Toxicology of *Asparagus laricinus* in rats

By

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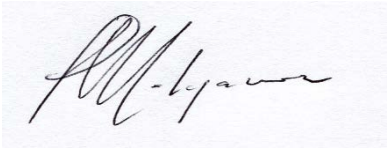
A declaration of independent work

I, Sekobane Daniel Mokgawa, student number 211152552 hereby declare that this dissertation submitted to the Central University of Technology, Free State; for the degree Master of Health Sciences in Biomedical Technology, is my own independent work that has not been submitted before to any institution, by myself or any other person in fulfilment (or partial fulfilment) for the requirements for the attainment of any qualification.



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Signature Date

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Dedications

This thesis is dedicated to my wife Masefako; for the undying support and encouragement throughout my research. To my sons and daughter, Mmushi, Mahlatse, Tshepho and Mahlogonolo.

In memory of my late son Tumelo, who suddenly passed on at a tender age.

I also dedicate this to my late father Ngwako Joseph; who always encouraged me to study further and strongly believed in me and my potential to succeed in life. To my late mother "Rennie" Ramasela Lydia; who has always been a pillar of my life. She taught me the basic principles of ubuntu and the importance of sharing.

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List of abbreviations

ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BASO	Basophils
BUN	Blood Urea Nitrogen
CASA	Cancer Association of South Africa
CHOL	Cholesterol
CREAT	Creatinine
EOS	Eosinophils
FBC	Full blood count
HB	Haemoglobin
HCT	Haematocrit
IARC	International Agency for Research in Cancer
IQR	Interquartile range
LYM	Lymphocytes
MCH	Mean Cell Haemoglobin
MCHC	Mean Cell Haemoglobin Content
MCV	Mean Cell Volume
MON	Monocytes
NEU	Neutrophils
PLT	Platelets
RBC	Red Blood Cells
WBC	White Blood Cells
WHO	World Health Organisation

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ABSTRACT

Traditional medicine has been a fertile source of revealing lead novel molecules which are then subjected to investigations using the techniques of the modern drug discovery. There are a number of conventional drugs that originate from plants, such as aspirin (from willow bark), digoxin (from foxglove), quinine (from cinchona bark), and morphine (from the opium poppy). The aim of the present work was to evaluate the possible toxic effects of the dried roots of *Asparagus laricinus* extracts using Sprague Dawley rats as animal models. In this study we investigated the use of *Asparagus laricinus* roots extracts for novel anticancer drug development, specifically looking at the safety and toxicology. Previous *in vitro* studies on *Asparagus laricinus* extracts have demonstrated anticancer activity against three human cell lines, namely, breast MCF7, renal TK10 and melanoma UACC62. These necessitated further studies on *Asparagus laricinus* extracts, such as toxicity, adverse effects investigations as well as *in vivo* biological studies using animal models. The objectives of the study was to evaluate variations in serum biochemical parameters, investigate possible deviations in haematological parameters, and also to assess histopathological variations on the liver, kidneys and spleen tissues of animals exposed to aqueous and ethanolic extracts of *Asparagus laricinus* roots.

The study was conducted at the Animal Research Unit at the University of the Free State, Bloemfontein, South Africa. Written approval for the final version of the protocol was obtained from the Interfaculty Animal Ethics Committee of the Faculty of Health Sciences at the University of the Free State. The dried plant roots were pulverized, 10g soaked in ethanol or distilled water and agitated for 72 hours at 120 rpms. The supernatant was filtered and the ethanol removed completely under vacuum. The aqueous sample was lyophilized to obtain dried powdered material. The powdered plant material was dissolved in distilled water to prepare 2%, 10% and 20% concentration. The material was also dissolved in ethanol and different concentrations were obtained by varying the volumes of the solution administered.

The study population consisted of a total of 54 Sprague Dawley rats (weighing between 180g and 250g), both male and female, obtained from the above research unit. The animals were divided into two groups of 24 and 30 rats for aqueous and

ethanolic extracts, respectively. The aqueous group was further divided into four subgroups of 6 rats which were exposed to 2%, 10% and 20% extracts and the control group (unexposed). The ethanolic group was divided into five subgroups of 6 rats which were exposed to increasing doses of 50, 100, 200 and 400mg/kg/day extracts and the last group served as controls (unexposed). The aqueous extracts were administered to the three subgroups for eight weeks ad libitum while the control group was exposed to tap water. Ethanol extracts were administered daily over a period of two weeks through gavage and the control group was administered water through gavage as well. Blood samples were collected, animals were sacrificed and organs/tissues excised for histological assessment.

Biochemical and haematological tests were selected as indicators of the damage to the tissue of organs, including the liver, kidney and spleen. A significant difference ($p<0.05$) was observed for platelets with the ethanol extract at a dose of 50g/kg/day. There were no statistical differences between the treatment groups and controls with regard to the rest of haematological variables and selected biochemical tests. Comparison of the controls (n=6) and treatment groups (n=6) revealed an average median change in weight of slightly above 50g over the entire eight-week period of experimentation with aqueous extracts. A significant difference ($p<0.05$) was observed for both haemoglobin and blood urea nitrogen results with the 20% water extract. There were no statistical differences between the treatment and control groups with regard to the rest of haematological variables and selected biochemical tests. Histological evaluation could not reveal any pathological changes in both the aqueous and ethanolic extracts across all levels of dosage.

The main purpose of the study was to establish whether *Asparagus laricinus* has any toxic or adverse effects on the tissue and organs of animal models. This was done by evaluating hepatotoxicity, nephrotoxicity, spleen and vascular damage to the animals. Dose-response assessment of the effect of the extract was done by analysis of the blood and tissue samples collected at the end of the research. Biochemical and haematological results could not show any patterns in abnormalities although we observed statistically significant results on few parameters. Histologically, no pathological changes were observed. In conclusion, we summarize that the toxicological evaluation of *Asparagus laricinus* extracts may be considered relatively

free of toxicity when given orally, as it did not cause death, damage or inflammation to the tissues, nor produced any remarkable biochemical and haematological adverse effects in both the male and female Sprague Dawley rats.

CHAPTER 1

INTRODUCTION

1.1 Background

Traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not (World Health Organization 2001; 2002). It is used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (World Health Organization 2001; 2002). Traditional systems in general have had to meet the needs of the local communities for many centuries. China and India, for example, have developed very sophisticated systems such as acupuncture and ayurvedic medicine. Traditional medicine is generally available, affordable, and commonly used in large parts of Africa, Asia, and Latin America.

Indigenous plant resources are often the source of medicine, nutrients and vitamins, or subsistence income available to the poorest in society (Shackleton *et al*., 1995). It is estimated that more than 80% of the world's population utilize plants as their primary source of medicinal agents (Cordell, 1995). Moreover, almost 65% of the world's population rely on medicinal plants for their primary health needs (Shanley & Luz, 2003; Calixto, 2005 ; Heibatullah *et al*., 2012). Estimates by the World Health Organisation (2008) state that about 80% of Africa's population rely on medicinal plants to totally or partially meet their health care needs (Addae-Mensah, 1992).

Sixty percent of South Africans subscribe to traditional medicine (van Wyk *et al.*, 1997) and there are over 300 000 traditional healers (Flint & Payne, 2013) which support 'a multi-million dollar hidden economy' (Cunningham, 1982; Mander & Le Breton, 2005) extending into neighbouring countries (Marshall, 1998). Traditional medicine is an integral part of the South African cultural life, a position that is unlikely to change to

any significant degree in years to come (Brandt *et al.*, 1995). In Southern Africa, the use and trade of traditional medicine is not restricted to rural areas (Williams *et al*., 1997). In Soweto, near Johannesburg, approximately 85% of the population consult traditional healers regularly (Gelfand, 1993). In the KwaZulu-Natal province (South Africa), approximately 80% of the population seeks medical advice from traditional healers, in preference or in addition to modern medicine.

Traditional medicine has been a fertile source for revealing novel lead molecules, which are then subjected to investigation using the techniques of the modern drug discovery (Balunus & Kinghorn, 2005). Reports indicate that medicinal plants possess bioactive compounds, which are responsible for many therapeutic effects (Singh *et al*, 2000). There are a number of conventional drugs that originate from plants, such as aspirin (from willow bark), digoxin (from foxglove), quinine (from cinchona bark), and morphine (from the opium poppy).

In fact, almost 122 drugs were discovered from 94 plant species through ethnobotanical leads (Fabricant & Farnsworth, 2001). Interestingly, of the 877 novel medicines that were developed in the period 1981-2002, 6% were natural products, 27% were derivatives of natural products and 16% were synthetics developed on the model of a natural product (Newman *et al*., 2003). This demonstrates that nature is an important source for developing novel leads for medicines (Cragg & Newmann, 2005). Even when new chemical structures are not found during drug discovery from medicinal plants, known compounds with new biological activity can provide important drug leads.

Modern isolation and screening technologies have enhanced the search for new lead molecules and increased interest in folk medicinal plant extracts by drug companies (Balunus & Kinghorn, 2005). Simultaneously, the enthusiasm for medicinal herbs and natural products have increased among the general public. There may be some safety concerns with traditional remedies, although they have been utilized in practice for hundreds of years.

Frequently used plants in traditional medicine are assumed safe, due to their long-term use (Elgorashi *et al*., 2002), and are considered to have no side effects because

they are ‘natural’ (Popat *et al*., 2001). This safety is based on their long-term usage in the treatment of diseases according to knowledge accumulated over centuries. This concept is largely circumstantial and it is important to determine the toxicology of plant extracts, especially those that are used frequently over long periods. Contrary to perceptions and general beliefs regarding the safety of medicinal plants, recent scientific research has shown that many plants used as food, or in traditional medicine, are potentially toxic, mutagenic and carcinogenic (Schimmer *et al*., 1988; Higashimoto *et al*., 1993; Schimmer *et al*., 1994; Kassie *et al*., 1996; De Sã Ferrira & Ferrão Vargas, 1999; Elgorashi *et al*., 2003; Marques *et al*., 2003).

The fact that medicinal plants may be toxic, mutagenic and carcinogenic is a clear warning that the consumption of medicinal plants without studies of efficacy and safety might be detrimental to our health. The danger is that continuous consumption may result in several side effects and complications that may affect different tissues and organs. Although consumption of such medicines may damage various tissues, the liver and kidneys are prime targets simply because they are involved in the degradation and excretion of a myriad of chemical compounds.

Mapanga & Masubayane (2010) have demonstrated the association between the use of medicinal plants for different disorders (including diabetes mellitus) with renal damage. There is still a lack of detailed documentation on the use of medicinal plants in South Africa. This is becoming an urgent issue, based on the fragility of traditional knowledge transmitted orally and the rapid pace of urbanisation and acculturation in this country (van Wyk *et al*., 1997). The informal (oral) traditional medical systems of the Khoi-San, the Nguni and the Sotho-speaking peoples of South Africa have not yet been systemized (van Wyk *et al.*, 1997).

Formal and informal (oral-tradition) systems of medicine exist together in South Africa, the first dating back only 300 years with the influx of European settlers and the latter possibly to Paleolithic times (van Wyk *et al.*, 1997).

In South Africa, and Africa as a whole, the evaluation and recognition of traditional medicine aims to improve its efficacy, safety, availability and wider application at low cost (Fennel *et al*., 2004). Despite the range of medicinal plants used and the rich

biodiversity of South Africa, only a relatively small number of plant species have been scientifically validated (Springfield *et al.,* 2005).

Despite deriving from foundations in the past, traditional medicine is not a static system, but is dynamic and adaptive. Although it reflects the value and perceptions of the people, it is under pressure from the introduction of the Western culture. The introduction of modern medicines and practices (including standardization of doses) as well as other socio-economic development processes (such as medical records and access to medical aid schemes) can improve the application of traditional healing.

Although Le Grand & Wondergem (1989) proposed that many users could benefit from the dissemination of existing knowledge, this has become a controversial subject. Intellectual property rights and the exploitation of developing countries' natural resources have become the focus of ethnobotany in the past few years.

Only a few clinical studies have been performed to verify the potency of traditional remedies against common diseases (Springfield *et al*., 2005). However, plant-derived natural products provide an interesting source for isolating and screening potent molecules to combat inflammatory diseases (Shale *et al*., 1999; Vigorita *et al*., 2001; Ojewole *et al*., 2007), hypertension (Kavitha *et al*., 2006) and cancer (Kucuk Guzel *et al*., 2006; Angelo & Edzard, 2009; Mashele & Kolesnikova, 2010). Several promising molecules have been identified recently, but there are still hurdles to overcome before they can become accepted as modern drugs.

There have been many validations of traditional remedies through scientific research, and the use of ethnomedical information has also contributed to health care worldwide through the isolation of bioactive compounds for direct use in medicine. The adverse effects of widely used plants are not well-documented in the literature. Based on their long-term use by man, one might expect the plants used in traditional medicine to have a low toxicity (Heibatullah *et al*., 2012).
However, recent investigations have indicated that many plants used as food or in traditional medicine have mutagenic effects in *in vitro* assay, which raises concerns about the potential mutagenic hazards resulting from the long-term use of medicinal plants (McGaw *et al*., 2000; Fabricant & Farnsworth, 2001).

1.2 Problem statement

Although treatments are targeted at tissues and organs in order to reduce morbidity and prevent mortality in the population, very often they lead to complications and adverse effects, especially when used over long periods. Almost all modern treatments have some side-effects which might be detrimental to the health of patients. These include, amongst others, chemotherapy, radiation therapy and surgery for the treatment of cancer.

In this study we were looking at the possibility of utilizing medicinal plants for novel anticancer drug development, specifically looking at the safety and toxicology of *Asparagus laricinus.* Previous *in vitro* studies on *Asparagus laricinus* extracts have demonstrated anticancer activity against three human cell lines, namely, breast MCF7, renal TK10 and melanoma UACC62 (Fouche *et al*., 2006; Mashele & Kolesnikova, 2010). These necessitate further studies on *Asparagus laricinus* extracts, such as toxicity, adverse effects investigations as well as *in vivo* biological studies using animal models.

Asparagus laricinus is monogeneric family, which was previously included within the Liliaceae family (Brummitt, 1992). It belongs to the family of Asparagaceae, a monocot and a member of the order Asparagales, that possess great diversity throughout Africa and especially in South Africa. *Asparagus* (Asparagaceae) is highly diverse with hermaphroditic and unisexual taxa, a variety of growth forms (herbs, shrubs, and vines), and vegetative morphology (phylloclade morphology, presence or absence of spines) (Dahlgren *et al*.,1985).

1.3 Justification of the study

Although *Asparagus laricinus* extracts have demonstrated anticancer activity *in vitro*; *in vivo* toxicity studies were never performed. Furthermore, antimutagenic activity in animal models also needs to be investigated. This study will pave a way to the next level which will be *in vivo* analysis of the extracts on animal models, which may probably lead to preclinical research; as well as phases I, II, and III of clinical trials in future.

1.4 Research aims and objectives

1.4.1 Aim of the Study

The aim of the present work was to evaluate the possible toxic effects of the dried roots of *Asparagus laricinus* extracts using Sprague-Dawley rats as animal models.

1.4.2 The objectives of this project

To investigate the toxicity of *Asparagus Laricinus* extracts by:

1. Evaluating variations in serum biochemical tests after exposing the rats to different concentrations of extracts in different solvents.
2. Investigating variations in haematological parameters after exposing the rats to different concentrations in different solvents.
3. Assessing histopathological variations of the liver, kidney and spleen tissues of rats exposed to different concentrations in different solvents.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of cancer

One of the most prominent diseases in humans today is cancer. Cancer is a generic term used for a large group of diseases that can affect any part or organs of the body. It is also referred to as malignant tumours or neoplasms. It is a group of diseases characterized by uncontrolled cell growth beyond their usual boundaries; which can invade adjoining parts of the body and spread to other organs. The process of spreading to other organs is referred to as metastasis and is the major cause of death from cancer (Murthy *et al.,* 1990).

Cancer arises from a single cell. The transformation from normal to a tumour is a multistage process, typically from pre-cancerous to malignant tumours. The changes are the results of the interaction between an individual's genetic makeup and three categories of external or environmental factors, viz.:

- biological carcinogens, such as viral, bacterial or parasitic infections,
- chemical carcinogens, e.g. asbestos, aflatoxin, components of smoke or arsenic; and
- physical carcinogens such as ultraviolet and ionizing radiation.

Generally, cancer begins after a mutational episode in a single cell and then it progressively transforms to malignancy in multiple stages through sequential acquisition of additional mutations (Khan & Pelengaris, 2006). In view of the fact that these initial events are the underlying causes of the whole progression of carcinogenesis, their inhibition would therefore be an efficient preventive measure (Bertram, 2001).

2.2 Phases in development of cancer

Cancer develops in several phases, depending on the type of tissue affected. Typically, these phases are: dysplasia, cancer *in situ*, localized invasive cancer, regional lymph node involvement, and distant metastases (WHO 2002). The first indication of abnormality is a change in the character of cells, known as dysplasia. The term "carcinoma *in situ*" is used when microscopic examination discloses cells with certain characteristics of cancer, that is, changes in the cell nuclei, but with no penetration of the underlying membrane that holds them in the tissue of origin. When the abnormal cell growth reaches areas underlying the tissue of origin, the cancer is regarded as invasive. With further growth, there is increasing invasion and destruction of adjacent tissues. Often, the cancer extends to the regional lymph nodes that drain the area. Cancer cells may also spread through the blood or lymphatic system to affect other organs (distant metastases).

With sufficient multiplication of abnormal cells, the cancer becomes apparent to the individual or to the physician. It commonly takes the form of a lump that may be seen or palpable in the organ involved, for example skin, breast, or prostate. Sometimes, even before detection, the cancer will have spread to lymph nodes or, if rapidly progressive, will have already caused detectable distant metastases. Figure 2.1 summarizes processes or stages in cancer development. The growth of the cancer can involve blood vessels and cause bleeding, which will be apparent if the cancer reaches part of an organ that is in direct or indirect contact with the exterior. For example, there may be blood in the sputum from lung cancer, blood in the stools from bowel cancer, or blood in the urine from bladder cancer. The growth of a cancer may also cause functional disturbances. For example, cancer of the brain may give rise to neurological symptoms and signs.

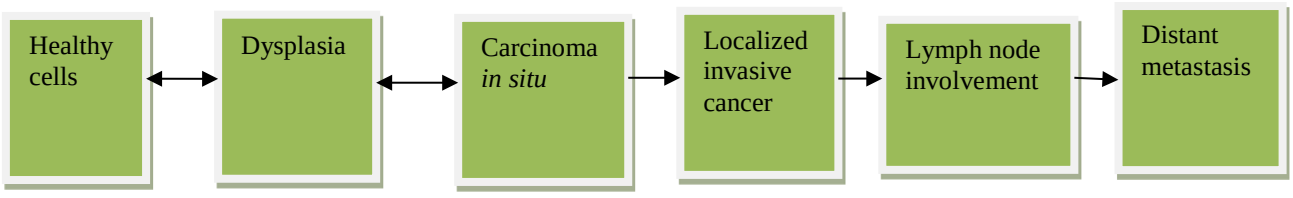

Figure 2.1 Typical phases or stages of cancer development.

2.3 Burden of cancer

According to the WHO, cancer is the leading cause of death worldwide and accounted for 8.2 million deaths (around 14% of all deaths) in 2012 (Globocan, 2012). The major causes of death are cancer of the lung, liver, stomach, breast and colorectal cancer. The distribution in terms of causes of mortality differs between males and females and also varies between developing and developed countries. Table 2.1 indicates the global and regional patterns of death by cause, as captured by the World Health Organization (2001).

Cancer remains a major obstacle to the overall public health in the more developed regions such as Europe and North America, as indicated in Table 2.1. More than 60% of the world's total new annual cases occur in Africa, Asia and Central and South America. These regions account for 70% of the world's cancer deaths (Globocan 2012). Although cancer is not a major problem for Africa, medicinal plants found throughout Africa may be a solution to developed countries in terms of providing plants (as herbs) or in identifying novel chemotherapeutic agents in plants.

Table 2.1 Global and regional patterns of annual deaths, by cause, 2000

	Deaths from all causes(thousands)	Deaths from infectious and parasitic diseases (%)	Deaths from cancer (%)	Deaths from circulatory diseases (%)	Perinatal deaths (%)	Deaths from injury (%)	Deaths from other causes (%)
World total	55694	25.9	12.6	30	4.4	9.1	18
More developed countries	13594	6.1	21.6	47.9	0.7	7.9	15.9
Less developed countries	42100	32.3	9.8	24.2	5.6	9.5	18.7
Africa	10572	61.7	5.1	9.2	5.5	7.1	11.5
South and Central America	3097	14.6	14.1	28.5	4.3	12.3	26.2
North America	2778	6.3	23.8	41.1	0.6	6.4	21.9
Middle East	4036	32.1	6.1	26.9	7.5	8.4	19.1
South East Asia	14157	29.9	8.1	28.9	7.1	9.7	16.4
Western Pacific	11390	10.6	18.6	31.2	2.8	10.7	26.1
Europe	9664	5.4	19.8	51.5	0.8	8.5	14.1

Source: The World Health Report 2001. WHO, Geneva

The following tables indicate the top 20 cancer deaths by cause for the population of South Africa for the year 2000. Table 2.2 clearly demonstrates that tracheal, bronchial and lung cancers are the major causes of death (16.5%) in the overall population, followed by oesophageal (13.3%) and cervical (8.3%) cancers respectively.

Table 2.2 Number of deaths due to different cancers in the year 2000 for South Africa

All persons		
Rank	**Cause of Death**	**Deaths**
1	Tracheal, Broncheal and Lung cancer	6885
2	Oesophageal cancer	5579
3	Cervical cancer	3498
4	Breast cancer	3206
5	Liver cancer	2651
6	Colorectal cancer	2567
7	Prostate cancer	2524
8	Stomach cancer	2348
9	Pancreatic cancer	1541
10	Leukaemia	1465
11	Mouth and Oropharyngeal cancer	1386
12	Lymphoma	1032
13	Laryngeal cancer	746
14	Bone and Connective tissue cancer	707
15	Ovarian cancer	691
16	Bladder cancer	673
17	Uterine cancer	638
18	Brain cancer	527
19	Melanoma	437
20	Kidney cancer	427
All cancers		**41657**

(Source: Cancer Association of South Africa)

Tables 2.3 and 2.4 categorize the causes of death by gender, male and female respectively. In Table 2.3 the picture remains the same for the number one and two causes of death, i.e. tracheal, bronchial and lung cancers; and oesophageal cancers which represent 21.9% and 16.7% of the male population respectively. Prostate cancer moves from the seventh position to third in the male population.

Table 2.3 Number of deaths due to different cancers in the year 2000 for males

Males		
Rank	Cause of Death	Deaths
1	Tracheal, Broncheal and Lung cancer	4669
2	Oesophageal cancer	3566
3	Prostate cancer	2524
4	Liver cancer	1666
5	Stomach cancer	1386
6	Colorectal cancer	1157
7	Mouth and Oropharyngeal cancer	985
8	Leukaemia	818
9	Pancreatic cancer	789
10	Laryngeal cancer	633
11	Lymphoma	601
12	Bladder cancer	469
13	Bone and Connective tissue cancer	360
14	Brain cancer	274
15	Kidney cancer	233
16	Melanoma	233
17	Non-Melanoma skin cancers	158
18	Breast cancer	50
All cancers		**21361**

(Source: Cancer Association of South Africa)

In Table 2.4, breast cancer leaps from the fourth to the first position in the female category. This represents 17.2% of the female population, followed by breast cancer (15.5%) and tracheal, bronchial and lung cancers (10.9%) in the third position. As mentioned earlier, Mashele & Kolesnikova (2010) have demonstrated *in vitro* antimutagenicity of *Asparagus laricinus* extracts against breast cancer (MCF7), which is the second major cause of death within the female population.

Table 2.4 Number of deaths due to different cancers in the year 2000 for females

Females		
Rank		Deaths
1	Cervical cancer	3498
2	Breast cancer	3156
3	Tracheal, Broncheal and Lung cancer	2216
4	Oesophageal cancer	2013
5	Colorectal cancer	1410
6	Liver cancer	986
7	Stomach cancer	962
8	Pancreatic cancer	752
9	Ovarian cancer	707
10	Leukaemia	647
11	Uterine cancer	638
12	Lymphoma	431
13	Mouth and Oropharyngeal cancer	401
14	Bone and Connective tissue cancer	331
15	Brain cancer	253
16	Bladder cancer	204
17	Melanoma	203
18	Kidney cancer	176
19	Laryngeal cancer	114
20	Non-Melanoma skin cancers	108
All cancers		**20296**

(Source: Cancer Association of South Africa)

2.4 Global cancer projections and strategies to curb escalating numbers.

It was projected that the annual cancer burden would rise from 14 million in 2012, to 22 million within the next two decades. The World Health Organization 2005 also projected an escalation in deaths from cancer, with an estimated 9 million deaths in the year 2015, and 11.4 million deaths by 2030.

Primary prevention measures, early detection mechanisms and cure are needed for Africa's cancer burden (715,000 new cases and 542, 000 deaths in 2008), a burden projected to double by 2030 due to demographic changes alone. Figure 2.2 illustrates the escalation in deaths caused by cancer in both developed and developing countries throughout the globe, as projected by the World Health Organization (Globocan, 2008).

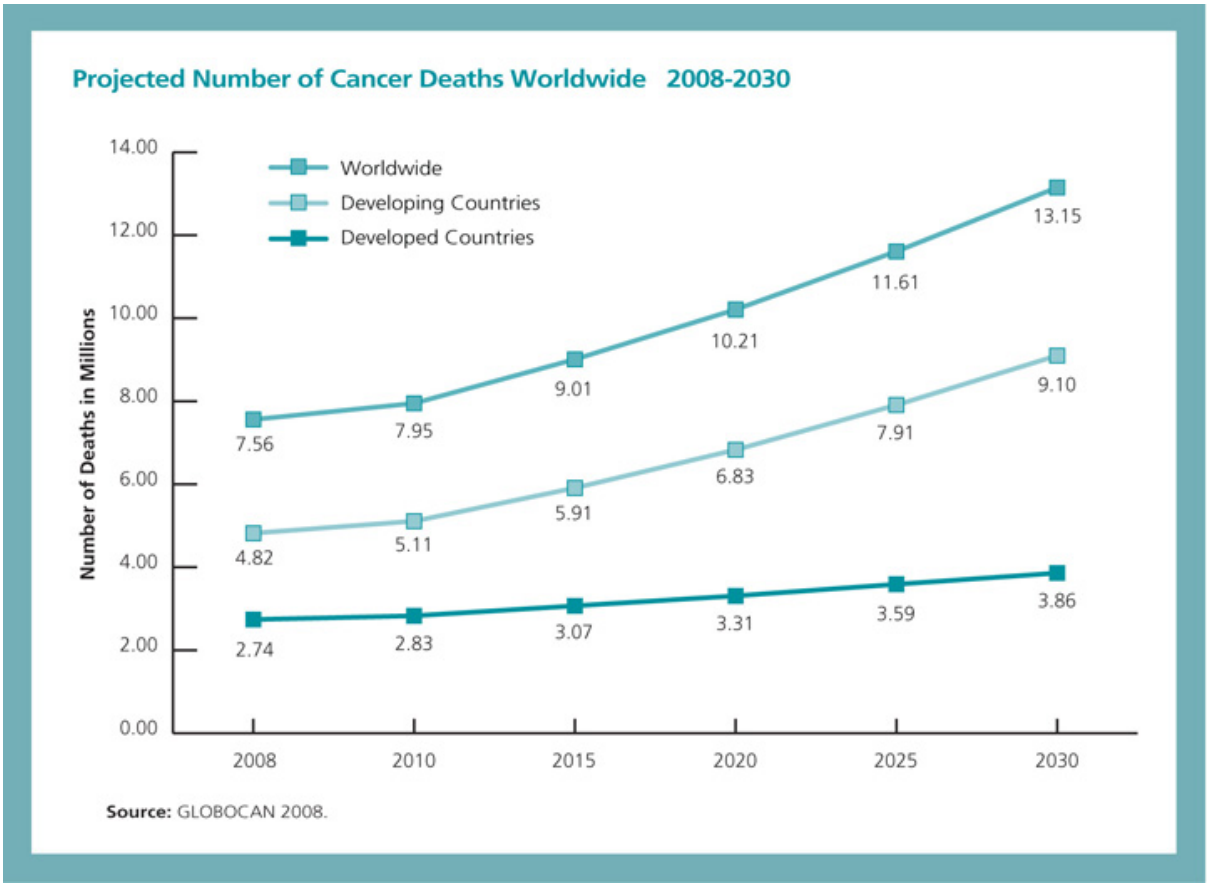

Figure 2.2 Global cancer death projections (2008 - 2030)

Currently, there is marked scientific and commercial interest in the continuing discovery of new anti-cancer agents from natural product sources (Kinghorn *et al.*,

2003). More than 50% of drugs used in clinical trials for anticancer activity, were isolated from natural sources or are related to them (Newman & Cragg, 2007). Hence, the search for natural products to be used in cancer therapy represents an area of great interest in which the plant kingdom is the most important source, providing many anti-tumour agents with novel structures and unique mechanisms of action (Chang *et al.*, 1999).

Present chemotherapy cancer treatments have proved to be ineffective as a result of their toxicity and cells developing resistant (McWhirter *et al*.,1996). Many conventional drugs also induce genetic damage that itself can be carcinogenic (Mashele & Fuku, 2011). A segment of the research community is thus focusing on identifying novel chemotherapeutic agents in plants that do not induce the destructive effects of conventional cytotoxic therapeutic agents. Table 2.5 below illustrates examples of side effects of some conventional drugs:

Table 2.5 Excerpt of Table of Major Chemotherapy Drugs and Hormones (modified from Mora & Potts, 2003)

Name and Use	Common Side Effects	Occasional Side Effects
Aminoglutethimide (Cytrdren, Elitpen). An aromatase inhibitor used in adrenal and prostate cancers. May be used as medical adrenalectomy in breast cancer. Given as a tablet.	Skin rash with fever, sluggishness and tiredness (usually goes away slowly within 4 to 6 weeks after treatment is finished).	Dizziness, swelling of face, weight gain, leg cramps, fever, chills and sore throat, loss of appetite, mild nausea and vomiting, leg cramps.
Cyclophosphamide(Cytoxan, Neosar, Endoxan) An alkylating agent used in lymphomas and Hodgkin's disease, myeloma, neuroblastoma, retinoblastoma, sarcomas, Wilms' tumor, cancers of the ovary, breast, prostate, head and neck, lung,	Nausea, vomiting, loss of appetite, loss of hair. (The patient) needs to drink extra liquids to prevent bladder problems. If (the patient) misses a dose, they *should not* double the	Blood in urine, pain when urinating, black tarry stools, fever, chills, nasal stuffiness and sore throat, cough and shortness of breath, dizziness, confusion, fast heartbeat, sterility (may be temporary), skin darkening, metallic taste during injection, blurred

Name and Use	Common Side Effects	Occasional Side Effects
bladder, cervix, stomach and uterus. Given IV or as a tablet.	next dose, but should talk with one's doctor.	vision, cataract, second cancers (leukemia, bladder).
Doxorubicin (Adriamycin, Rubex, Adriamycin RDF, PFS or MDV) An antitumor antibiotic used in leukemias, lymphomas, Wilm'stumor, neuroblastoma, multiple myelomas, sarcomas, cancers of the breast, ovary, bladder, thyroid, stomach, cervix, endometrium, liver, esophagus, head and neck, pancreas, prostate, testes and lung. Given IV.	Nausea and vomiting, red urine (usually lasts one or two days after each dose), hair loss, loss of appetite, heart problems.	Mouth sores, darkening of soles, palms or nails, may reactivate skin reactions from past radiation, fever, chills and sore throat, diarrhea, eye problems, fast or irregular heartbeat, shortness of breath, pain in joint, side or stomach, burning pain at injection site.
Fluorouracil (Adrucil, 5-FU, 5-Fluorouracil, Efudex) An antimetabolite used in cancers of the stomach, colon, rectum, breast, pancreas, bladder, cervix, endometrium, esophagus, head and neck, liver, lung, ovary and skin. Usually given IV, except for skin, where a cream is used.	Nausea, mouth sores, diarrhea, skin darkening (sensitive to sun).	Mouth, tongue or lip sores, hair loss, skin rash or dryness, vomiting, poor muscle coordination, swelling of palms and soles, nail loss or brittle nails, eye irritation, increase of tears, blurred vision, headache, euphoria.
Methotrexate (Folex, Folex PFS, Mexate, Mexate-AQ, Abitrexate, Rheumatrex) An antimetabolite used in choriocarcinoma, hydatiform mole, multiple myeloma, leukemia, lymphomas, sarcomas, cancers of the breast, head and neck, lung, bladder, brain, cervix, esophagus, kidney, ovary, prostate, stomach and testes. Given IV	Mild nausea and vomiting, diarrhea, mouth sores. (The patient) should not take more or less than the amount prescribed by the doctor. If a dose is missed, the next dose should *not* be doubled and the physician should be consulted.	Loss of appetite, stomach pain, yellowing of eyes or skin, fever, chills and sore throat, cough, shortness of breath, blood in urine or dark urine, hair thinning, headache, dizziness, blurred vision, drowsiness or confusion, joint pain, skin rash, reddening of skin (sensitive to sun) anemia, flank pain, blurred vision, confusion, seizures.

Name and Use	Common Side Effects	Occasional Side Effects
most commonly, in the muscle, or as a tablet.	(The patient) may need to drink extra liquids to prevent kidney problems. (The patient) should *not* take aspirin or other medicine for swelling or pain without first checking with the physician. When very high doses are given, it is followed by the drug leucovorincalcium to counteract life-threatening side effects (called leucovorin rescue).	
Tamoxifen (Nolvadex, taxomifen citrate) Anantiestrogen used in breast cancer. Given as a tablet.	Hot flashes, vaginal discharge. (The patient) should not take more or less than the amount prescribed by the physician. If (the patient) misses a dose, she should not take the missed dose at all and should not double the next dose; she should consult with her doctor. The patient should use birth control while taking tamoxifen, but she should not take birth control pills since they may change the effects of the tamoxifen. If she should become pregnant while taking tamoxifen, she should consult with her physician immediately.	Vaginal bleeding, dryness or itching, nausea, and vomiting, loss of appetite, irregular menstrual periods, hot flashes, endometriosis, bone and tumor pain, visual changes skin rash and itchiness, dizziness, loss of hair, depression, light-headedness, confusion, fluid retention, headache, anemia, swelling of legs, loss of appetite, blood clots, increased risk of uterine cancer.

Name and Use	Common Side Effects	Occasional Side Effects
Vincristine (Oncovin, Vincasar PFS, leurocristine) A plant alkaloid used in leukemia, lymphomas, sarcomas, neuroblastoma, Wilms' tumor, melanoma, multiple myeloma, cancers of the colon, rectum, brain, breast, cervix, ovary, lung and thyroid. Given IV.	Hair loss, numbness or tingling in hands or feet.	Pain in arms, legs, jaw or stomach, pain in testicles, mouth sores, fever, chills and sore throat, severe constipation, metallic taste, hoarseness, agitation, confusion, light-headedness, dizziness, drooping eyelids, jaw or joint pain, blurred or double vision, anemia, stomach cramps.
Temodal Brain cancer	Blood and bone marrow problems, hair loss, nausea vomiting, diarrhoea	
Vectibix Colorectal cancer	Metabolic problems, blurred vision, back pain, hair loss, coughing, breathing difficulties, difficulty in sleeping	
Cisplatin Ovarian, testicular and bladder cancer	Neurotoxicity, deafness	

2.5 Cancer and medicinal plants

Knowledge about the medicinal value of many plants that form part of the rich biodiversity in South Africa is largely contained in the oral traditions of the various ethnic groups that constitute the indigenous people of South Africa (van der Merwe *et al.*, 2001).

Several medicinal plants are traditionally used in the treatment of a variety of ailments, including cancer in many communities of South Africa and neighbouring countries. Since ancient times, herbal medicine has always been one of the main components

of the healthcare system. Despite the range of medicinal plants used and the rich biodiversity of South Africa, only a relatively small number of plant species have been scientifically validated for safety and efficacy (Springfield *et al*., 2005).

Traditional medicine has been a fertile source for revealing novel lead molecules, which are then subjected to investigation using the techniques of modern drug discovery (Balunus & Kinghorn, 2005). In fact, modern pharmaceutics have benefited from medicinal plants (Table 2.6). Although discovered through serendipitous laboratory observation, three of the major sources of anticancer drugs on the market, or completing clinical trials, were derived from North American plants used medicinally by Native Americans: the Papaw (Asimina spp), the Western Yew Tree (Taxus brevifolia), effective against ovarian cancer and the May-apple (Podophyllum peltatum) used to combat leukaemia, lymphoma, lung and testicular cancer (Gurib-Fakim, 2006).

Table 2.6 Botanical drugs used in traditional medicine, and from which useful modern drugs were produced.

Botanical names	English names	Indigenous use	Origin	Uses in Biomedicine
Adhatodavasica	-	Antispasmodic, antiseptic, insecticide, fish poison	India, Sri Lanka	Antispasmodic, oxytocic, cough suppressant
Catharanthusroseus	Periwinkle	Diabetes, fever	Madagascar	Cancer chemotherapy
Condrodendrontomentosum	-	Arrow poisoning	Brazil, Peru	Muscular relaxation
Gingko biloba	Gingko	Asthma, anthelmintic (fruit)	Eastern China	Dementia, cerebral deficiencies
Harpagophytumprocumbens	Devil's claw	Fever, inflammatory conditions	Southern Africa	Pain, Rheumatism
Piper methysticum	Kava	Ritual stimulant, tonic	Polynesia	Anxiolytic, mild stimulant
Podophyllumpeltatum	May apple	Laxative, skin infections	North America	Cancer chemotherapy, warts
Prunusafricana	African plum	Laxative, 'Old man's disease'	Tropical Africa	Prostate hyperplasia

Source: A. Gurib-Fakim / Molecular aspects of medicine 27 (2006) 1-93

Interestingly, of the 877 novel medicines that were developed in the period 1981-2002, 6% were natural products, 27% were derivatives of natural products and 16% were synthetics developed on the model of a natural product (Newman *et al*., 2003). This demonstrates that nature is an important source for developing novel leads for medicines. Even when new chemical structures are not found during drug discovery from medicinal plants, known compounds with new biological activity can provide important drug leads.

Currently, over 85, or 48.6%, of drugs used in clinical trials for anticancer activity, are actually derived from natural products or are natural products (Newman & Cragg, 2012). This demonstrates the rationale for the search for novel drug molecules in medicinal plants, especially when literature shows that plant-derived compounds have provided attractive possibilities for treatment strategies (Jain & Jain, 2011).

2.6 *Asparagus laricinus*

In this study *Asparagus laricinus,* belonging to Asparagaceae family, was investigated. *Asparagus laricinus* is a monogeneric family, which was previously included within the Liliaceae family (Brummitt, 1992). It belongs to the family of Asparagaceae, a monocot and a member of the order Asparagales, and possesses great diversity throughout Africa, especially in South Africa. This plant is commonly known as Lesitwane among the Batswana clans in South Africa.

Asparagus (Asparagaceae) is highly diverse with hermaphroditic and unisexual taxa, a variety of growth forms (herbs, shrubs, and vines), and vegetative morphology (phylloclade morphology, presence or absence of spines) (Dahlgren *et al*.,1985).
The genus *Asparagus* comprises approximately 100 species and consists of herbs, shrubs and vines.

Asparagus laricinus is part of the traditional medicine used in many parts of South Africa for the treatment of several ailments. Examples of its use include its roots for the treatment of tuberculosis, and its use as a diuretic in the Khoi-San and Cape Dutch ethnobotany (van Wyk, 2008). In the North West province, the roots are used for treatment of sores, redwater, urinary infections, umblical cord inflammation and general ailments by Setswana-speaking people (van Der Merwe *et al*., 2001). The leaves and stem are medicinally used in South West parts of Gauteng (Dzerefos & Witkowski, 2001).

Many of these herbs, including *Asparagus laricinus,* have not yet been scientifically assessed for their efficacy or safety to tissue or organs of recipients *in vivo*. Mashele & Fuku, (2011) showed that *Asparagus laricinus* polyphenol extract exhibited a dose-dependent antimutagenic ability. The plant extract showed no mutagenic effect on all tested *Salmonella typhimurium* bacteria strains *in vitro*. Previous *in vitro* studies on *Asparagus laricinus* extracts have demonstrated anticancer activity against three human cell lines namely, breast MCF7, renal TK10 and melanoma UACC62 (Fouche *et al*., 2006; Mashele & Kolesnikova, 2010).

These cell lines were selected because of their high sensitivity to detect anticancer activity. Thus, investigation of traditionally used medicinal plants is valuable as a source of potential chemotherapeutic agents, and also to assess the safety of the continuous use of medicinal plants. As a result, most studies are being directed at popular medicine, with the aim of identifying natural products which exhibit therapeutic properties (Hamburger & Hostettmann, 1991).

As some of the active ingredients are potentially toxic, there is a need to evaluate the safety of plant preparations. These necessitate further studies on *Asparagus laricinus* extracts, such as toxicity, adverse effects investigations, as well as *in vivo* biological studies using animal models.

2.7 Previous toxicology studies on medicinal plants

Although plant extracts have been used in the treatment of diseases according to knowledge accumulated over centuries, scientific research has shown some substances present in these medicinal plants to be potentially toxic and carcinogenic (De Sã Ferrira & Ferrão,1999). Investigation of traditionally used medicinal plants is thus valuable on two levels: firstly, as a source of potential chemotherapeutic drugs, and secondly, as a measure of safety for the continued use of medicinal plants (Verschaeve *et al.*, 2004). The latter is the area of concern for the researcher, since *Asparagus laricinus* has been used in folk medicine for the treatment of cancer for years.

Literature does not reveal any previous toxicological studies for *Asparagus laricinus* per se, but several studies were performed on other medicinal plants used for the treatment of cancer. Arun & Tangpu (2006) demonstrated that *Lithocarpus dealbata* showed no mortality or any visible signs of acute toxicity, while serum biochemistry tests did not reveal any noticeable changes in aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol (CHOL) and protein levels in animals. Toxicological studies on *Moringa oleifera* have indicated an absence of severe hepatotoxicity and organ damage, except in very high doses. The acute lethality (LD_{50}) test has been found to be relatively safe with subchronic toxicity studies, eliciting no significant difference in sperm quality, haematological and biochemical parameters in

the treated rats, as compared to the controls (Isitua & Ibeh, 2003; Awodele *et al.,* 2012; Ugwu *et al*., 2013). These studies engaged the use of laboratory experimental animal models to evaluate the toxicology of plant extracts.

CHAPTER 3

RESEARCH DESIGN AND METHODOLOGY

3.1 Introduction

Toxicology is the study of how chemical substances interact with living systems and affect normal processes. The information obtained from toxicological studies is utilized to predict safe exposure levels of chemicals. Although *in vitro* analysis (e.g. tissue cultures and organs) of toxicity is preferred, animal models are necessary to validate the results of non-living or *in vitro* tests. The *in vitro* tests are performed as the last protective step before exposure of humans and animals to potentially dangerous substances. The advantage of animal testing is to evaluate the effect when the entire system is involved, which cannot be evaluated by *in vitro tests*; as hormones, enzymes and other systemic influences are not available.

In this study, Sprague Dawley rats were utilized to determine the toxicity of *Asparagus laricinus* extracts. The animals were exposed to different concentrations of ethanol and water extracts of *Asparagus laricinus* over a maximum period of eight weeks. The rats were sacrificed at the end of the experiment, blood collected and tissues excised for biochemical, haematological and histological analysis.

3.2 Study design

A case control study that involved experimental animals, where cases were exposed to different concentrations of *Asparagus laricinus* extracts utilising water, dichloromethane and ethanol as solvents. Controls were not exposed to the extract but supplied with water, which served as placebo.

3.3 Study population

Sprague Dawley rats of either sex were reared at the Animal Research Unit of the University of the Free State, Bloemfontein, South Africa. The rats were three months

old; weighed between 180g and 250g at the beginning of the experiment; and were fed with standard pelleted food.

3.4 Sample size

A total of 78 Sprague Dawley rats, both male and female, obtained from the Animal Research Unit at the University of the Free State in 2013, were divided into 3 major groups which were exposed to water, ethanol and dichloromethane extracts of *Asparagus laricinus.* The three groups consisted of 24 rats for the water extract, 30 rats for ethanol extract and 24 rats for dichloromethane extract. A total of 12 rats were used as controls and they were included in the numbers supplied for the water and ethanol extracts. The six used for the water extract were utilised for the entire 8-week period, while the other six controls were used for two weeks of exposure to ethanol and dichloromethane extracts.

The dichloromethane extract was discontinued when two rats died due to adverse effect after administration of the extract. Due to the adverse effects mentioned, the dichloromethane group was discontinued and this resulted in only 54 rats being used for the entire experiment.

3.5 Inclusion and exclusion criteria

Rats weighing between 150g and 250g were included in the study to ensure homogeneity amongst the study population.
Rats weighing above or below the above-mentioned range were excluded.

3.6 Ethical consideration

Written approval for the final version of the protocol was obtained from the Interfaculty Animal Ethics Committee (Ethics number 16/2012 – Appendix A) of the Faculty of Health Sciences at the University of the Free State, before the extracts could be administered to the rats. The study was conducted at the animal facility by the researcher, under the guidance of personnel who are skilled and trained in the handling of experimental animals, and the ethical guidelines were followed at all times.

At the end of the study the rats were administered with Halothane (anaesthetic) and blood samples collected. They were finally sacrificed by administration of higher doses of halothane.

3.7 Materials

3.7.1 Plant authentication (validation)

The plant was authenticated by scientists at the National Botanical Gardens in Bloemfontein, Free State, South Africa. Voucher specimen number MASH 200 was allocated to the plant.

3.7.2 Consumables and instruments utilized in the study

The apparatus used in the study were serviced according to schedule and properly maintained. Instruments for biochemical and haematological analysis were calibrated accordingly, and the quality control results were within reference ranges. Table 3.1 indicates the instruments used during the procedures, the supplier and test(s) performed.

Table 3.1 Instruments / equipment used during the procedures.

Instrument	Supplier	Tests performed
Dimension®Xpand®Plus	Siemens	Aspartate aminotransferase (AST)
		Alanine aminotransferase (ALT)
		Alkaline Phosphatase (ALP)
		Blood Urea Nitrogen (BUN)
		Creatinine
		Cholesterol
ABX Pentra 60	The Scientific Group	Full Blood Count (FBC)
H2500 Microprocessor	Energy Beam Sciences Inc.	Tissue processing
Shandon Histocentre	Optolabor (Pty)(Ltd)	Tissue embedding
Leica Waterbath	Leica Biosystems Nussloch GmbH	Tissue floating
Leica Microtome	Leica Biosystems Nussloch GmbH	Tissue slicing
Oven	Labcon	Dewaxing
Zeiss Microscope.	Zeiss South Africa	Histological investigations
Macsalab Mill	Eriez South Africa	Pulverizing of roots material
VIRTIS 5L Freeze Dryer	SP Scientific	Lyophilisation

Tables 3.2, 3.3, 3.4 display reagents consumed for haematological, biochemical and histological investigations respectively.

Table 3.2 Consumables for haematologic analytes.

Controls/ reagents	Catalogue No.	Supplier
ABX control L	2062207	The Scientific Group
ABX control H	2062208	The Scientific Group
ABX control N	2062203	The Scientific Group
ABX Diluent	0901020	The Scientific Group
ABX Eosinofix	0206010	The Scientific Group
ABX Basolyse	0906003	The Scientific Group
ABX Alphalyse	0906013	The Scientific Group
ABX Cleaner	0903010	The Scientific Group

Table 3.3 Consumables for biochemical analytes.

Reagent/consumable	Catalogue No. (Reagent)	Catalogue No. (Calibrator)	Supplier
ALP	DF15A	DC19	Siemens
ALT	DF143	DC143	Siemens
AST	DF41A	DC19	Siemens
BUN	DF21	DC18D	Siemens
CHOL	DF27	DC16	Siemens
CREATININE	DF33A	DC18D	Siemens
ABS (CONTROL)	DF79	-	Siemens

Table 3.4 Consumables for histological investigations.

Reagent/consumable	Catalogue No.	Supplier
Ethanol	1044735	UniVar
Isopropanol	5075040LC	Saarchem
Paraffin wax	CLSC4902000EM	Lasec
Xylene	7220220LC	Saarchem
Haematoxylin	3403747	BDH
Eosin	34197	BDH
Entellan	1.07961.0500	Merck
Coverslips	C9802	Sigma Aldrich
Slides	S8902	Sigma Aldrich

3.8 Methods

3.8.1 Plant Extraction Methods

The plant material (*A. laricinus*) was authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa. The collected root materials were dried at room temperature, pulverised by a Macsalab Mill (Model 200, LAB) and weighed. The powder was then stored at room temperature until analysis. Plant material (10 grams of the dried roots) was soaked in a volume of 500 ml of ethanol, dichloromethane or purified water for 72 hours under shaking conditions (120 rpms). The supernatant was filtered passively through a Whatman® filter paper, 11 cm in diameter. The solvent (ethanol) was removed completely under vacuum, by using a speed evaporator (Univapo 100H) at 50°C.The aqueous sample was lyophilised for 72 hours in the VIRTIS 5 L freeze dryer (VIRTIS New York, USA) to obtain a dried

powdered plant extract. The dried samples were then reconstituted in either water or ethanol.

3.8.2 Plant extracts administration method

Seventy eight Sprague- Dawley rats were divided into three groups; 24 for the water extract, 30 for ethanol and 24 for dichloromethane extracts.

Group 1: Water extract

It consisted of 24 rats which were further subgrouped into 4 groups of 6 rats per subgroup. Although we had six rats per group, they were further grouped into two rats per cage (see Figure 3.1) as per rules and regulations governing the use of laboratory animals. Water extracts were diluted to prepare 2%, 10% and 20% concentrations. The different concentrations were administered to three of the four groups *ad libitum*. The last group served as controls, where tap water was administered instead of the extract. The extracts were administered over a period of eight weeks, using a 200ml feeding bottle per cage. Bottles were cleaned and replaced with a fresh extract after every two days.

Figure 3.1 Setup for different water extracts and control group (each column represents a group and each cage contains two rats).

Group 2: Ethanol extract

It consisted of 30 rats which were further subgrouped into 5 groups of 6 rats per subgroup. Although we had six rats per group, they were further grouped into two rats per cage as per rules and regulations governing the use of laboratory animals. Ethanol extracts were administered once per day for two weeks. The extracts were administered through gavage by varying the volumes, resulting in the amounts as indicated in the table below:

Table 3.5 Animal groups and doses of ethanol extracts per day

Extract dose(mg/kg/day)	50	100	200	400	Controls
Number of rats per group	**6**	**6**	**6**	**6**	**6**

Although the controls had water *ad libitum*, water was administered by gavage every time the exposed groups were administered with the extracts.

Group 3: Dichloromethane extract

According to the proposal, different amounts as per table below were supposed to be administered intramuscularly. Due to the nature of pain and ulcerations resulting from repetitive intramuscular injections on the tails of rats over a two-week period, scientists at the Animal Research Unit recommended a switch over to gavage feeding. The process was immediately stopped due to the death of two rats within a few minutes after administration of the extract through gavage. Administration of dichloromethane was discontinued and this group was completely excluded from the research project.

Table 3.6 Animal groups and doses of dichloromethane extracts per day

Amount of extract per day	50mg/kg	100mg/kg	200mg/kg	400mg/kg
Number of rats per group	6	6	6	6

3.8.3 Sample collection

The rats were placed in a desiccator with Halothane (Safeline Pharmaceuticals) soaked in cotton wool for anaesthetic purposes until they were completely unconscious. Blood samples were collected in EDTA anticoagulated tubes

(haematological analysis) and non-anticoagulated tubes (clotted blood for biochemical analysis) through insertion of the needle into the heart. The rats were administered with further halothane for euthanization. The blood collection process was done by scientist employed by the Animal Research Unit at the University of the Free State. Liver, spleen and kidneys were excised (by the researcher) and immediately preserved with 10% Neutral Buffered Formalin for histological investigations.

3.8.4 Laboratory investigations

Substances ingested by animals including food, medication and fluids (extracts in our case) are absorbed in the gastrointestinal tract after digestion. Absorbed substances are transported to the liver via the portal vein for detoxification and further processing and distribution throughout the body tissues and organs via the vascular system (blood). Blood passes through the kidneys where it is filtered in the nephrons, and where unwanted and waste products are excreted in urine.

The presence of toxic substances in ingested material may cause damage to tissues and organs such as the liver, kidneys and blood cells in circulation. The extent of the damage may also be associated with the period of exposure and the amount/ concentration of substances in circulation.

Selected biochemical tests include enzymes such as aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (ALP) which assesses the extent of the damage to hepatic tissues. Blood urea nitrogen and creatinine are specifically selected for the assessment of kidney function, and would therefore reflect damage to the nephrons and other tissues of the kidney. Blood urea nitrogen (BUN) and creatinine clearance are well established biomarkers of renal function that can be measured cheaply and easily using an enzyme/oxidation reaction assay and high performance liquid chromatography (HPLC), respectively (Mouton & Holder, 2006). Lastly, cholesterol is necessary for the assessment of damage to the heart and vascular system.

Haematological tests would reflect damage to the blood cells (red blood cells, white blood cells and platelets) because these cells would be exposed to toxic substances

during transportation in the vascular system. Apart from the above-mentioned analytes, histological investigation of the organs/ tissues excised will assess the damage caused by such substances, as well as the extent of the damage.

Blood samples for biochemical analysis were allowed to clot, followed by centrifugation at 3000rpm for 10 minutes. Serum was collected in 2ml tubes and analysed within five hours, and some aliquots stored in a refrigerator set at 4°C.The samples were kept for one week in order to allow repeat analysis in case of unreliable, doubtful or inaccurate results.

Haematology samples were analyzed within five hours using the ABX Pentra 60. All three levels of quality control samples were run in conjunction with the rats' blood for every batch of samples.

3.8.4.1 Biochemical parameters

Quantitative analysis of Aspartate aminotransferase (AST); Alanine aminotransferase (ALT); Alkaline phosphatase (ALP); Cholesterol (CHOL); blood urea nitrogen (BUN) and Creatinine were performed using Dimension XpandPlus™ auto analyzer (supplied by Siemens).

3.8.4.1.1 Principle for the determination of Aspartate aminotransferase

Aspartate aminotransferase (AST) catalyzes the transamination from L-aspartate to α-ketogluterate, forming L-glutamate and oxaloacetate. The oxaloacetate formed is reduced to malate by malate dehydrogenase (MDH), with simultaneous oxidation of reduced nicotinamide adenine dinucleotide (NADH). The change in absorbance with time due to the conversion of NADH to NAD is directly proportional to the AST activity and is measured using a bichromatic (340nm, 700nm) rate technique (Bergmeyer *et al.,* 1978).

3.8.4.1.2 Principle for the determination of Alanine aminotransferase

Alanine aminotransferase catalyzes the transamination of L-alanine to α-ketogluterate, forming L-glutamate and pyruvate. The pyruvate formed is reduced to lactate by

lactate dehydrogenase (LDH) with simultaneous oxidation of reduced NADH. The change in absorbance is directly proportional to the alanine aminotransferase activity and is measured using a bichromatic (340nm, 700nm) rate technique (Bergmeyer *et al.,* 1978).

3.8.4.1.3 Principle for the determination of Alkaline phosphatase

Alkaline phosphatase catalyzes the transphosphorylation of p-nitrophenylphosphate (p-NPP) in the presence of the transphosphorylating buffer, 2-amino-2-methyl-1-propanol (AMP). The reaction is enhanced through the use of magnesium and zinc ions. The change in absorbance at 405 nm due to the formation of p-nitrophenol (p-NP) is directly proportional to the ALP activity, since the reactants are present in non-rate-limiting quantities and is measured using a bichromatic (405 nm, 510 nm) rate technique (Bowers & McComb,1966).

3.8.4.1.4 Principle for the determination of Cholesterol

Cholesterol esterase (CE) catalyzes the hydrolysis of cholesterol esters to produce free cholesterol, which, along with pre-existing free cholesterol, is oxidized in a reaction catalyzed by cholesterol oxidase (CO) to form cholest-4-ene-3-one and hydrogen peroxide. In the presence of horseradish peroxidase (HPO), the hydrogen peroxide thus formed is used to oxidize N,Ndiethylaniline-HCl/ 4-aminoantipyrine (DEA-HCl/AAP) to produce a chromophore that absorbs at 540 nm. The absorbance due to oxidized DEA-HCl/AAP is directly proportional to the total cholesterol concentration, and is measured using a polychromatic (452, 540, 700 nm) endpoint technique (Rautela & Liedtke, 1978).

3.8.4.1.5 Principle for the determination of BUN (Blood Urea Nitrogen)

Urease specifically hydrolyzes urea to form ammonia and carbon dioxide. The ammonia is used by the enzyme glutamate dehydrogenase (GLDH) to reductively aminate α-ketoglutarate, with simultaneous oxidation of reduced NADH. The change in absorbance at 340 nm due to the disappearance of NADH is directly proportional to the BUN concentration in the sample and is measured using a bichromatic (340 nm, 383 nm) rate technique (Burtis & Ashwood, 2001).

3.8.4.1.6 Principle for the determination of Creatinine

In the presence of a strong base such as NaOH, picrate reacts with creatinine to form a red chromophore. The rate of increasing absorbance at 510 nm, due to the formation of this chromophore is directly proportional to the creatinine concentration in the sample and is measured using a bichromatic (510, 600 nm) rate technique. Bilirubin is oxidized by potassium ferricyanide to prevent interference (Knapp & Mayne, 1987).

3.8.4.2 Haematological parameters

An ABX Pentra 60 analyzer was used to determine the haematological parameters. Haematological parameters determined in this study included Red Blood Cell count (RBC), Haemoglobin (Hb), Haematocrit (Hct), Mean Cell Volume (MCV), Mean Cell Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), White Blood Cell count (WBC), differential count and platelet count (Plt).

The analyzer uses the multi distribution sampling system (MDSS) where one cycle is distributed into 3 blood samples for RBC/Plt, BASO/WBC and LMNE matrix which are channelled to the three respective chambers with reagents. The different full blood count parameters where analysed using current impedance changes; spectrophotometry; double hydrodynamic sleeving coupled with cytochemistry; and measuring of transmitted light; to measure the different parameters of the full blood count (Nakamine , 2004:2-13).

3.8.4.2.1 Principle for the detection of RBC and Platelets

The analyzer uses the measurement of impedance variation generated by the passage of cells through a calibrated micro aperture. The specimen is diluted in electrolytic diluents (good conductor of current) and pulled through the calibrated micro-aperture. Two electrodes are placed on either side of the aperture. Electric current passes through the electrodes continuously. When the cell passes through the aperture, electric resistance between the two electrodes increases proportionately with the cell volume.

3.8.4.2.2 Principle for the detection of Haemoglobin

Red blood cells are lysed and the haemoglobin released combines with potassium cyanide to form a chromogenous cyanmethaemoglobin compound. The compound is measured through the optical part of the first dilution chamber using a spectrophotometric technique at a wavelength of 550nm. The absorbance obtained is multiplied by the coefficient of calibration in order to get the actual concentration in g/100ml.

3.8.4.2.3 Principle for the detection of Haematocrit

The height of the impulse generated by the passage of a cell through the micro-aperture is directly proportional to the volume of the analyzed RBC. This data is utilised for calculation of MCV, which is used for calculation of haematocrit. The haematocrit is measured as a function of numeric integration of the MCV.

3.8.4.2.4 Calculations for MCV, MCH, and MCHC

- MCV (Mean Cell Volume) is calculated directly from the RBC histogram.
- MCH (Mean Cell Haemoglobin) is calculated from the Hb value and the RBC number.

  The mean haemoglobin weight in each RBC is given by the formula:
  MCH (pg) = Hb/RBC X10

- MCHC (Mean Corpuscular Haemoglobin Contained) is calculated according to the Hb and Hct values. Mean Hb concentration in the total volume of RBC is given by the formula:

  MCHC= Hb/Hct X100

3.8.4.2.5 WBC and differential count

General principles
The WBC count is carried twice by two different sensors:

- In the BASO count chamber at the same time as BASO count.

- In the optical chamber during the acquisition of the LMNE matrix.

  The reference count is the one obtained in the WBC and BASO count chamber.

3.8.4.2.6 BASO/WBC count

- Detection principle is the same as RBC.

  Differentiation between BASOs and other leukocytes is obtained by means of BASOLYSE II specific lysing action.

- All the WBCs are counted between the electrical threshold <0> and threshold <BA3>.

  The basophils are located from threshold <BA2> to threshold <BA3>.

Actual results are obtained as follows:
WBC: Number of cells per volume x coefficient of calibration.
BASO: Number of cells per volume x coefficient of calibration in percentage regarding the total number of leukocytes (BASO + WBC nuclei).

LMNE matrix

The WBC and Differential count are based on 3 essential principles:

- The double hydrodynamic sleeving (DHSS).
- The volume measurement impedance changes.
- The measurement of transmitted light with 0° angle, which permits a response according to the internal structure of each element and its absorbance by means of incident light diffusion.

25µl of whole blood is delivered to the LMNE chamber in a flow of EOSINOFIX. This reagent lyses the RBC, stabilizes the WBC in their native forms and stains the eosinophil nuclei with specific colouration. The solution is then stabilized and transferred to the measuring chamber. Each cell is then measured both in absorbance (cytochemistry) and resistivity (volume).

Results

A matrix is drawn up with the volumes on the X-axis and optical transmission on the Y-axis from the above measurements. The study of the matrix image permits the clear differentiation of 4 out of the 5 leukocyte population.

3.8.4.3 Histological Investigations

The liver, spleen and kidneys are prime targets for histological investigations because they are involved in the degradation and excretion of a myriad of chemical compounds. Renal damage has been associated with the use of the medicinal plants in the treatment of different disorders, including diabetes mellitus (Mapanga & Musabayane, 2010).

3.8.4.3.1 Preservation/fixation

Excised liver, kidney and spleen tissues were immediately placed in 10% Formalin for fixation. They were stored in separate specimen bottles at room temperature until processed.

3.8.4.3.2 Processing

The samples were sliced into smaller tissues, stored in labelled histology cassettes before processing. They were processed with a microwave technique (Leong, 1991), using a H2500 microwave processor supplied by Energy Beam Sciences, Inc.

Tissue samples were immersed in a 100% ethanol container and placed in the microwave at 67°C for 16 minutes. Ethanol was discarded and the procedure repeated for a further 16 minutes. This was followed by immersion of the samples in 100% isopropanol and incubation at 60°C for 15 minutes. The step was repeated with fresh 100% isopropanol for a further 15 minutes. In all steps mentioned above, the container was loosely covered and agitated at all times. In the next two steps of the procedure, tissue samples were immersed in paraffin wax for 15 minutes each and the temperature was set at 65°C and 80°C respectively.

3.8.4.3.3 Embedding and tissue slicing

The samples were embedded in paraffin wax using the Shandon HistoCentre, distributed by Optolabor (PTY) (LTD) in South Africa, according to the following procedure:

The cassettes containing processed tissues were removed from the wax container in the last processing step and placed on the heat section of the embedding instrument. The metal mould was heated slightly for each tissue, filled with molten paraffin wax and the tissue positioned in the molten wax using forceps. The labelled plastic mould of the cassette was placed onto the metal mould, filled completely with molten paraffin wax and placed on the cold plate for the wax to harden.

Samples were cut into thin slices (5µm) using Leica^{TM} microtome, floated in a water bath and picked with slides and labelled according to the plastic mould.

The wax was allowed to melt in an oven set at 57°C for 30 minutes and the slides were stained.

3.8.4.3.4 Staining

The slides were stained with Hematoxylin and Eosin (H&E) procedure as depicted in Table 3.7 below**.**

Table 3.7: Hematoxylin and Eosin stain

Step	Process	Solution	Timing
1	De-wax	Xylene	5 minutes
2	De-wax	Xylene	3 minutes
3	Hydrate	100% ethanol	1 minutes
4	Hydrate	95% ethanol	30 seconds
5	Hydrate	70% ethanol	30 seconds
6	Hydrate	water	30 seconds
7	Nuclear stain	Mayer's Haematoxilyn	5 minutes
8	Rinse	Water	30 seconds
9	Differentiate	1% acid alcohol	30 seconds
10	Blueing	Scott's tap water	1 minute
11	Cytoplasmic stain	Eosin	5 minutes
12	Dehydrate	70% ethanol	30 seconds
13	Dehydrate	90% ethanol	30 seconds
14	Dehydrate	100% ethanol	1 minute
15	Clearing	Xylene	1 minute
16	Clearing	Xylene	1 minute

Slides were mounted with a coverslip using Entellan.

Cases and controls were diagnosed microscopically at 40X magnification by a pathologist at the Universitas Academic Complex, Bloemfontein. A total of 486 slides were microscopically evaluated because three tissues were sliced from different anatomical regions of the organs The pathologist specifically undertook macrosopic and microscopic investigations for variations on the tissues excised.

Macroscopically, he investigated the presence of abnormal architecture of the tissue parenchyma on all specimens. Microscopically, he investigated any signs of dysplasia in terms of cellular changes, viz, nuclear pleomorphism, high nuclei-cytoplasm ratio, nuclear hyperchromasia, necrosis and abnormalities observed during mitosis. Any signs of inflammation were also investigated microscopically.

3.9 Statistical analysis

Data was captured in Microsoft Excel® by the researcher. Any further analysis was done by a statistician using SAS Version 9.2. Descriptive statistics, namely frequencies and percentages, were calculated for categorical and medians, and percentiles were calculated for numerical data. The normality of the numerical variables were tested using Shapiro-Wilk's test, and if a variable was skewed ($p < 0.05$) the median and inter-quartile range (IQR) was reported. Analytical statistics,

namely the Kruskal-Wallis test, was used to compare median values between the control and experimental groups, as well as to compare the median values between the different extract concentrations. A significance level of 0.05 was used.

CHAPTER 4

RESULTS

4.1 Introduction

The aim of the study was to evaluate the possible toxic effects of *Asparagus laricinus* extracts using Sprague Dawley rats as the animal model. The rats were exposed to different plant extracts in ethanol, dichloromethane and water at different concentrations and doses. The dichloromethane extract procedure was discontinued due to adverse events as mentioned in the methodology section.

In this chapter, we focused closely at statistical analysis of different laboratory results with reference to haematological and biochemical parameters in blood samples, as well as the histological assessment of tissues excised from the kidney, liver and spleen. Haematological and biochemical variables were compared, firstly, between exposed and unexposed groups, and secondly, also within groups in both ethanol and aqueous extracts. Macroscopic and microscopic assessment of tissues excised were done by investigations of morphologic and functional changes by comparing organs as well as histological slides preparation between exposed and unexposed animals.

4.2 Comparison of results obtained for the control group and the experimental population

The results of the combined control and experimental groups are presented in this section, starting with mass, followed by haematological, clinical chemistry parameters and wrapping it up with histological investigations. Statistical analysis for comparing controls and treatment groups for both aqueous and ethanolic groups at increasing dosages are also presented. In addition, the difference within the different extract concentrations for aqueous and ethanol groups is illustrated in the section.

4.2.1 Mass

The median weight of all controls (n=12) and the experimental group combined (n=42) were statistically compared using Kruskal-Wallis test. No significant differences (p>0.05) between the median weights of unexposed and exposed rats at the start and termination of the experiment was observed, as illustrated by Table 4.1.

Table 4.1 Median change in weight of the controls (unexposed) and experimental (exposed) rats.

		Median	IQR	Min – Max	p-value
Start mass (g)	Control	180.0	178.0-188.5	158.0-201.0	0.252
	Experimental	188.0	179.0-193.0	164.0-204.0	
End mass (g)	Control	216.5	211.5- 238.0	209.0-271.0	0.102
	Experimental	225.0	220.0-244.0	201.0-265	
Change in Mass	Control	41.5	33.0-54.5	19.0-70.0	0.700
	Experimental	44.0	33.0-56.0	7.0-74.0	

IQR (Interquartile Range), **Min** (Minimum), **Max** (Maximum) **g** (Grams)

4.2.2 Haematological parameters

Comparison studies for full blood count parameters between controls (n=12) and combined experimental group (n=42) were performed. There were no significant differences (p>0.05) between combined controls and experimental animals regarding haematological parameters of the combined ethanol and aqueous extracts at different levels/doses (Table 4.2 & 4.3).

Table 4.2 Comparison of RBC parameters between combined controls and experimental groups.

		RBC ($x10^9$ /l)	HB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)
Median	Control	7.67	15.10	42.20	56.00	19.80	35.40
	Exposed	7.87	15.5	43.60	56.00	19.80	35.65
IQR	Control	7.5-8.1	14.8-15.3	41.4-43.6	54.0-56.0	19.0-19.9	35.3-35.9
	Exposed	7.4-8.1	14.9-16.0	41.5-45.0	55.0-56.0	19.6-20.1	35.4-35.8
p-value		0.820	0.183	0.320	0.537	0.655	0.516

IQR (Interquartile Range), **RBC** (Red Blood Cells), **HB** (Haemoglobin g/dl), **HCT** (Haemoglobin Content %), **MCV** (Mean Cell Volume) fl (Femtoliters), **MCH** (Mean Cell Haemoglobin), pg (Picograms), **MCHC** (Mean Cell Haemoglobin Content g/dl)

Table 4.3 Comparison of white cell parameters and platelet between combined controls and experimental groups.

		WBC	LYM	MON	NEU	EOS	BAS	PLT
Median	Control	3.70	2.83	0.32	0.48	0.03	0.03	734.0
	Exposed	4.25	3.45	0.36	0.54	0.02	0.03	795.0
IQR	Control	2.6-5.1	1.91 - 3.80	0.16 - 0.45	0.37 – 0.57	0.02 - 0.05	0.02 - 0.04	669.0-753.0
	Exposed	3.2-5.8	2.60 - 4.47	0.17 - 0.43	0.35 - 0.84	0.01 - 0.09	0.02 - 0.03	690.0-826.0
p-value		0.436	0.443	0.848	0.429	0.737	0.3500	0.057

WBC (White Blood Cells $x10^9$ /l), **LYM** (Lymphocytes $x10^9$ /l), **MON** (Monocytes $x10^9$ /l), **NEU** (Neutrophils $x10^9$ /l), **EOS** (Eosinophils $x10^9$ /l), **BAS** (Basophils $x10^9$ /l), **PLT** (Platelets $x10^9$ /l)

4.2.3 Biochemical parameters

Liver enzymes, cholesterol, urea and creatinine results for the combined groups of ethanol and aqueous extract controls (n=12); in comparison with combined different treatment groups (n=42) were statistically analysed using a Kruskal-Wallis test . Again, no significant statistical differences (p>0.05) across the different extracts and levels of concentrations were observed (Table 4.4).

Table 4.4 Comparison of biochemical parameters between combined controls and experimental groups.

		CHOL	BUN	CREA	ALP	AST	ALT
Median	Control	2.00	6.25	47.00	107.5	125.00	58.50
	Exposed	2.00	6.00	29.00	131.0	123.50	53.00
IQR	Control	2.00-2.00	5.85-6.85	14.50-74.50	92.00-141.50	101.50-185.50	51.00-86.00
	Exposed	2.00-2.00	5.40-6.50	12.00-71.00	91.00-151.00	102.00-164.00	45.00-66.00
p-value		0.061	0.215	0.876	0.365	0.9254	0.219

(P<0.05) statistically significant**; CHOL** (Cholesterol)**. BUN** (Blood Urea Nitrogen)**, CREAT** (Creatinine), **ALP** (Alkaline Phosphatase), **AST** (Aspartate Aminotransferase), **ALT** (Alanine Aminotransferase).

4.3 Comparative study of the control group versus the various ethanol extract group

In this section, the statistical analysis for comparing controls and treatment groups of ethanoic groups, at increasing dosages, are presented. This was done to determine the effects of lower and higher ethanol extract concentration on the parameters.

4.3.1 Mass

Table 4.5 below represents the statistical analysis of the comparison of the weight of rats (in grams) for the control (n=6) and treatment groups, at increasing dosages of ethanoic extracts ranging from 50mg/kg/day to 400mg/kg/day. Significant differences were observed for the initial mass of rats exposed to 200mg/kg/day (p=0.0100), and also for the termination mass of rats exposed to 400mg/kg/day (p=0.0303). There was no difference in the change of mass for the rest of the dosages administered.

Table 4.5 Median weight of controls (unexposed) and experimental (exposed) rats at different levels of ethanol extract

		Control	Amount of ethanol extract (mg/kg/day)			
			50	**100**	**200**	**400**
Start mass	Median	180.0	183.0	186.0	193.0	187
	IQR	179.0-183.0	173.0-191.0	184.0-189.0	190.0-197.0	183.0 - 189.0
	p-value		0.8717	0.1978	0.0100*	0.2265
End mass	Median	214.0	219	222.0	222.5	230.50
	IQR	211.0-217.0	217.0-220.0	220.0-224.0	217.0-225.0	223.0-244.0
	p-value		0.2281	0.0782	0.921	0.0303*
Change in mass	Median	33.0	36.0	39.5	31.5	47.5
	IQR	30.0-37.0	29.0-44.0	33.0-43.0	24.0-33.0	34.0-55.0
	p-value		0.873	0.333	0.420	0.078

*(P<0.05) statistically significant.

Figure 4.1 below illustrates the graphical presentation of the differences between the initial and termination median masses at increasing ethanol extract doses. These clearly illustrates that the median change in weight over the two weeks experimental period is slightly below 50mg. It is also observed that rats exposed to 200mg/kg/day dose had the least weight change, while those exposed to a 400mg/kg/day dose had the most. These changes were not statistically significant.

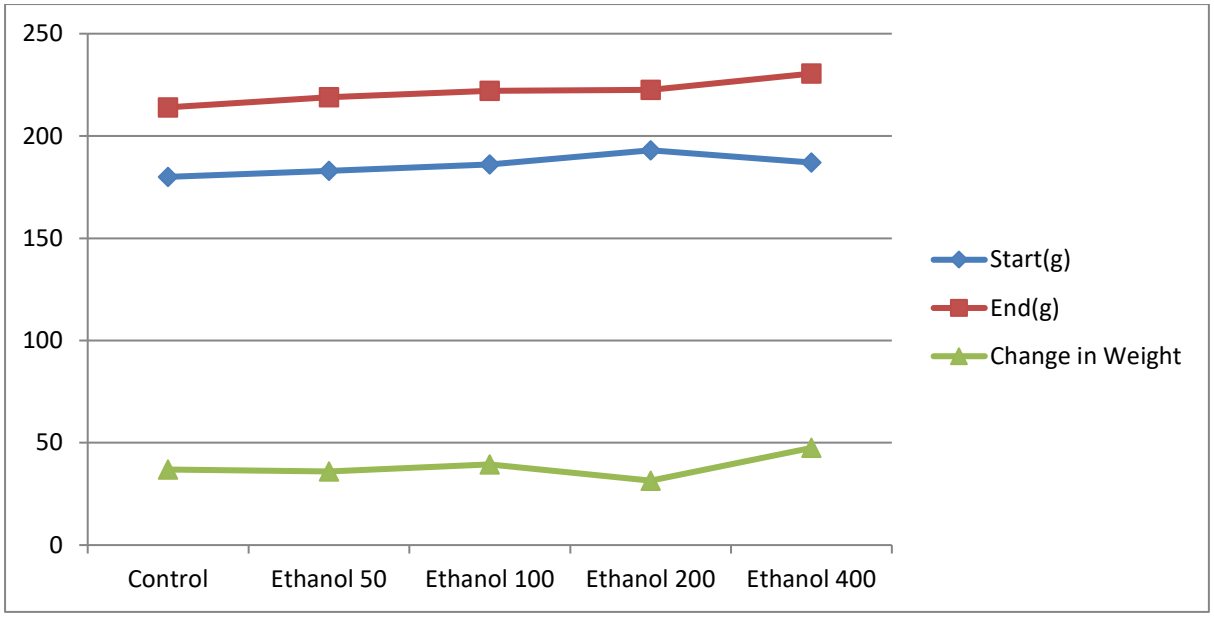

Figure 4.1 Graphical representation of the median change in mass for the rats on ethanol extracts.

4.3.2 Haematological parameters

Full blood counts were determined for the rats treated with ethanol extracts at different doses ranging from 50mg/kg/day to 400mg/kg/day. A Kruskal-Wallis test for comparing median results was performed and the results showed no significant difference between the control (n=6) and treatment groups (n=6) across most levels of ethanoic extracts.

Table 4.6 Comparison of WBC and PLT between controls and rats exposed to ethanol extracts across all doses.

		WBC	LYM	MON	NEU	EOS	BAS	PLT
Median	Control	3.80	3.01	0.40	0.46	0.03	0.04	730.00
	Exposed 50mg/kg	4.20	3.43	0.36	0.53	0.02	0.03	850.00
p-value		0.233	0.144	0.465	0.584	0.465	0.097	0.011*
	Exposed 100mg/kg	3.60	2.78	0.33	0.42	0.01	0.03	762.00
p-value		0.410	0.855	0.584	0.170	0.462	0.139	0.201
	Exposed 200mg/kg	4.95	4.03	0.33	0.58	0.02	0.03	783.00
p-value		0.394	0.201	0.394	1.00	1.00	0.159	0.394
	Exposed 400mg/kg	5.75	3.93	0.40	0.60	0.02	0.03	839.00
p-value		0.575	0.748	0.200	1.00	0.872	0.059	0.0547

*($P<0.05$) statistically significant; **WBC** (White blood cells $x10^9$ /l), **LYM** (Lymphocytes $x10^9$ /l), **MON** (Monocytes $x10^9$ /l), **NEU** (Neutrophils $x10^9$ /l), **EOS** (Eosinophils $x10^9$ /l), **BAS** (Basophils $x10^9$ /l), **PLT** (Platelets $x10^9$ /l)

Table 4.6 illustrates significant differences (p=0.0106) in the median results of platelets at the dosage of 50mg/kg/day. It must be noted that the percentage change from the control median (730 x 10^9/L) to 50mg/kg (850 x 10^9/L), was 16%, while the human refence range is 150 – 410 x10^9/L (Bates & Lewis, 2011). The human range spans over a range of 63%. Furthermore, it must be noted that the 400mg/kg group's platelet median was 839 x 10^9/L, which showed a 13% change with no statistical significant difference. The remaining parameters in both Tables 4.6 and 4.7 could not demonstrate any significant differences statistically (p>0.05).

Table 4.7 Comparison of red blood cells parameters between unexposed and exposed rats for ethanol extracts across all doses.

		RBC	HB	HCT	MCV	MCH	MCHC
Median	Control	7.50	15.05	42.10	56.00	19.85	35.70
	Exposed 50mg/kg	7.95	15.70	44.10	56.00	19.80	35.80
p-value		0.273	0.1190	0.143	0.827	0.700	0.855
Median	Control	7.50	15.05	42.10	56.00	19.85	35.70
	Exposed100mg/kg	7.06	14.40	40.10	56.00	20.10	35.80
p-value		0.273	0.583	0.464	0.392	0.573	0.855
Median	Control	7.50	15.05	42.10	56.00	19.85	35.70
	Exposed 200mg/kg	7.57	15.15	42.40	56.00	20.10	35.80
p-value		0.831	0.915	1.00	0.221	0.051	0.669
Median	Control	7.50	15.05	42.10	56.00	19.85	35.70
	Exposed 400mg/kg	7.59	14.95	41.45	55.00	19.80	35.60
p-value		0.749	0.810	0.748	0.212	0.315	0.809

IQR (Interquartile range), **RBC** (Red blood cells x10^9 /l), **HB** (Haemoglobin g/dl), **HCT** (Haemoglobin content %), **MCV** (Mean Cell Volume in microtoliters), **MCH** (Mean Cell Haemoglobin Picograms), **MCHC** (Mean Cell Haemoglobin Content g/dl)

It is of utmost importance to emphasize that the results for full blood count parameters are expressed in varying units, and therefore fewer figures for comparison of their medians would not be practical at this stage.

4.3.3 Biochemical parameters

Median results for biochemical tests were compared between the controls (n=6) and experimental group (n=6) using a Kruskal-Wallis test.There was no statistical difference between the controls and increasing doses of ethanol extracts in all biochemical results, as illustrated by Table 4.8.

Table 4.8 Comparison of biochemical parameters between controls and rats exposed to ethanol across all concentrations.

		CHOL	BUN	CREA	ALP	AST	ALT
Median	Control	2.00	6.55	14.50	141.50	149.00	69.50
	Exposed 50mg/kg	2.00	6.15	8.50	167.00	165.50	72.50
p-value		1.000	0.688	0.514	0.0542	0.936	0.873
	Exposed 100mg/kg	2.00	6.90	24.00	146.50	119.50	47.50
p-value		1.00	0.749	0.199	0.630	0.229	0.078
	Exposed 200mg/kg	2.00	6.25	14.00	132.00	164.00	65.00
p-value		1.000	0.422	0.867	0.7479	0.7483	0.747
	Exposed 400mg/kg	2.00	6.10	23.00	145.50	121.50	54.00
p-value		1.000	0.127	0.295	0.261	0.229	0.192

This is further illustrated in figures 4.2, 4.3 and 4.4, which summarizes median results for cholesterol and blood urea nitrogen; creatinine and enzymes respectively. These constituents were grouped together according to the units in which the results are expressed. Table 4.8 illustrates the p-values for biochemical parameters.

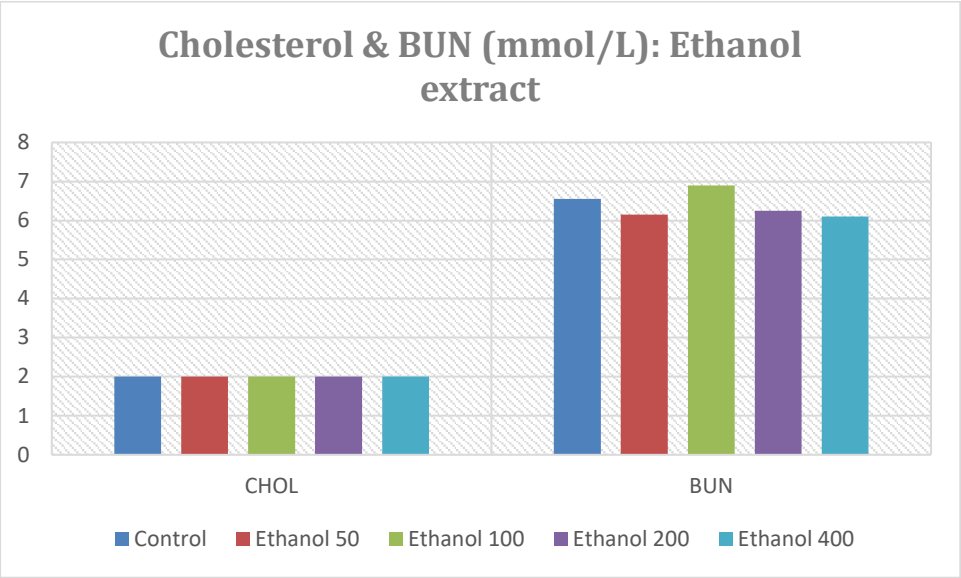

Figure 4.2 Median results of cholesterol and blood urea nitrogen at increasing doses of ethanol extracts.

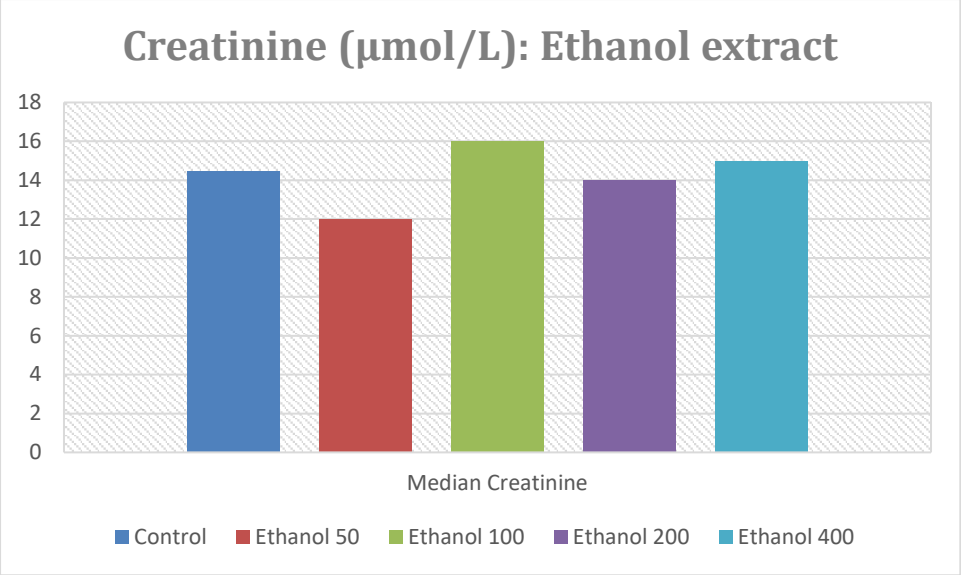

Figure 4.3 A comparative study of the median creatinine results at increasing doses of ethanol extracts.

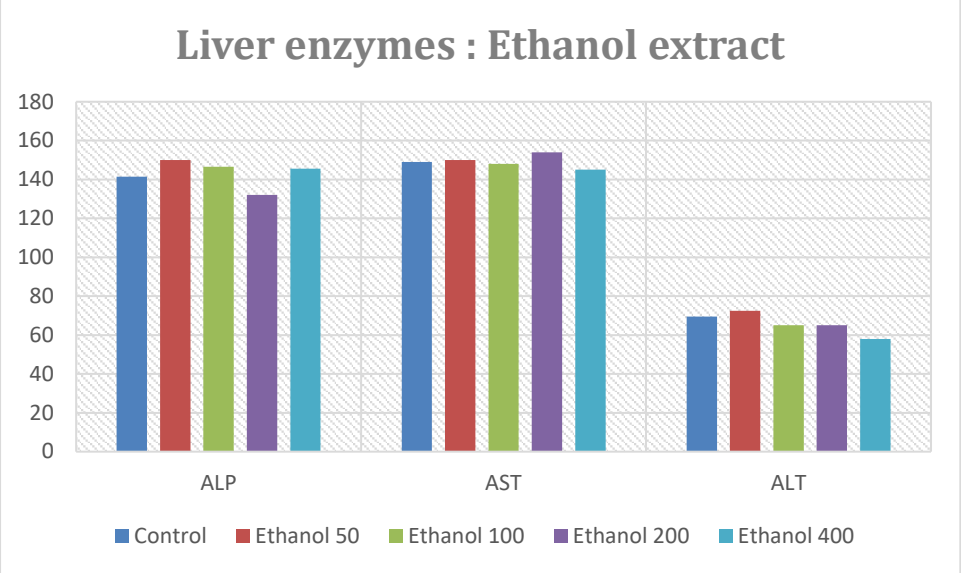

Figure 4.4 A comparative study of median liver enzymes results at increasing doses of ethanol extracts.

4.4 Comparative study of the control group versus various aqueous extracts group

4.4.1 Mass

The median change in mass for both treated (experimental) and untreated (control) groups over the experimental period (8 weeks), were recorded. Results for controls and experimental groups were compared using a Kruskal-Wallis test. There was no significant difference ($p>0.05$) in the median mass between the controls (n=6) and all experimental groups (n=18), as illustrated in Table 4.4. Furthermore, the change in median weights throughout the entire experimental period was approximately 50g, as illustrated by Figure 4.5 below.

Table 4.9 Median weight of controls (unexposed) and experimental (exposed) rats at different levels of aqueous extracts.

		Control	Concentration of the aqueous extract		
			2%	**10%**	**20%**
Start mass	Median	181.0	188.0	189.0	181.5
	IQR	176.0-197.0	182.0-188.0	168.0-192.0	173.0-197.0
	p-value		0.5189	0.8728	0.9358
End mass	Median	235.5	246.5	236	236.0
	IQR	215.0-251.0	245.0-252.0	220-242.0	220.0-251.0
	p-value		0.5218	1.000	0.8099
Change in mass	Median	54.5	60.0	49.5	51.0
	IQR	46.0-66.0	59.0-63.0	48.0-56.0	44.0-54.0
	p-value		0.336	0.631	0.748

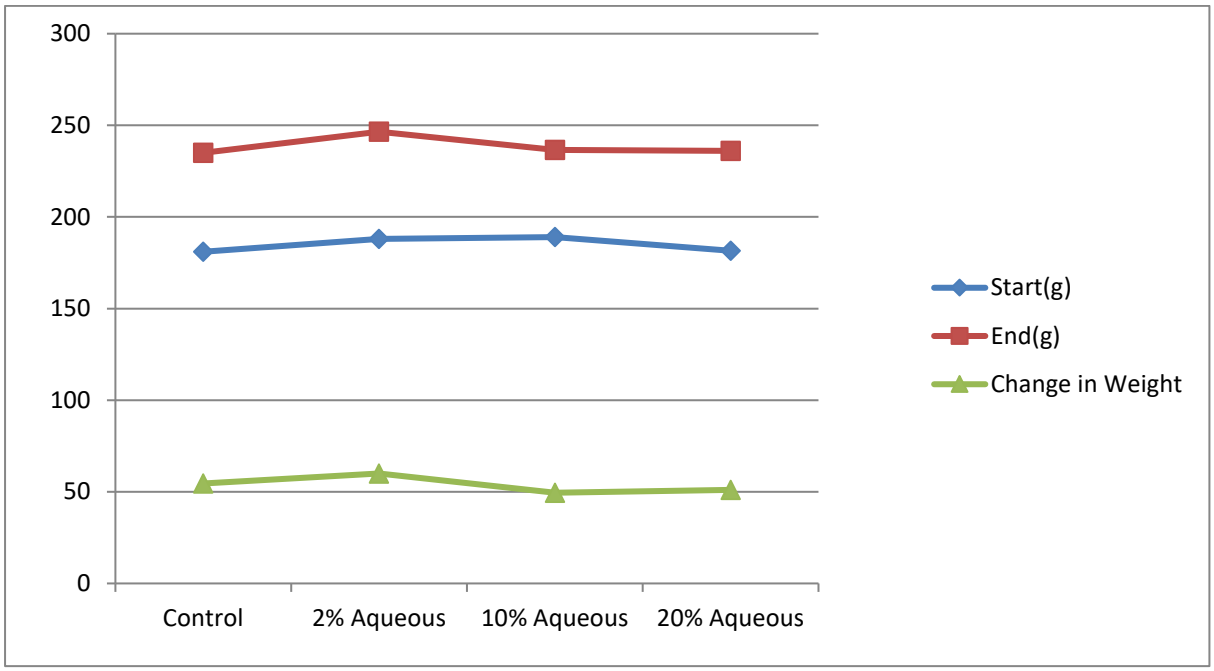

Figure 4.5 Graphical representation of the median change in mass of the rats treated with aqueous extracts over a period of eight weeks.

The change in weight was similar in all groups, with 2% aqueous extract having a slightly higher mass, which was not significant.

4.4.2 Haematological parameters

Tables 4.10 and 4.11, that compare the controls (n=6) and experimental groups (n=6) at different concentrations of aqueous extracts, specifically with regard to full blood count parameters and p-values, were prepared. There were no significant differences in all concentrations ranging from 2% to 10% (p>0.05) in FBC parameters. A significant difference was observed for haemoglobin (p=0.0353) in subjects exposed to 20% aqueous extracts, as illustrated in Table 4.10. It must be noted that the percentage change from control median haemoglobin concentration (15.1g/dl) to 20% aqueous (15.85g/dl) was 5%, while the human refence range for males is 13 – 17g/dl (Bates & Lewis, 2011). The human range spans over a range of 24%. Furthermore, it must be noted that 10% of the aqueous group's haemoglobin concentration was 15.95g/dl, which showed a 6% percentage change with no statistical significant difference.

Table 4.10 Comparison of RBC parameters between controls and exposed rats across all aqueous extracts.

		RBC	HB	HCT	MCV	MCH	MCHC
Median	Control	8.04	15.10	42.60	54.00	19.00	35.30
	Exposed 2%	7.99	15.45	43.75	55.50	19.70	35.55
p-value		0.784	0.234	0.314	0.453	0.357	0.409
	Exposed 10%	8.08	15.95	45.20	56.00	19.65	35.55
p-value		0.715	0.361	0.465	0.344	0.359	0.4059
	Exposed 20%	7.98	15.85	44.70	55.50	19.60	35.40
p-value		0.521	0.035*	0.082	0.396	0.356	0.348

RBC (Red Blood Cells x10^9 /l), **HB** (Haemoglobin g/dl), **HCT** (Haemoglobin Content %), **MCV** (Mean Cell Volume in Microtoliters), **MCH** (Mean Cell Haemoglobin picograms), **MCHC** (Mean Cell Haemoglobin Content g/dl), *(P<0.05) statistically significant

Table 4.11 Comparison of WBC and PLT parameters between unexposed and exposed rats across all aqueous extracts.

	WBC	LYM	MON	NEU	EOS	BAS	PLT

Median	Control	3.40	2.3	0.17	0.52	0.02	0.02	734
	Exposed 2%	3.40	2.63	0.26	0.48	0.01	0.02	721.50
p-value		0.855	0.855	0.359	1.000	0.645	0.130	0.465
	Exposed 10%	3.90	2.83	0.29	0.60	0.03	0.02	710.50
p-value		0.715	0.144	0.464	0.143	0.578	0.082	0.855
	Exposed 20%	5.25	4.11	0.33	0.66	0.05	0.03	724.00
p-value		0.201	0.855	0.714	0.583	0.782	0.121	1.000

WBC (White Blood Cells $x10^9$), **LYM** (Lymphocytes $x10^9$ /l), **MON** (Monocytes $x10^9$ /l), **NEU** (Neutrophils $x10^9$ /l), **EOS** (Eosinophils $x10^9$ /l), **BAS** (Basophils $x10^9$ /l), **PLT** (Platelets $x10^9$ /l)

4.4.3 Biochemical parameters

Several figures were created for the comparison of the median results of controls (n=6), and experimental results (n=6) for the increasing doses of aqueous extracts. Table 4.12 summarizes the medians and p-values across increasing aqueous extracts. No significant differences were observed throughout increasing doses of aqueous extracts in biochemical parameters, except for the blood urea nitrogen (p=0.0081) for animals exposed to 20% aqueous extract. Figures 4.7 to 4.8 illustrates a summary of comparisons of median results between the controls (n=6) and experimental groups (n=6), at increasing doses of aqueous extracts for biochemical parameters.

Table 4.12 Comparison of biochemical parameters between controls and exposed rats across all aqueous extract.

		CHOL	BUN	CREA	ALP	AST	ALT
Median	Control	2.00	6.15	74.5	92.00	101.5	56.5
	Exposed 2%	2.00	5.85	80.00	89.50	100.50	47.50
p-value		0.3173	0.4217	0.1488	0.7471	0.5745	0.5738
	Exposed 10%	2.00	5.95	69.00	88.00	136.00	53.50
p-value		0.3137	0.9356	0.1978	0.8726	0.4233	0.3350
	Exposed 20%	2.00	5.05	73.00	98.00	111.00	53.00
p-value		0.3173	0.0081*	0.7483	0.8726	0.5189	0.9361

*(P<0.05)statistically significant **;** **CHOL** (Cholesterol), **BUN** (Blood Urea Nitrogen), **CREAT** (Creatinine), **ALP** (Alkaline Phosphatase), **AST** (Aspartate Amino Transferase), **ALT** (Alanine Aminotransferase).

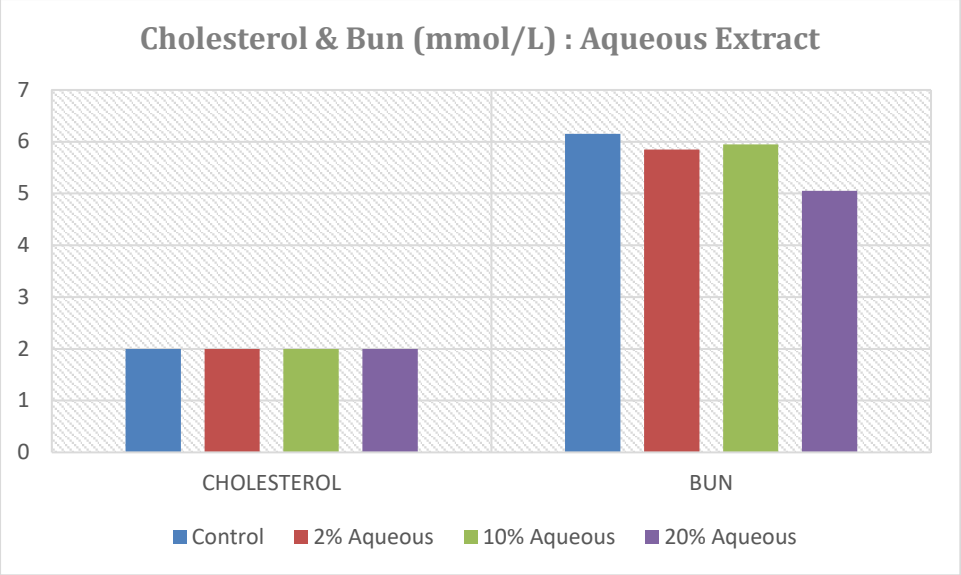

Figure 4.6 A comparative study of median cholesterol and BUN results at 2%, 10% and 20% of water extracts.

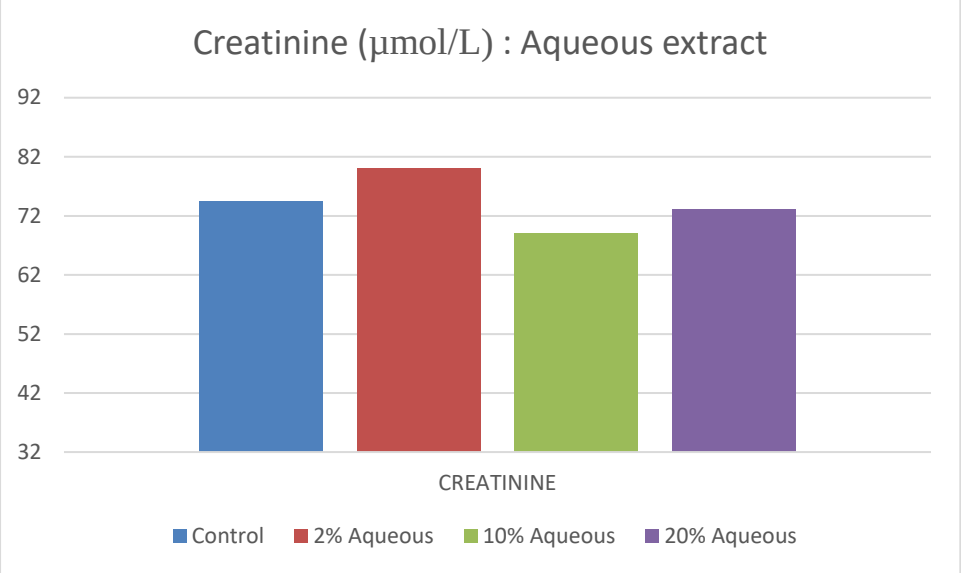

Figure 4.7 A comparative study of median creatinine results at increasing doses of water extracts.

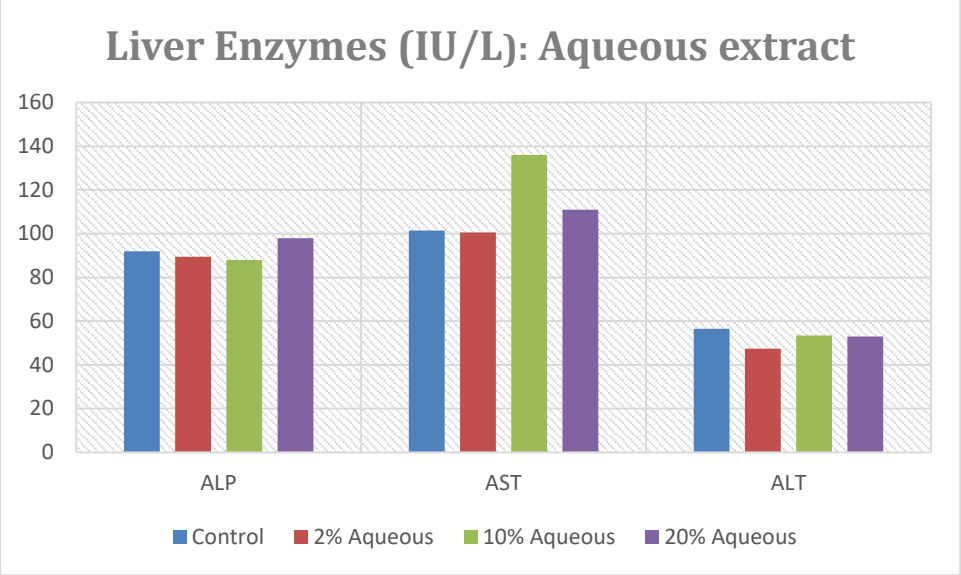

Figure 4.8 A comparative study of median liver enzymes results at increasing doses of water extracts.

4.5 Histological investigations

Macroscopic examination of the kidney, liver and spleen tissue could not demonstrate any pathological variations between the treatment groups and controls in both aqueous and ethanol extracts, as assessed by the pathologist. A histopathological study of the rats' kidney, liver and spleen tissue indicated a normal architecture of tissue in both the controls and treatment groups, as illustrated in Figures 4.9 to 4.11, respectively.

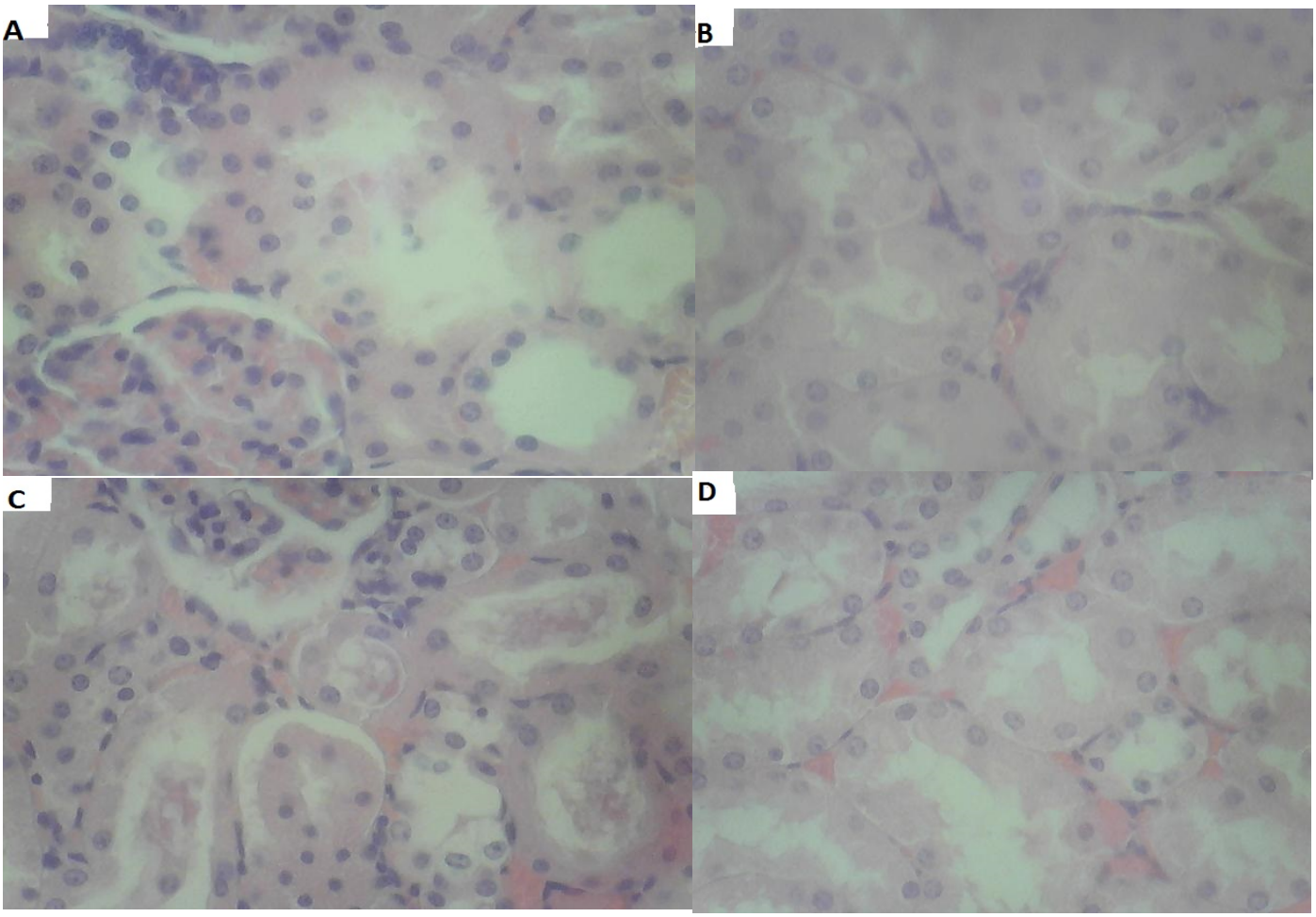

Figure 4.9 Photomicrographs of the longitudinal sections of the renal cortex region of control and treated animals. (A) Renal cortex of control (aqueous extract) rats showing glomeruli (40×). (B) Treated with 2% aqueous extract of *Asparagus laricinus*.(40×). (C) Renal cortex of a control (ethanol extract) rat showing glomeruli (40×). (D) Treated with 50mg/kg/day ethanol extract (40×).

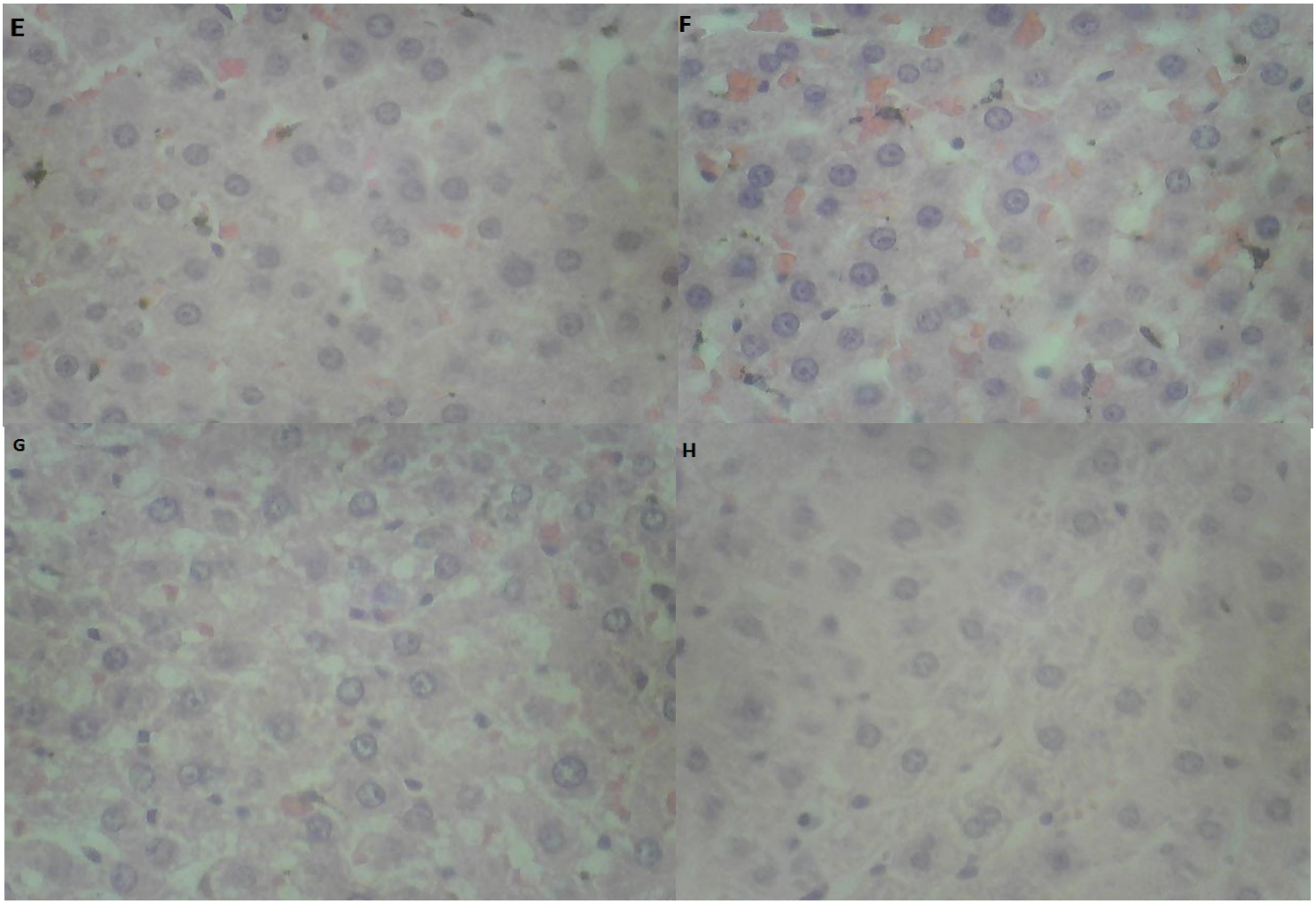

Figure 4.10 Effect of aqueous and ethanol extracts of *Asparagus laricinus* on the liver tissue of Sprague Dawley rats. (E) Control tissue for aqueous extracts (40×). (F) Treated with 10% aqueous extract. (40×). (G) Control rats for ethanol extract (40×). (H) Treated with 100mg/kg/day ethanol extract (40×).

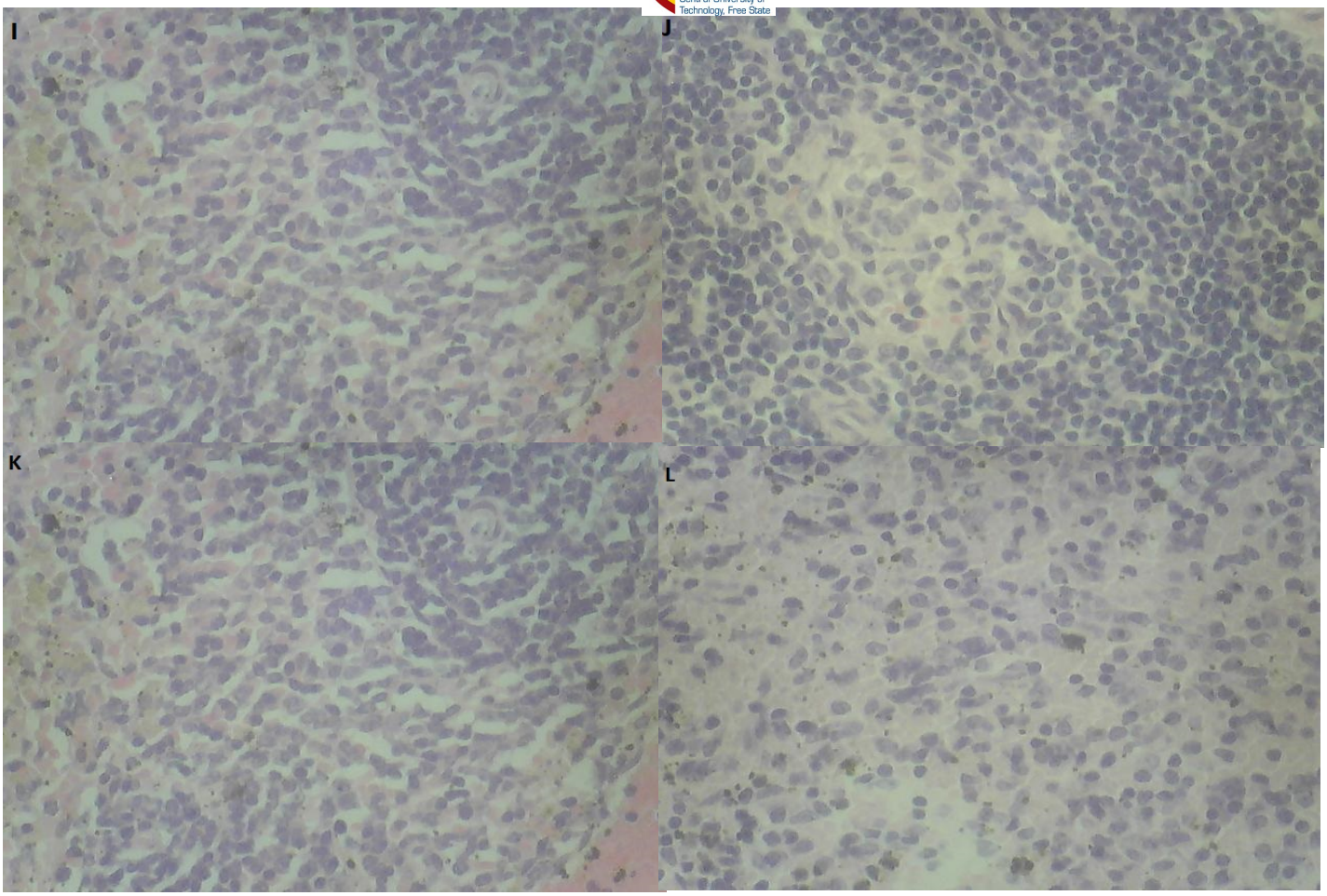

Figure 4.11 Photomicrographs of the spleen for control and treated animals. (I) Control tissue for aqueous extracts (40×). (J) Treated with 10% aqueous extract of *Asparagus laricinus*. (40×). (K) Control rats for ethanol extract (40×). (L) Treated with 200mg/kg/day ethanol extract (40×).

4.6 Overall summary

Biochemical and haematological tests were selected as indicators of the damage to the tissue of organs, including the liver, kidney and spleen. Extremely high or low results would then estimate the severity of the damage to such tissues. Histological results would demonstrate the type of tissue damage, as well as the extent of the damage.

When the combined experimental group (n=42) was compared with the combined controls (n=12), no significant difference was observed for the mass and selected haematological and biochemical variables.

Further comparison of treatment groups (n=6) and controls (n=6) across all ethanol extracts showed significant differences in the starting median change in weight at the 200g/kg/day dosage, as well as the median termination weight at 400g/kg/day. The median change in weight remained slightly below 50g over the entire two-week period of experimentation. A significant difference ($p<0.05$) was observed for platelets with the ethanol extract at a dose of 50g/kg/day. There were no statistical differences between the treatment groups and controls with regard to the rest of haematological variables and selected biochemical tests.

Comparison of the controls (n=6) and treatment groups (n=6) revealed an average median change in weight of slightly above 50g over the entire eight-week period of experimentation with aqueous extracts. A significant difference ($p<0.05$) was observed for both haemoglobin and BUN results with the 20% water extract. There were no statistical differences between the treatment and control groups with regard to the rest of haematological variables and selected biochemical tests.

Histological evaluation could not reveal any pathological changes in both the aqueous and ethanolic extracts across all levels of dosage.

CHAPTER 5

DISCUSSION

5.1 Introduction

One of the unique aspects attributed to planet earth is plants. A variety of plant species have been scientifically and traditionally identified for their medicinal properties and values respectively. Accessibility and affordability have always made traditional medicine convenient for healthcare and nutritional use in Africa (World Health Organization., 2002; Tchacondo *et al.,* 2012).

The family/genus asparageceae has also been used for treatment of various ailments. The species *Asparagus laricinus* has been extensively studied for its anticarcinogenic properties (Mashele & Konesnikova, 2010), and the active ingredients identified (Fuku *et al.,* 2013). The accumulated rich knowledge of this species have encouraged us to continue with further studies in the form of *in vivo* assessment of the toxicology of both aqueous and ethanolic extracts of the plant.

Although medicinal plants may have biological activities that are beneficial to humans, the potential toxicity of these bioactive substances has not been well established (Rosidah *et al*., 2009; Idoh *et al.*, 2016). Moreover, despite the widespread use, few scientific studies have been undertaken to ascertain the safety and efficacy of traditional remedies (Graça *et al.,* 2007).

The main purpose of the study was to establish whether *Asparagus laricinus* has any toxic or adverse effects on the tissue and organs of animal models (Sprague Dawley rats). This was done by administering both aqueous and ethanol extracts of *Asparagus laricinus* to groups of animals, and also varying the concentrations of these extracts. Dose-response assessment of the effect of the extract was done by analysis of the blood samples collected at the end of the research. Haematological analysis consisted of a full blood count; clinical chemistry analysis was done by measuring selected liver enzymes; creatinine and urea for renal function; and histologically we investigated any damage or inflammation of the liver, spleen and kidney tissue.

5.2 Comparison of haematological parameter of control and different concentration of all extracts

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. However, as indicated in Table 4.6 in chapter 4, significant differences were noted with ethanolic extracts for platelets at a dose of 50mg/kg/day ($p=0.011$). It must be noted that this statistical significant change produced a 15% increase in the median concentration, in comparison with the control. Although it seems significant, it must be noted that the reference range for platelets in humans spans 63%, and the 400mg/kg/day group showed a 13% increase without a statistically significant change.

Based on the above, it seems that even though a statistical significant change was obtained, it does not translate to a clinically significant change.
In the aqueous extracts, statistically significant results were observed for 20% of the extracts for Hb, as indicated in Table 4.10. This increase in the haemoglobin concentration produced a 5% change, while the human range spans 37% and the 10% aqueous extracts had a 6% change without a statistical significant change. Based on these facts it seems that the statistical significant change does not translate to a clinically significant change.

5.3 Comparison of chemical parameter of control and different concentration of all extracts

Generally, biochemical results could not conclusively indicate the presence of toxicity, as the majority of the results were not significantly different in both the treated and untreated groups of rats. Table 4.10, however, paints a different picture, as it shows significant differences between the controls and rats exposed to 20% aqueous extracts, with regard to the BUN. The results show no specific trend or pattern, and therefore may mislead the researcher. The results might be genuine or spurious, and therefore further investigations may be suggested.

5.4 Comparison of histological changes between control and different concentration of all extracts

There were no traces of damage or inflammation of the tissue excised from the kidney, liver and spleen of the rats exposed or unexposed to *Asparagus laricinus* extracts. Figures 4.9 to 4.11 compare haematoxilyn and eosin stains (H&E) from both exposed and unexposed tissue.

Literature does not reveal any toxicological studies on *Asparagus laricinus*, however, similar studies were conducted for anticancer plants using either mice, rats or rabbits. There are reports on acute toxicity studies on *Hyptis suaveolens* Poit. (Lamiaceae) leaves (Danmalam *et al., 2009*); *Moringa oleifera* Lam. (Moringaceae*)* (leaves) (Isitua & Ibe, 2003; Sreelata *et al.,* 2011; Ugwu *et al.,* 2013; Berkovich *et al.,* 2013); *Newbbouldia laevis (Bignoniaceae)* (stem leaves and roots) (Azuine *et al.,* 1995) and *Nigella sativa (Ranunculaceae)* (seeds) (Ali & Blunden, 2003; Ekanen & Yusuf, 2008;) to cite but a few.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Introduction

The main purpose of the study was to assess the safe use of the *Asparagus laricinus* plant extracts by undertaking toxicological studies. This was done by evaluating hepatotoxicity, nephrotoxicity, spleen and vascular damage to the animals. Selected liver enzymes and other tests; renal dysfunction indicators and haematological parameters were investigated by statistically comparing treated and untreated animals. Furthermore, the excised liver, spleen and kidney tissue were examined histologically for any damage or inflammation caused by exposure to the extracts.

6.2 Conclusion

6.2.1 Haematological parameter

There was no significant difference between the treatment and control groups with regard to the full blood count.There were, however, clinically non-significant changes that can be deemed as sporadic or anomalic results, regarding the haemoglobin levels while using 20% aqueous extracts, and with regard to platelets while using ethanolic extracts administered at 50mg/kg/day. The results could not demonstrate any specific pattern across the aqueous or ethanol extracts.

6.2.2 Clinical chemistry parameters

There was no significant difference between the treatment and control groups with respect to the liver enzymes, blood urea nitrogen, cholesterol and creatinine results. There were, however, sporadic or anomalic results regarding BUN, while using 20% aqueous extracts., The fact that the results could not demonstrate any specific pattern or trend across varying concentrations warrants further investigation.

6.2.3 Histological changes

The histological assessment has proven that both aqueous and ethanolic extracts of *Asparagus laricinus* had no detrimental or adverse effects on the vital organs of the Sprague Dawley rats. Tissue damage, lesions or inflammation were not observed on

the kidney, liver or spleen of the treatment groups as compared to the control group. The pattern was observed across increasing doses of aqueous and ethanolic extracts.

6.3 Limitations

Although the collection and analysis of blood and tissue samples needs ample time, the actual experimental time was conducted over a maximum period of eight weeks, which perhaps was not sufficient for the tissue damage to occur. The number of rats was limited as a result of the of laws and policies governing the use of experimental animals for research purposes. We currently do not have the reference or normal ranges for biochemical and haematological parameters for rats, and therefore relied on comparing the results to those of the control group.

6.4 Recommendations

The period of experimentation should be increased and motivation for a bigger sample size would also be necessary, for the researcher to improve the quality of the results. Normal ranges for the haematological and biochemical tests should be established.

6.5 Overall conclusion

In conclusion, we summarize that the toxicological evaluation of *Asparagus laricinus* extracts may be considered relatively free of toxicity when given orally, as it did not cause death, damage or inflammation to the tissues, nor produced any remarkable biochemical and haematological adverse effects in both the male and female Sprague Dawley rats. Further studies may also be conducted to demonstrate *in vivo* efficacy against cancer, because thus far studies were conducted using cell lines (*in vitro* studies).

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APPENDICES

APPENDIX A

Ethics approval

Central University of Technology, Free State

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS UV

Internal Post Box / Interne Posbus G40
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Ms H Strauss

2012-11-12

AREC Ref: AREC-181111-005

MR SD MOKGAWA
DEPT OF HEALTH TECHNOLOGY
CENTRAL UNIVERSITY OF TECHNOLOGY
FREE STATE
BLOEMFONTEIN
9300

Dear Mr Mokgawa

ANIMAL EXPERIMENT NR 16/2012
RESEARCHER: MR SD MOKGAWA, HEALTH TECHNOLOGY, CUT
PROJECT TITLE: "TOXICOLOGY OF ASPARAGUS LARICINUS EXTRACTS IN RATS."

You are hereby kindly informed that the approval of the above study was condoned at the Interfaculty Animal Ethics Committee meeting on 8 November 2012.

ANIMAL	*NUMBER*	*EXPIRY DATE*
SPRAGUE DAWLEY	72	SEPTEMBER 2013

Kindly take note of the following:

1. ***Fully completed and signed applications have to be submitted electronically to StraussHS@ufs.ac.za and a hard copy has to be submitted too.***
2. ***A signed progress report with regard to the above study has to be submitted electronically to StraussHS@ufs.ac.za while a hard copy has to be submitted to Ms H Strauss, Room D115, Francois Retief building, Faculty of Health Sciences. A report has to be submitted when animals are physically involved and after completion of the study. Guidelines with regard to progress reports are available from the secretary and on the Faculty Intranet.***
3. ***Researchers that plan to make use of the Animal Experimentation Unit must request a quotation from the Head, Mr Sob Lamprecht***
4. ***Contract research: Fifty (50%) of the quoted amount is payable when you receive the letter of approval.***

Regards

.....
CHAIR:
INTERFACULTY ANIMAL ETHICS COMMITTEE

APPENDIX B

Language editing

Quinton Oosthuizen
LANGUAGE PRACTITIONER
(B.Tech Language Practice – CUT)

DECLARATION

19 January 2017

To whom it may concern

This serves to inform you that I have linguistically revised, in English, the document entitled, *Toxicology of Asparagus Laricinus in rats*, and that all necessary changes to the document have been made.

You are more than welcome to contact me should you require any further information.

Kind regards

Quinton Oosthuizen

Contact Details
Cellphone: 061 491 4828
E-mail: quinton.oosthuizen@gmail.com