Vero cells. Their ability to show more cytotoxicity on cancer cells than on non-cancerous cells, their low IC_{50} as well as the cell cycle analysis prompted further investigation. The dichloromethane extract of *Senecio asperulus DC.* appeared to arrest cells in the G2 as well as in the early mitotic (M) phase of the cell cycle on PC3 cells, while on the MCF7 cells, cell arrests were more in the S phase. These findings show that cytotoxicity of this extract against PC3 and MCF-7 cancer cell lines was not due to apoptosis, but rather due to cell arrest at the G2 and early mitosis phase. The methanol extract of *Asparagus laricinus Burch.* revealed cell arrest in the early M phase of the cell cycle but also shows a high percentage of MCF-7 apoptotic cells. Therefore, this extract induced MCF-7 cell death through apoptosis at an acceptable growth inhibition concentration dose and therefore, can be considered as a suitable candidate for future chemotherapeutic drugs.

Declarations

Author contribution statement

MFENGWANA, PH: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

MASHELE, S.S, MANDUNA, IT: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Akarolo-Anthony, S.N., Ogundiran, T.O., Adebamowo, C.A., 2010. Emerging breast cancer epidemic: evidence from Africa. Breast Canc. Res. 12 (4), S8.
Alimbetov, D., Askarova, S., Umbayev, B., Davis, T., Kipling, D., 2018. Pharmacological targeting of cell cycle, apoptotic and cell adhesion signaling pathways implicated in chemoresistance of cancer cells. Int. J. Mol. Sci. 19, 1690.
Ayoub, I.M., El-Shazly, M., Lu, M.C., Singaba, A.N.B., 2014. Antimicrobial and cytotoxic activities of the crude extracts of Dietes bicolor leaves, flowers and rhizomes. South Afr. J. Bot. 95, 97–101.
Azwanida, N.N., 2015. A review on the extraction methods use in medicinal plants, principle, strength and limitation. Medical and Aromatic Plants 4 (3), 196.
Brummitt, R.K., 1992. Vascular Plant Families and Genera, 8. Royal Botanical Gardens, Kew, pp. 14–18.
Chang, D.C., Xu, N., Luo, K.Q., 2003. Degradation of cyclin B is required for the onset of anaphase in Mammalian cells. J. Biol. Chem. 278, 37865–37873.
Crowley, L.C., Marfell, B.J., Waterhouse, N.J., 2016. Analyzing cell death by nuclear staining with Hoechst 33342. Cold Spring Harb. Protoc. 1 (9).
DiPaola, R.S., 2002. To arrest or not to G2-M cell-cycle arrest. Clin. Cancer Res. 8, 3311–3314.
Dirsch, V.M., Kirschke, S.O., Estermeier, M., Steffan, B., Vollmar, A.M., 2004. Apoptosis signalling triggered by the marine alkaloids ascidiidemin is routed via caspase-2 and JNK to mitochondria. Oncogene 23, 1586–1593.
Eichhorn, J.M., Alford, S.E., Sakurikar, N., Chambers, T.C., 2014. Molecular analysis of functional redundancy among anti-apoptotic Bcl-2 proteins and its role in cancer cell survival. Exp. Cell Res. 322 (2), 415–424.
Ferreira, C.G., Epping, M., Kruyt, F.A., Giaccone, G., 2004. Apoptosis: target of cancer therapy. Clin. Cancer Res. 8, 2024–2034.
Freshney, R.I., 2005. Culture of Animal Cells: a Manual of Basic Techniques, 5 th. John Wiley & sons Inc., Hoboken, New Jersey.
Jemal, A., Bray, F., Forman, D., et al., 2012. Cancer burden in Africa and opportunities for prevention. Cancer 118, 4372–4384.
Kose, L.S., Motsetse, A., Van Vuuren, S., 2015. Ethnobotanical survey of medicinal plants used in the Maseru district of Lesotho. J. Ethnopharmacol. 170, 184–200.
Krishan, A., 1975. Rapid flow cytofluorometric analysis of cell cycle by propidium iodide staining. JCB (J. Cell Biol.) 66, 188–193.
Lee, J.Y., Kim, E.Y., Jung, K.W., Shin, A., Chan, K.K., Aoki, D., et al., 2014. Trends in gynecologic cancer mortality in East Asian regions. Journal of Gynecologic Oncology 25, 174–182.
Luqmani, Y.A., 2005. Mechanisms of drug resistance in cancer chemotherapy. Med. Princ. Pract. 14, 35–48.
Mashele, S.S., Kolesnikova, N., 2010. In vitro anticancer screening of Asparagus laricinus extracts. Pharmacologyonline 2, 246–252.
Moore, A., Mercer, J., Dutina, G., Donahue, C., Bauer, K., et al., 1997. Effects of temperature shift on cell cycle, apoptosis and nucleotide pools in CHO cell batch cultures. Cytotechnology 23, 47–54.
Mugomeri, E., Chatanga, P., Hlapisi, S., Rahlao, L., 2014. Phytochemical characterization of selected herbal products in Lesotho. Lesotho Medical Association Journal 12, 38–47.
Nojima, H., 2004. G1 and S-phase checkpoints, chromosome instability, and cancer. Methods Mol. Biol. 280, 3–49.
Ogbole, O.O., Segun, P.A., Adeniji, A.J., 2017. In vitro cytotoxicity of medicinal plants from Nigeria ethnomedicine on Rhabdomyosarcoma cancer cell line and HPLC analysis of active extracts. BMC Complement Altern. Med. 17, 494.
Planchais, S., Glab, N., Inzé, D., Bergounioux, C., 2000. Chemical inhibitors: a tool for plant cell cycle studies. FEBS (Fed. Eur. Biochem. Soc.) Lett. 476, 78–83.
Porter, L.A., Donoghue, D.J., 2003. Cyclin B1 and CDK1: nuclear localization and upstream regulators. Prog. Cell Cycle Res. 5, 335–347.
Quattrocchi, U., 2016. CRC World Dictionary of Medicinal and Poisonous Plants: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology. CRC, Taylor & Francis Group, Boca Raton, Fla.
Seigel, R.L., Miller, K.D., Jemal, A., 2018. Cancer statistics. CA. A Cancer Journal for Clinicians 68, 7–30.
Selvan, D.A., Mahendiran, D., Kumar, R.S., Rahiman, A.K., 2018. Garlic, green tea and turmeric extracts-mediated green synthesis of silver nanoparticles: phytochemical, antioxidant and in vitro cytotoxicity studies. J. Photochem. Photobiol. B Biol. 180, 243–252.
Shapiro, G.I., Supko, J.G., Patterson, A., Lynch, C., Lucca, J., Zacarola, P.F., Muzikansky, A., Wright, J.J., Lynch Jr., T.J., Rollins, B.J., 2001. A Phase II trial of the cyclin-dependent kinase inhibitor flavopiridol in patients with previously untreated stage IV non-small cell lung cancer. Clin. Cancer Res. 7, 1590–1599.
Sylla, B.S., Wild, C.P., 2012. A million Africans a year dying from cancer by 2030: what can cancer research and control offer to the continent? Int. J. Cancer 130, 245–250.
Van der Merwe, D., Swan, G.E., Botha, C.J., 2001. Use of ethnoveterinary medical plants in cattle by Setswana-speaking people in the Madikwe area of the North West Province of South Africa. J. S. Afr. Vet. Assoc. 72 (4), 189–196.
WHO, 2013. Traditional Medicine Strategy 2014-2023. http://www.searo.who.int/entity/health_situation_trends/who_trm_strategy_2014-2023.pdf?ua=1. (Accessed 20 June 2018).
Wildlife Wholesale Nursery, 2018. Asparagus Laricinus. https://wildflowernursery.co.za/indigenous-plant-database/asparagus-laricinus/. (Accessed 25 June 2018).
Wolf, F., Sigl, R., Geley, S., 2007. The end of the beginning': cdk1 thresholds and exit from mitosis. Cell Cycle 6, 1408–1411.
World Health Organization, 2017. Regional Office for Africa. Cancer. https://www.afro.who.int/health-topics/cancer. (Accessed 25 June 2018).
Yoshimoto, K., Hanaoka, H., Sato, S., Kato, T., Tabata, S., Noda, T., Ohsumi, Y., 2004. Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. Plant Cell 16, 2967–2983.

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In vitro Antibacterial, Antioxidant and Anti-Inflammatory Effects of *Senecio asperulus* and *Gunnera perpensa* from Mohale's Hoek, Lesotho

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ABSTRACT

Background: Traditional medicinal plants have been widely used to treat or manage various ailments for centuries in Lesotho. With an increase in multi drug resistance and undesired adverse events to current drugs challenges, there is a need for alternative drugs. **Aim:** In this study we aimed at the investigation of antibacterial, antioxidant and anti-inflammatory effects of *Senecio asperulus* and *Gunnera perpensa* roots extracted in three solvents of different polarities. **Materials and Methods:** Antibacterial activity was determined using the disc diffusion method, while antioxidant activity was determined using free radical scavenging of 2,2-diphenyl-1-picrylhydrazyl and the ferric reducing antioxidant power assay. The Lipopolysaccharide (LPS) stimulated RAW 264.7 mouse macrophage *in vitro* model was used to evaluate the anti-inflammatory activity of both plants. Resveratrol was used as a positive control. **Results:** Methanol extracts of *Senecio asperulus* inhibited microbial growth even at the lowest concentration of 50 µg/ml. *Senecio asperulus* dichloromethane extract was active on most bacteria with MIC's between 50 µg/ml and 500 µg/ml. However, the water and methanol extracts of *Gunnera perpensa* had no activity against all organisms tested. Aqueous extracts of *Senecio asperulus* and *Gunnera perpensa* showed free radical scavenging activity yielding EC_{50} values of 100 µg/ml and 25 µg/ml, respectively. The aqueous extracts of *Senecio asperulus* showed moderate anti-inflammatory activity from 50 to 200 µg/ml. while the methanol extract was at 200 µg/ml and with no cytotoxicity. No anti-inflammatory activity was observed from all *Gunnera perpensa* extracts using LPS-induced macrophages, this suggests that this species may be using other mechanisms for anti-inflammatory activity. **Conclusion:** The antibacterial, antioxidant and anti-inflammatory activities observed from water extracts of *Senecio asperulus* support its ethnomedicinal use for the management of inflammation related diseases.

Key words: Antibacterial activity, Antioxidant activity, Anti-inflammatory activity, *Senecio asperulus*, *Gunnera perpensa*, Medicinal plants.

key messages: In Mohale's Hoek, Lesotho, *Senecio asperulus* and *Gunnera perpensa* are used for the treatment or management of various ailments especially since access to western health care facilities is limited. We are reporting on the efficacy of *Senecio asperulus* aqueous and methanol extracts as good candidates for potent novel anti-inflammatory, multi-drug resistant bacteria and antioxidant drugs development, as they had no cytotoxicity effects on tested RAW 264.7 cells. It is important to validate the ethnopharmacology and pharmacognosy of crude extract instead of isolated compounds as most active ingredients from medicinal plants work in synergy, hence crude extracts were used in this study.

INTRODUCTION

Inflammation is the protective response to injury of body cells and tissues due to exposure to various factors like infections, chemicals, heat and mechanical injuries. Chronic inflammation could be considered the basis of disease and this is a typical feature of many chronic disorders. Infections by organisms which are resistant to killing and clearing by the body, tend to cause chronic inflammation. Cell walls of gram-negative bacteria have lipopolysaccharides (LPS), which are endotoxins that have the ability to cause inflammation. The host response to LPS is known to be mediated by reactive oxygen species (ROS) as they lead to Rac activation and IL-1 expression, which are required to induce inflammation response.[1-3] Excessive reactive oxygen species are closely involved in various human diseases such as inflammation, cancer, heart disease, aging, atherosclerosis, rheumatoid arthritis and Alzheimer's disease. Antioxidants have an ability to neutralize excessive ROS which can induce the oxidative stress that causes cell damage and culminates in inflammation.

Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterised by persistent/chronic inflammation ultimately resulting in tissue dysfunction. The inflammation process triggers the release of mediators; histamine, kinins and prostaglandins, by damaged tissues which in

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turn involves cell membrane alterations, vascular permeability and increased protein denaturation.[4] Inflammation mediators are released as a result of induced cyclooxygenase 2 (COX-2), nitric oxide (NOS) and lipoxygenase (LP). Most non-steroidal anti-inflammatory drugs inhibit pathways that lead to the generation of inflammation mediators and this is essential for the treatment of inflammation. A good non-steroidal anti-inflammatory drug (NSAIDs) acts through the inhibition of induced nitric oxide synthase (iNOS), which is a key enzyme for the generation of nitic oxide (NO) and cyclooxygenase (COX) isozymes. There is dissatisfaction with existing anti-inflammatory drugs, as they impose toxicity and adverse side effects, particularly affecting the gastrointestinal and cardiovascular systems.[5] Moreover, they lead to much dependence on them, as disorders reoccur after treatment has been discontinued. The bad side effects from the synthetic anti-inflammation drugs that are currently available have triggered a global trend of returning to natural sources of medicines. Thus, there is a significant increase in scientific and commercial interest in the discovery of new anti-inflammatory therapeutic and preventative agents from natural product sources.

Medicinal plants play a vital role in Lesotho's primary healthcare system as they are components of the most diverse and oldest African therapeutic systems. People in Lesotho use medicinal plants for self-medication and they can diagnose and treat minor ailments without even consulting a traditional doctor or herbalist.[6] This occurs particularly in remote rural areas with limited access to health facilities. Local people in these areas rely on a variety of medicinal plants such as *Senecio asperulus* and *Gunnera perpensa* to manage different ailments including inflammation related diseases.[7,8] *Senecio asperulus* is a member of the Asteraceae family and is known as *Moferefere* or *Letapisa* by the Basotho people in Lesotho. *Gunnera perpensa* known as *Qobo* belongs to the Gunneraceae family. The dried roots of both plants are extensively used, independently or as a mixture with other medicinal plants, to treat a variety of ailments. Their uses include, but not limited to; colds and flu, urinary tract infections, sexual transmitted diseases, reproductive healthcare, umbilical cord inflammation, as vasodilating agents and circulation improvers.[8,9] However, there is limited scientific data to validate these ethnobotanical claims is scarce. Thus, this study was carried out to validate and justify the traditional claims for the use of *Qobo* and *Moferefere* in the treatment of inflammation related diseases.

METHODS AND MATERIALS

Plant collection

The study received a plant collection and export permit from the Ministry of Tourism Environment and culture, Lesotho and import approval (NC.553/2017) from The Department of Economic Development, Tourism and Environmental Affairs, South Africa. Plant material was collected from the mountains of Lesotho (Mohale's Hoek district) and authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa where voucher specimens, PHM01 for *Senecio asperulus* and PHM02 for *Gunnera perpensa* were deposited.

Preparation of plant extracts

The roots of both plant species were washed, air dried at room temperature and then ground into fine powder using an electric blender. The powdered roots of *Senecio asperulus* and *Gunnera perpensa* were soaked separately with distilled water (DH_2O), methanol (MeOH) and dichloromethane (DCM) for 72 h with occasional stirring. After filtration, the aqueous extracts were freeze dried and the organic solvent extracts were concentrated with rocket evaporator. Thereafter, the extracts were reconstituted in dimethyl sulphoxide (DMSO) to give a final concentration of 100 mg/mL respectively. Stock solutions were sonicated were solubility was an issue. All samples were then stored at 4°C.

Cell culture

The mouse macrophage cell line, RAW 264.7 were cultured and maintained in RAW 264.7 growth medium 1 (RAWGM1) at 37°C in a humidified atmosphere with 5% CO_2. Suspensions of RAW 264.7 monolayer culture were seeded into 96 well microtiter plates at a density of 25,000 cells per well using a volume of 50 µl in each well. The microtiter plates were then incubated at 37°C, 5% CO_2 and 100% relative humidity for 24 h prior to addition of test compounds to allow for cell attachment. The culture medium was then removed and the samples were added to give final concentrations of 12.5 and 50 µM.

Microorganisms

The American Type Culture Collection (ATCC) microorganisms used in this study were obtained from the Pathcare microbiology laboratory in Welkom South Africa. Four Gram positive bacteria (Staphylococcus aureus ATCC BAA-1026, Staphylococcus saprophyticus ATCC BAA-750, Enterococcus faecalis ATCC 29212 and Streptococcus pneumoniae ATCC 49619) and four Gram negative bacteria (Enterobacter cloacae ATCC 700323, Escherichia coli ATCC 25922, Proteus mirabilis ATCC 35659 and Klebsiella pneumoniae ATCC 700603) were inoculated onto Muller-Hinton (MH) agar and later used in this study. All bacterial suspensions were prepared using sterile saline to obtain an optical density comparable to the density of 0.5 McFarland barium sulphate standard (turbidity =108 CFU/mL).

Phytochemical screening assay

The qualitative phytochemical screening for the presence of flavonoids, tannins, saponins, terpenoids and alkaloids was carried out following methods by Shanmugam *et al.*[10]

In vitro antibacterial assay

The antibacterial activity was conducted using the disc diffusion method as adopted from Thitilertdecha *et al.*[11] and Su *et al.*[12] To determine the minimum inhibition concentration (MIC) of each plant extract, selected Gram positive and Gram-negative micro-organisms where treated with 4 concentrations of plant extracts from serial dilution namely: 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml. After serial dilutions were prepared, 6 mm filter paper discs were impregnated with the extract at each concentration. The extract-loaded discs were then placed on the surface of the agar inoculated with different micro-organisms as mentioned previously. Plates were incubated aerobically at 37°C and zones of inhibition were measured after 24 h. The zones of inhibition were measured in mm using a calliper and the lowest concentration at which growth inhibition resulted was considered as minimum inhibition concentration (MIC). Discs (6 mm filter paper discs) dipped in DMSO were used as negative controls, while commercial chloramphenicol discs (25 µg) and ampicillin (10 µg), were used as positive controls. The tests were performed in triplicates and mean was calculated and reported.

In vitro anti-oxidant assay

The DPPH (2,2-diphenyl-1picrylhyrazyl) free radical scavenging assay was performed using a method by Najafabad and Jamei.[13] Two hundred micro-litres (200 µL) of 0.1 mM DPPH prepared in methanol was added to 100 µL of the plant extract diluted in different concentrations. The mixture was incubated in the dark at room temperature for 30 min. Absorbance was then measured at 517 nm. Ascorbic acid was used as a positive control. The experiments were performed in triplicates and percentage inhibition of the DPPH radical scavenging activity was calculated using the formula:

% Inhibition=((Ac-A_1)/Ac) X 100

Where Ac is the absorbance of the control and A_1 is the absorbance of the sample.

Estimation of reducing power

The reducing power of the plant extracts was estimated using the method by Najafabad and Jamei,[13] whereby 500 µl of 0.2 M phosphate buffer (pH-6.6), 500 µl of ferricyanide (1% w/v) were mixed with 200 µl of each extract and the mixture was then incubated for 20 min at 50°C. After incubation, 500 µl of TCA (10% w/v) was added to the extract mixture and centrifuged for 10 min at 3000 rpm. Thereafter, 500 µl of the supernatant was collected using an automated pipette and added to 500 µl of distilled water together with 100 µl of ferric chloride (0.1% w/v) and mixed thoroughly. Absorbance was then measured at 700 nm wavelength. Percentage inhibition was calculated for the determination of reducing power:

% Inhibition= (Ac-A_1)/Ac X 100

Where A_1 is the absorbance of the sample, Ac is the absorbance of control.

Determination of anti-inflammatory activity in RAW 264.7 macrophages

To assess the anti-inflammatory activity of the plant extracts, 50 µl of Lipopolysaccharide (LPS) containing medium was added to the 96-wells plates with RAW 264.7 treated with plant extracts (as mentioned above under cell culture). Cells were then incubated for 20 h. To quantify nitric oxide (NO) production, 50 µl of the above culture medium with LPS was transferred to a new 96-well plate and 50 µl Griess reagent added. Absorbance was then measured at 540 nm wavelength and the results were expressed relative to the appropriate untreated control. A well-known inhibitor of Inducible nitric oxide synthase (iNOS) expression, resveratrol, was used as a positive control.

MTT assay

To confirm that toxicity was not a contributory factor for anti-inflammatory activity, cell viability was assessed using the MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) assay.[14] RAW 264.7 cells were seeded in a 96 well microtiter plates at a density of 25,000 cells per well using a volume of 50 µl in each well and allowed to adhere for 24 h at 37°C in a CO_2 incubator. Media was then carefully aspirated from adherent cell cultures, discarded and replaced with fresh media. Cells were then treated with various concentrations of the plant extracts and re-incubated for 24 h at 37°C. After incubation, the culture media was aspirated again and replaced with equal volumes of fresh media and MTT working solution (5 mg/mL in phosphate buffer solution). Plates were then incubated further for 4 h at 37°C. The media with MTT was removed and 100 µl of DMSO solution was added into each well to solubilize the formed MTT formazan crystals (purple colour). Absorbance was measured at 570 nm wavelength and cell inhibition percentage was determined using the formula:

Percentage Cell Inhibition = [100- Abs (sample)/Abs (control)] X 100.

Statistical analysis

All assays were performed in triplicate in all independent and separate experiments. The data is presented as means ± SD from three independent analyses and separate experiments. One-way analysis of variance (ANOVA) was applied to determine the statistical significance in various anti-inflammatory and cytotoxicity markers level between the control and the tested samples. Microsoft EXCEL software was used. The level of significance will be set at 0.05 and 0.01.

RESULTS AND DISCUSSION

Phytochemical analysis

Phytochemicals are naturally produced by plants for their protection and as a defence mechanism against microbes and insects. These phytochemicals can either be primary compounds such as, chlorophyll, proteins and common sugars, or be secondary compounds such as terpenoids, alkaloids, flavonoids, reducing sugars, tannins and phenols.[15] Secondary metabolites are known to have important medicinal benefits to humans.[16] Moreover, many studies have proved the diverse pharmacological activities of terpenoids as anti-bacterial, anti-oxidant, anti-inflammatory, anticancer, anti-viral, anti-malarial and anaesthetic agents.[17-20] Geographical properties, natural conditions and bioconstituents of soil, does influence quality of secondary metabolites.[21,22] This is indeed true, as *Senecio asperulus* collected from Mohale's Hoek, Lesotho (more rural area) was found to be rich in secondary metabolites when compared with phytochemistry findings by Mugomeri *et al.*[7] using the same plant but collected from Maseru, Lesotho (which is the capital city of the Basotho country). However, our findings corroborated with Mugomeri *et al.*[7] findings as *Senecio asperulus* methanol extracts had flavonoids, of which the aqueous extracts of the same plant showed no presence of flavonoids. This simply means that *Senecio asperulus* methanolic extracts contain flavonoids, regardless of their point of collection. From our results, terpenoids were present from all extracts of both plant species, extracted in different solvents, thus supporting the traditional medicinal values of these plants (Table 1). Aqueous extracts of *Senecio asperulus* showed the presence of more phytochemicals when compared to methanolic and dichloromethane extracts of the same plant species. *Gunnera perpensa* water extracts showed only the presence of flavonoids, terpenoids and tannins. However, its methanolic and dichloromethane extracts revealed the presence of almost all investigated phytochemicals (Table 1). This discovery indicates that active ingredients from this plant are more hydrophobic thus extracted better with solvents which are non-polar.

Antibacterial activity

To determine the antibacterial activity and minimum inhibitory concentration (MIC) of each plant extract, selected Gram-positive and Gram-negative micro-organisms where treated with different concentrations of plant extracts. The zone of inhibition was measured and compared to that of controls. Two known antibiotics namely; ampicillin (for the Gram-positive organisms) and chloramphenicol (for the Gram-negatives) were used as positive controls, while saline was used as a negative control. MIC of each active extracts were presented in Table 2. Antibacterial bioactive plant compounds affect the integrity of the bacterial cell wall permeability so the bacteria leak to death, thus, inhibiting bacterial growth.[23] The methanolic extracts of both *Senecio asperulus* and *Gunnera perpensa* failed to inhibit the growth of the Gram positive and negative microorganisms selected for this study even at higher concentrations of 500 µg/ml, except for *S. faecalis* (Table 2). The most active plant extract was *Senecio asperulus* aqueous extract as it managed to inhibit growth of all strains tested against. *Gunnera perpensa* dichloromethane extracts also inhibited most microorganisms and only one organism (*S. faecalis*) was resistant even at the highest concentration. The antibacterial activity of (*Senecio asperulus* aqueous extract and *Gunnera perpensa* dichloromethane extract) relates bioactivity to the phytochemical content (Tables 1 and 2).

Minimum inhibition concentration (MIC) required to inhibit *S. faecalis* was 500 µg/ml for *Senecio asperulus* methanol extract and 50 µg/ml for the aqueous and DCM extracts. Aqueous extracts further showed MIC to be 50 µg/ml for most tested Gram-negative's and Gram-positive's, except for *S. aureus* and *S. saprophyticus*, as their MIC 's were at 100 µg/ml and 250 µg/ml, respectively (Table 2). *Senecio asperulus*

Table 1: Phytochemical screening analysis of *Senecio asperulus* and *Gunnera perpensa*. (+) Indicate the presence and degree (due to color intensity) of phytochemicals and (-) Indicate the absence of phytochemicals.

Plant extract	Flavonoids	Tannins	Saponins	Terpenoids	Steroids	Alkaloids
Senecio asperulus (MeOH)	++	++	-	+	+	-
Senecio asperulus (H_2O)	-	+	+	+	++	+
Senecio asperulus (DCM)	+	-	-	++	+	+
Gunnera perpensa (MeOH)	+	+	+	++	-	+
Gunnera perpensa (H_2O)	+++	+	-	+	-	-
Gunnera perpensa (DCM)	+++	-	+	++	-	++

Table 2: Antibacterial analysis of *Senecio asperulus* and *Gunnera perpensa*.

Micro-organisms	Concentration (µg/ml)	S1	S2	S3	G1	G2	G3	Control A	Control B
S. faecalis	500	+	++	+	-	-	-	++	-
	250	-	+	+	-	-	-		
	100	-	+	+	-	-	-		
	50	-	+	+	-	-	-		
S.pneumoniae	500	-	+	-	-	-	+	++	-
	250	-	+	-	-	-	+		
	100	-	+	-	-	-	+		
	50	-	+	-	-	-	+		
P.mirabilis	500	-	++	+	-	-	++	-	++
	250	-	+	+	-	-	+		
	100	-	+	+	-	-	+		
	50	-	+	+	-	-	-		
K.pneumoniae	500	-	++	+	-	-	++	-	++
	250	-	+	+	-	-	++		
	100	-	+	+	-	-	+		
	50	-	+	+	-	-	+		
S.saprophyticus	500	-	+	-	-	-	+	++	-
	250	-	+	-	-	-	+		
	100	-	-	-	-	-	+		
	50	-	-	-	-	-	+		
S.aureus	500	-	+	-	-	-	+	++	-
	250	-	+	-	-	-	+		
	100	-	+	-	-	-	-		
	50	-	-	-	-	-	-		
E. coli	500	-	+	+	-	-	+	-	++
	250	-	+	+	-	-	+		
	100	-	+	+	-	-	+		
	50	-	+	-	-	-	+		
E.cloacae	500	-	+	+	-	-	++	-	++
	250	-	+	-	-	-	+		
	100	-	+	-	-	-	+		
	50	-	+	-	-	-	+		

Abbreviations: ***S1:*** *Senecio asperulus* methanol extract; ***S2:*** *Senecio asperulus* aqueous extract; ***S3:*** *Senecio asperulus* dichloromethane extract; ***G1:*** *Gunnera perpensa* methanol extract; ***G2:*** *Gunnera perpensa* aqueous extract; ***G3:*** *Gunnera perpensa* dichloromethane extract; [++] : sensitive with zone of inhibition ≥ 16 mm; [+] : sensitive with zone of inhibition ≤ 16 mm; [-] : resistant with no zone of inhibition. **Control A:** Ampicillin; **Control B:** Chloramphenicol. MIC of each active extracts are presented with **"Bold"** cross signs (+) and yellow colouring.

further showed the MIC to be at 50 µg/ml for *S. faecalis*, *P. mirabilis* and *K. pneumoniae* and at 100 µg/ml for *E. coli*. *E. cloacae* resisted the DCM extract on all other concentrations, besides the highest tested concentration of 500 µg/ml, however, the zone of inhibition was > 16 mm. *Gunnera perpensa* DCM extract was very active with the MIC of 50 µg/ml for most organisms, besides *P. mirabilis* and *S. aureus* as their MIC's were 100 µg/ml and 250 µg/ml, respectively (Table 2).

Anti-oxidant activity

Biological oxidant reactions of exogenous and endogenous factors generate active oxygen species or oxidants such as free radicals in a human body. Unfortunately, these unstable produced oxidants give rise to oxidative stress and are found to be responsible for many diseases due to their ability to cause DNA damage. Phenols have redox properties which acts as hydrogen donors, oxygen quenchers and reducing agents, thus, delocalize the unstable and unpaired electron within the aromatic structure.[24] Antioxidants from natural sources are important in safeguarding human health and as protection from diseases resulting from oxidative stress. Phytochemical antioxidants such as flavonoids have an aptitude to reduce oxidative stress by neutralizing these reactive oxygen species. Moreover, natural antioxidants are considered to be more active and safer to health than synthetic antioxidants, as the latter turns to have detrimental side effects when used *in vivo*.[17]

The DPPH assay was used to investigate the free radical-scavenging activities of *Senecio asperulus* and *Gunnera perpensa*. Activities were expressed as percentages in order to determine plant extract concentration required to achieve a 50% DPPH scavenging activity (EC_{50}). The dose-dependent antioxidant activity of the tested extracts are summarized in Figure 1. The aqueous extract of *Senecio asperulus* had an EC_{50} of 100 µg/ml and managed to scavenge about 78% DPPH at the highest concentration of 500 µg/ml. *Gunnera perpensa* aqueous extract EC_{50} was observed at the lowest concentration of 25 µg/ml, whereas ascorbic acid EC_{50} was only at 50 µg/ml, which is two-folds. This was very impressive as it suggests *Gunnera perpensa* to be a very strong antioxidant agent.

The genus Senecio is known to be rich in flavonoids[25] and *Gunnera perpensa* is the only species from the Gunnera genus which has flavonoids.[26] Flavonoids are known to have very strong antioxidant activities.[27,28] Tannins are known as water-soluble phenolic compounds and were present in both plant species aqueous extracts. According to Karamac´ *et al.*[29] tannins can chelate metal ions and interfere with the formation of hydroxyl free radicals, thus, cancels oxidation. There was a directly proportional relationship between the presence of both flavonoids and tannins and the antioxidant activity of *Senecio asperulus* methanol and *Gunnera perpensa* aqueous extracts. Thus, indicating that these phenols; flavonoids and tannins, were the leading contributors to the observed antioxidant activities.

The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity as it proves the electron transfer ability of the extract. The reduction of oxidized intermediates of lipid peroxidation process was indicated by the formation of ferrous chloride and potassium ferrocyanide which produced a green to blue colour. The intensity of the formed colour was dependent on the reducing power of the extract used. *Senecio asperulus* and *Gunnera perpensa* aqueous and methanol extracts showed increased ferric reducing power with the increasing concentration as shown in Figure 2. The aqueous extracts from *Gunnera perpensa* proved to be a better source of antioxidants as their reducing power were 50% at 100 µg/ml, followed by the methanolic extracts as these extracts also showed increased reducing power. The dichloromethane extracts of both plants showed less reducing power than ascorbic acid and the other extracts. These results did indicate that *Senecio asperulus* and *Gunnera perpensa* have very strong free radical scavenging and antioxidant properties (Figure 1) possibly due to their hydrogen donor ability as confirmed by the estimation of ferric reducing power.

Anti-inflammatory activity

Macrophages represent a highly heterogeneous group of hematopoietic cells present in almost all tissues including adipose. Moreover, they diversely perform complex microbicidal functions, such as; surveillance, chemotaxis, phagocytosis and destruction of pathogenic micro-organisms. Therefore, the development of therapeutics which can non-specifically enhance the innate immune response represents a promising strategy to combat classical and emerging infectious agents.[30] However, depending on the trigger, macrophage responses can be divided into two distinct and mutually exclusive activation programs termed classical and alternative. Classical activation results in a highly inflammatory phenotype and mainly occurs in response to bacterial products such as lipopolysaccharides (LPS) and interferon gamma (IF-γ). These classically activated macrophages produce a myriad of pro-inflammatory signals which can alter the functionality of its surrounding cells.

In addition, these activated cells produce various highly reactive oxidants including nitric oxide (NO), a product of the catalysis of arginine by the enzyme inducible nitric oxide synthase (iNOS).[31] The mouse macrophage cell line, RAW 264.7, is a well characterised and popular model to investigate the anti-inflammatory potential of test samples. As cells were cultured in multi-well plates and activated by exposure to LPS which induces the expression of iNOS with concomitant nitric oxide formation. Changes in NO production were determined by measuring the levels of nitrate in the culture medium. Figure 3 illustrates the levels of nitrate macrophage cells produce nitrite and nitrate in response to LPS formed after macrophages were exposed to plant extracts.

High anti-inflammatory activity was observed from the DCM extract *of Senecio asperulus*, even when compared with the activity of the control. *Senecio asperulus* showed an ability to suppress NO production by down-regulating the expression of iNOS in RAW 264.7 macrophages. According to Chen *et al.*[32] non-lectin glycoprotein (PCP-3A) inhibits LPS-induced production of NO through the down-regulation of iNOS via a transcription factor NF-JB mechanism. Thus, this could suggest that anti-inflammatory activity observed from *Senecio asperulus* (Figure 3) may be through this mechanism involving the transcription factor NF-JB, however, this remains to be elucidated through further investigations. Furthermore, it is known that LPS induces the expression of iNOS through redox-sensitive signalling pathways where superoxide anions act as second messengers. Antioxidants inhibit redox-active signalling by blocking radical producing enzymes.[33] Thus, the antioxidant potential of this *Senecio asperulus* could be responsible for the observed anti-inflammatory activity.

Senecio asperulus methanol and aqueous extracts exhibited anti-inflammatory potential, which was moderate when compared with that of the positive control, resveratrol. All *Gunnera perpensa* extracts showed no detectable inhibition, even at the highest concentration of 200 µg/ml. This reported lipopolysaccharide induced anti-inflammatory activity assay was carried out simultaneous with the evaluation of cell viability (MTT assay) to confirm the absence of cytotoxicity of the test sample.

Cell viability

To confirm the absence of toxicity as a contributory factor, cell viability was assessed using MTT. The MTT assay is a colorimetric assay is used to reflect the number of viable cells present through the assessment of cell metabolic activity after treatment with test compounds. MTT, a yellow tetrazole, is reduced to purple formazan in living cells.[34] A solubilization

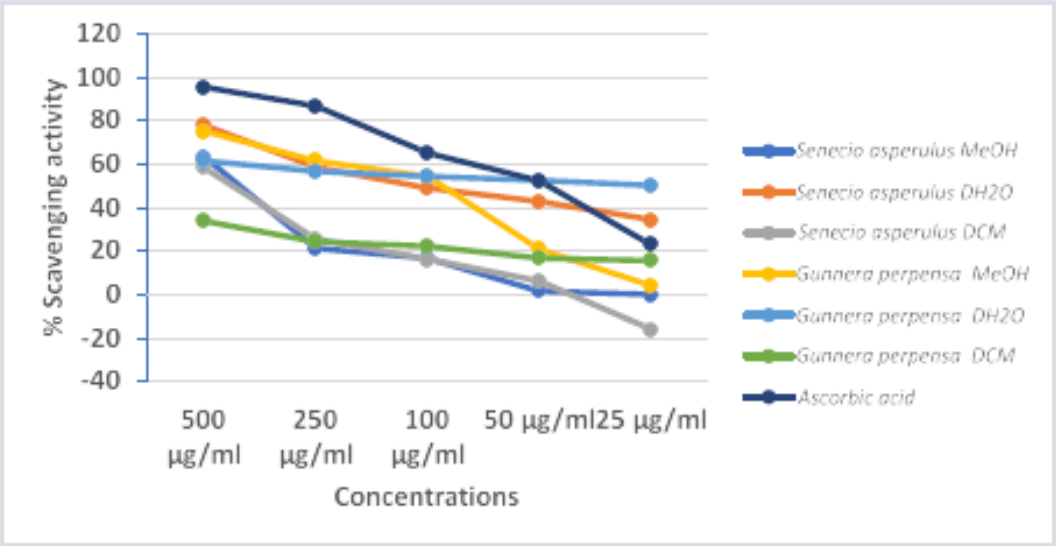

Figure 1: Antioxidant activity with DPPH assay.

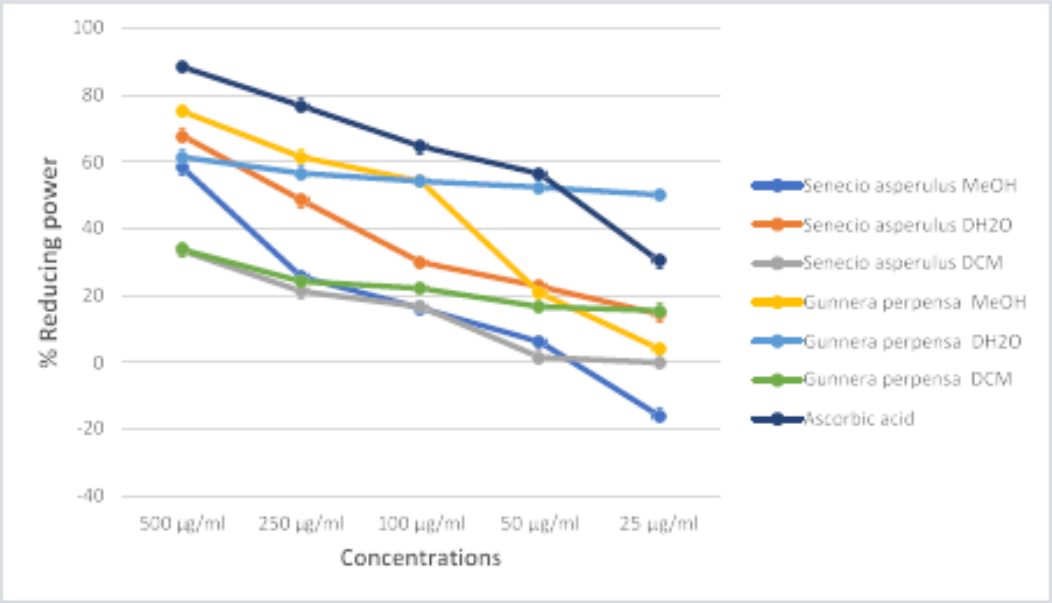

Figure 2: Ferric reducing power of *Senecio asperulus* and *Gunnera perpensa* extracts compared with ascorbic acid.

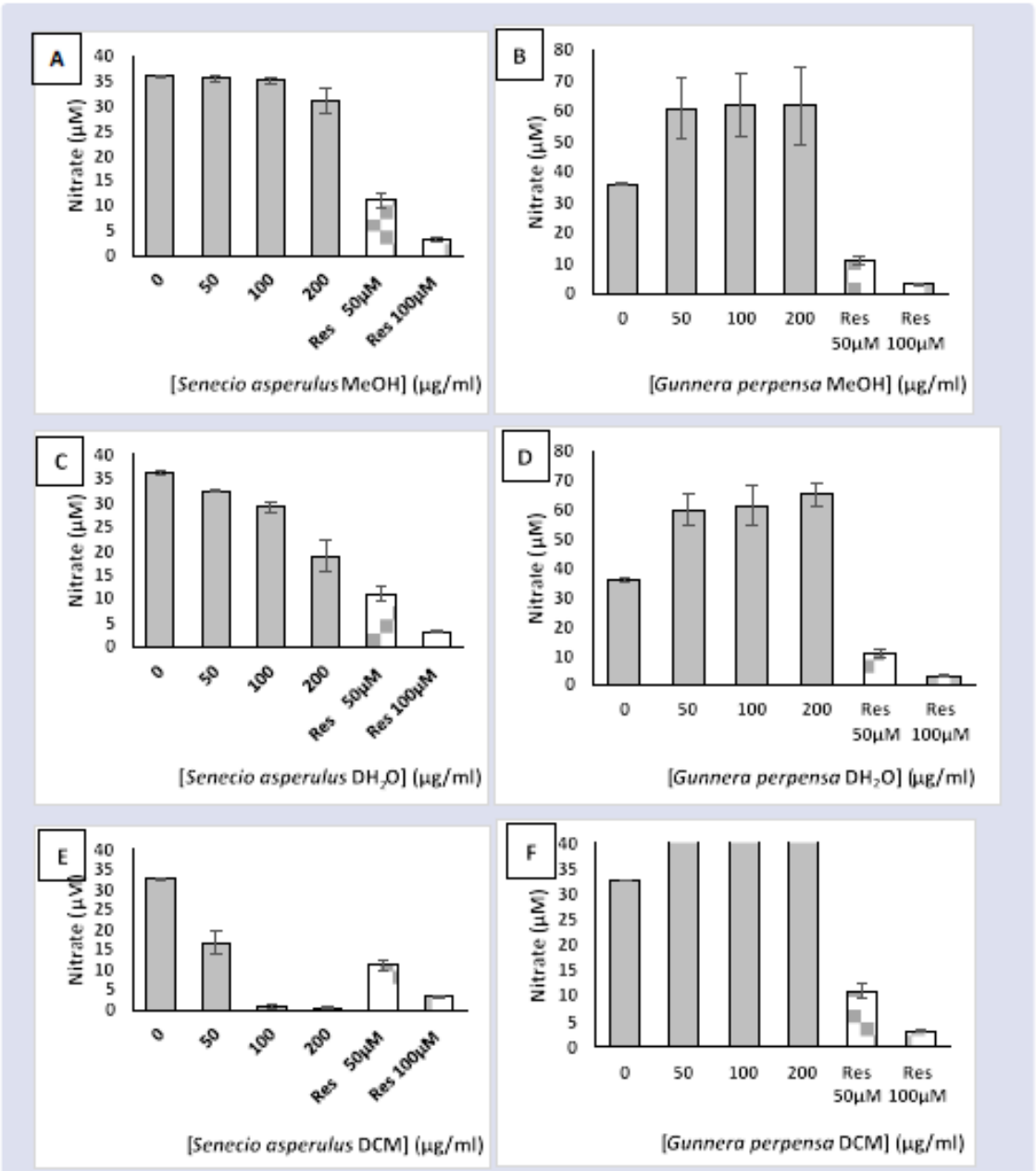

Figure 3A-F: Nitric oxide production in LPS activated macrophages treated with different concentrations of *Senecio asperulus* and *Gunnera perpensa* extracts compared with resveratrol.

solution is then added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution is quantified spectrophotometrically by measuring light absorbance at a wavelength of 600 nm. The degree of light absorption depends on the solvent. NAD(P)H-dependent oxidoreductase enzymes in the cytosolic compartment of the cell influences the reduction of tetrazolium dye.[35] Thus, MTT reduction depends on the cellular metabolic activity due to NAD(P)H flux.

Methanol and aqueous extracts of *Senecio asperulus* showed no significant toxicity towards RAW 264.7 cells (Figures 4A and 4C). However, the toxicity of the DCM extract of *Senecio asperulus* did override the meaningful prediction of anti-inflammatory potential from Figure 3E. Thus, re-evaluation at lower concentrations below 50 µg/ml may provide more conclusive decision however toxicity remains a potential risk for continual use of any medicine, not excluding natural medicinal plants.

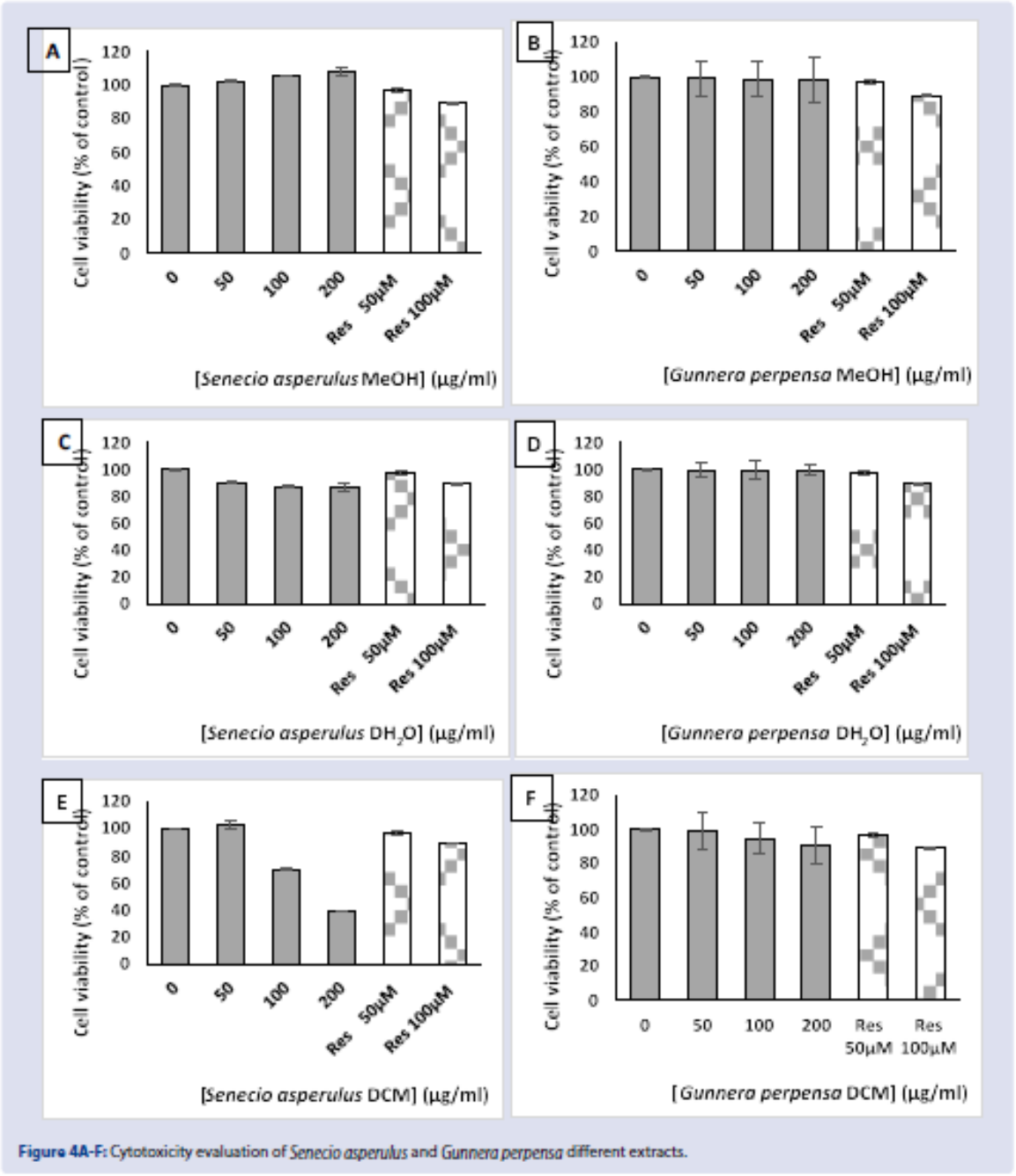

Figure 4A-F: Cytotoxicity evaluation of *Senecio asperulus* and *Gunnera perpensa* different extracts.

CONCLUSION

This study demonstrated that, *Senecio asperulus* and *Gunnera perpensa* are both rich in phytochemicals especially flavonoids, tannins, saponins and alkaloids. These phytochemicals are known for their pharmacological activities such as anti-oxidant, antidiabetic, antibacterial, antiviral, anti-inflammatory, anti-diarrheal, hepatoprotective and radioprotective effects, amongst others. Thus, based on our results, we suggest that *Senecio asperulus* aqueous and methanol extracts can be good candidates for potent anti-inflammatory, multi-drug resistant bacteria and novel antioxidant drugs development, as they had no cytotoxicity effects on tested cells. Furthermore, our results validated the Basotho traditional medicinal use of *Senecio asperulus* and *Gunnera perpensa* for the treatment of inflammation related conditions and for the treatment of wounds (due to their antimicrobial, antioxidant and anti-inflammatory activity). However, further studies for the purification of bioactive compounds (so as to synthesise these active compounds in order to promote their conservation) and *in vivo* studies, are recommended for the evaluation of these active extracts as effective anti-inflammatory, antibacterial and antioxidant agents.

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CONFLICTS OF INTEREST

No conflicts of interest to be declared by authors.

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ABBREVIATIONS

µL: Microlitre; µM: Micromolar; ATCC: American Type Culture Collection; CO_2: Carbon dioxide; COX-2: Cyclooxygenase 2; DCM: Dichloromethane; DMSO: Dimethyl sulphoxide; DNA: Deoxyribonucleic acid; DPPH: 2,2-diphenyl-1picrylhyrazyl; EC_{50}: Half maximal effective concentration; IF-γ: Interferon gamma; IL-1: interleukin-1; iNOS: induced nitric oxide synthesis; LP: lipoxygenase; LPS: lipopolysaccharides; MeOH: Methanol; MIC: minimum inhibition concentration; mM: Millimolar; MTT: 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide; nm: Nanometre; NOS: Nitric oxide; NSAIDs: Non-steroidal anti-inflammatory drug; ROS: reactive oxygen species; TCA: Trichloroacetic acid; w/v: Weight per volume.

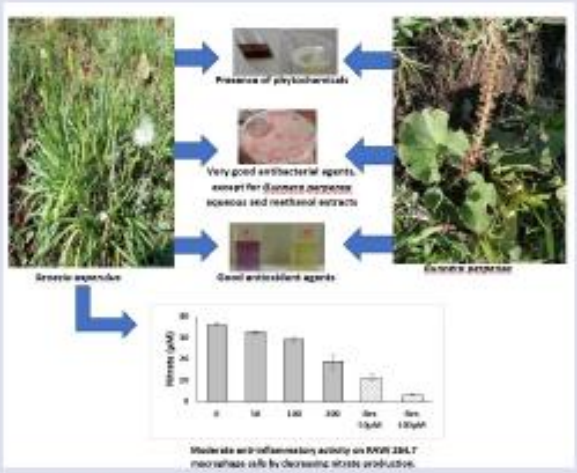
REFERENCES

1. Hsu HY, Wen MH. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. J Biol Chem. 2002;277(25):22131-9.
2. Hsu BG, Yang FL, Lee RP, Pang TC, Harn, HJ, Chen HI. N-acetylcysteine ameliorates lipopolysaccharide-induced organ damage in conscious rats. J Biomed Sci. 2004;11:152-62.
3. Antonicelli F, Brown D, Parmentier M, Drost EM, Hirani, N, Rahm, D K, MacNee W. Regulation of LPS-mediated inflammation *in vivo* and *in vitro* by the thiol antioxidant nacystelyn. Am J Physiol Lung Cell Mol Physiol. 2004;286:1319-27.
4. Leelaprakash GS, Mohan Dass SM. *In vitro* anti-inflammatory activity of methanol extract of *Enicostemma axillare*. Int J Drug Dev Res. 2011;3(3):189-96.
5. Bruno, Patrignani. New insights into the use of currently available non-steroidal anti-inflammatory drugs. J Pain Res. 2015;8:105-18.
6. Moteetee A, Van Wyk B. The medical ethnobotany of Lesotho: A review. Bothalia. 2011;41(1):209-28.
7. Mugomeri E, Chatanga P, Hlapisi S, Rahlao L. Phytochemical characterization of selected herbal products in Lesotho. Lesotho Med Asso J. 2014;12:38-47.
8. Kose LS, Moteetee A, Van Vuuren S. Ethnobotanical survey of medicinal plants used in the Maseru district of Lesotho. J Ethnopharmacol. 2015;170:184-200.
9. Van der Merwe D, Swan GE, Botha CJ. Use of ethnoveterinary medical plants in cattle by Setswana-speaking people in the Madikwe area of the North West Province of South Africa. J S Afr Vet Assoc. 2001;72(4):189-96.
10. Shanmugam S, Sathish KT, Panneer SK. Laboratory handbook on Biochemistry.2nd ed. PHI Learning Pvt. Ltd; 2010.
11. Thitilertdecha N, Teerawutgulrag A, Rakariyatham N. Antioxidant and antibacterial activities of *Nephelium lappaceum L.* extracts. LWT-Food Sci Technol. 2008;41(10):2023-35.
12. Su P, Yang C, Yang J, Su P, Chuang L. Antibacterial activities and antibacterial mechanism of polygonum cuspidatum extracts against nosocomial drug-resistant pathogens. Molecules. 2015;20(6):11119-30.
13. Najafabad AM, Jamei R. Free radical scavenging capacity and antioxidant activity of methanolic and ethanolic extracts of plum (*Prunus domestica L.*) in both fresh and dried samples. Avicenna J Phytomed. 2001;4(5):343-53.
14. Van Meerloo J, Kaspers GJL, Cloos J. Cell sensitivity assays: The MTT assay. Methods Mol Biol. 2011;731:237-45.
15. Abdul W, Mehreen G, Syed BJ, Muhammad N, Ajmal K, Rukhsana G, Asnad. Phytochemical analysis of medicinal plants occurring in local area of mardan. Biochem. Ana. Biochem. 2013;2(4):1-4.
16. Krishnaiah D, Rosalam S, Awang B. Phytochemical antioxidants for health and medicine A move towards nature. Biotechnol Mol Biol Rev. 2007;1(4):97-104.
17. Wagner KH, Elmadfa I. Biological relevance of terpenoids: Overview focusing on mono-di and tetraterpenes. Ann Nutr Metab. 2003;47:95-106.
18. Kappers IF, Aharoni A, van Herpen TW, Luckerhoff LL, Dicke M, *et al.* Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. Science. 2005;309:2070-2.
19. Rabi T, Bishayee A. Terpenoids and breast cancer chemoprevention. Breast Cancer Res Treat. 2009;115:223-39.
20. Negi JS, Singh P, Rawat B. Chemical constituents and biological importance of Swertia: A review. Curr Res Chem. 2011;3:1-15.
21. Inbathamizh L, Padmini E. Effects of geographical properties on the phytochemical composition and antioxidant potential of *Moringa oleifera* flowers. BioMedRx. 2013;1(3):238-47.
22. Santhi K, sengottuvel R. Qualitative and quantitative phytochemical analysis of *Moringa concanensis* nimmo. Int J Curr Microbiol App Sci. 2016;5(1):633-40.
23. Yildirim A, Mavi A, Kara AA. Determination of antioxidant and antimicrobial activities of *Rumexcrispus L.* extracts. J Agric Food Chem. 2001;49:4083-9.
24. Ross JA, Kasum CM. Dietary flavonoids: Bioavailability, metabolic effects and safety. Annu Rev Nutr. 2002;22:19-34.
25. Singh D, Satish CS, Manisha DS. Chemical and potential biological perspectives of genus Senecio (Asteraceae). Eur J Pharm Med Res. 2017;4(11):200-22.
26. Maroyi A. From traditional usage to pharmacological evidence: Systematic review of *Gunnera perpensa L.* Evid Based Complement Alternat Med. 2016:14-25.
27. Hollman PCH. Evidence for health effects of plant phenols: Local or systemic effects? J Sci Food Agric. 2001;81:842-52.
28. Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. Flavonoids: A review of probable mechanisms of action and potential applications. Am J Clin Nutr. 2001;74(4):418-25.
29. Karamac' MA. Kosinska RA. Chelating of Fe(II), Zn(II) and Cu(II) by tannin fractions separated from hazelnuts, walnuts and almonds. Food Chem Toxicol. 2006;39:257-60.
30. Mifsud EJ, Tan AC, Jackson DC. TLR agonists as modulators of the innate immune response and their potential as agents against infectious disease. Front Immunol. 2014;5:1-10.
31. Sangwon K and Prem P. Effects of interferon-g and lipopolysaccharide on macrophage iron metabolism are mediated by nitric oxide-induced degradation of iron regulatory protein 2. J Biol Chem. 2000;275(9):6220-6.
32. Chen JN, De Mejia EG, Wu JSB. Inhibitory effect of a glycoprotein isolated from golden oyster mushroom (*Pleurotus citrinopileatus*) on the lipopolysaccharide-induced inflammatory reaction in RAW 264.7 macrophage. J Agric Food Chem. 2011;59(13):7092-7.
33. Jedinak A, Dudhgaonkar S, Wu Q, Simon J, Sliva D. Anti-inflammatory activity of edible oyster mushroom is mediated through the inhibition of NF-B and AP-1 signaling. Nutr J. 2011;10:52-9.

34. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1–2):55-63.

35. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnol Annu Rev. 2005;11:127-52.

GRAPHICAL ABSTRACT



SUMMARY

The antibacterial and antioxidant activities observed from *Senecio asperulus* aqueous extracts and *Gunnera perpensa* dichloromethane extracts related with the phytochemical contents observed from these extracts as well as the anti-inflammation activity of *S. asperulus*. To the best of the author's knowledge, there was no report on the anti-inflammation ability of *S. asperulus* roots extracts on lipopolysaccharide induced RAW 264.7 macrophages. This research study further provides evidence that antioxidants do suppress iNOS expression and subsequently nitric oxide production, thus inhibit inflammation. Moreover, these findings support the ethnomedicinal use of *Senecio asperulus* for the management of inflammation related diseases.

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Prof. Mashele is currently the Dean of the Faculty of Health and Environmental Sciences. He is also the Director of the Drug Discovery Unit at CUT. He is serving on the editorial boards of many international journals and as panel member in research foundations. He has supervised many students and published extensively in high impact factor journals. His Drug Discovery Unit currently has more than 50 Masters and Doctoral students. His Unit's latest discovery saw widespread media coverage. He is collaborating with Universities overseas, the CSIR and the National Cancer Institute (USA). Prof Mashele has been nominated for the 'Who's who in the World' by International experts.

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