Histopathology induced by a medicinal plant indigenous to South Africa that has shown in vitro anti-microbial activity against drug resistant strains of *Mycobacterium Tuberculosis*

by

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In accordance with Rule G4.6.3, I hereby declare that the above-mentioned treatise/ dissertation/ thesis is my own work and that it has not previously been submitted for assessment to another University or for another qualification.

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ABSTRACT

Histopathology induced by a medicinal plant indigenous to South Africa that has shown anti-microbial activity against both drug-sensitive and drug-resistant strains of *Mycobcterium tuberculosis. Mycobacterium tuberculosis* is the causative agent of pulmonary tuberculosis (TB). It is responsible for more deaths annually than any other pathogen globally. (Kathleen *et al.,* 1993).

Pulmonary tuberculosis is a global burden with new cases being reported annually. There are challenges that compromise the conventional treatment of this disease such as drug resistance among TB patients. The ethno-botanical discovery of medicinal plants extracts is bringing a new hope in the fight against the drug resistant strains of *Mycobacterium tuberculosis*. Some researchers in South Africa have ethno-botanically identified some plants which are being used by traditional healers to cure ailments related to tuberculosis. However, there is a threat of toxicity associated with compounds isolated from these medicinal plants (Lall and Meyer, 1999).

The aim of this study was to investigate the histopathology that may be induced by the plant extract that has shown in-vitro inhibition against both drug- resistant and drug-sensitive strains of *Mycobacterium tuberculosis*. The objectives of the study: carry out a 7 day pilot repeated oral toxicity study on albino Swiss mice to: Establish the dosages to be administered in the 28 day study and standardise methodologies for assessing animal weight, feed consumption, clinical observations and histopathology. To carry out a 28 day repeated oral toxicity study using the dosages obtained from the 7 day study on Swiss albino mice.

In the two studies no dose dependent lesions were observed. All lesions observed are spontaneous, age related to laboratory animals. Therefore the NOAL/NOAEL/MTD for this study is 500mg/kg.b.wt.

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ABBREVIATIONS

A

AB.OW	Absolute Organ Weight
AFGSTN	Afghanistan
AIDS	Acquired Immune Deficiency Syndrome
ALB	Albumin
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance

B

BNGDSH	Bangladesh
BRZL	Brazil
BW	Body Weight

C

CHOL	Cholesterol
CLO	Cloride
CLT	Clotting Time
CMC	Car boxy Methyl Cellulose
CPCSEA	Committee for the Purpose of Control Supervision of Experiments on Animals
CRE	Creatinine

D

DMSO	Dimethyl Sulphur Oxide
DRC	Democratic Republic of Congo

E

E. natalensis	Euclea natalensis
EDTA	Ethyl-Diamine Tetra Acetic Acid
ETD	Extensive Drug-Resistant

ETHIOP	Ethiopia
Et al.	And others
EEZZ	Ethanol Extract Zerumbet Rhizomes
G	
G	Group
GLP	General Laboratory Practice
Glu	Glucose
GOT	Glutamic oxaloacetatic transaminase
GPT	Glutamic pyruvate transaminase
H	
H & E	Haematoxylin- Eosin
HD	High Dose
HG	Haemoglobin
HIV	Human Immune Deficiency Virus
I	
INDO	Indonesia
L	
LD	Lower Dose
Lymph	Lymphocytes
M	
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MD	Mid Dose
MDR	Multi-Drug Resistance
MRC	Medical Research Council
MTD	Maximum Tolerated Dose

MZBQ	Mozambique
N	
NBISA	National Biodiversity Institute of South Africa
NDDP	Novel Drug Development Platform
Neutr	Neutrophil
NMMU	Nelson Mandela Metropolitan University
NOAEL	No Observed Adverse Effects Level
NOEL	No Observed Effects Level
NRDP	National Research and Development Platform
O	
OECD	The Organization for Environmental Co-operation and Development
P	
PCV	Packed Cell Volume
PHOS	Phosphorus
Plt	Platelet
Pop	Population
PTB	Pulmonary Tuberculosis
R	
RBC	Red Blood Cell
RDTS	Repeated Dose Toxicity Study
ROTS	Repeated Oral Toxicity Study
ROW	Relative Organ Weight
S	
SA	South Africa
T	
TB	Tuberculosis
TGL	Triglycerides

Tot	Total
TP	Total Protein
U	
U.R. Tanzania	United Republic of Tanzania
URE	Urea
V	
V. NAM	Vietnam
W	
WBC	White Blood Cell
WHO	World Health Organization
WSU	Walter Sisulu University
Z	
ZIM	Zimbabwe

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CHAPTER 1

LITERATURE REVIEW AND STUDY OBJECTIVES

1.1 INTRODUCTION

Tuberculosis (TB) still remains a health problem globally with over a million new infections and a mortality rate of 1.5 million individuals annually (Hawn *et al.*, 2014). The emerging multi-drug resistant (MDR) strains that accompany human immune deficiency virus (HIV) infection in high-incidence populations contribute significantly to the health burden of TB (Areeshi *et al.*, 2014). The standard treatment that is advocated by the World Health Organization (WHO) for active tuberculosis includes long-term therapy that incorporates the use of isoniazid, rifampicin, pyrazinimide and ethambutol as front line drugs (WHO, 2013). Drug resistance against established treatment options for TB makes research into new forms of therapy an imperative in health care (Ntulela *et al.*, 2009).

South Africa is currently witnessing a high number of cases of drug-resistant TB. In some parts of the country, one in ten cases of TB is resistant to treatment. It is therefore essential to have new anti-tuberculosis agents, which can be readily and simply produced from some local source (Warner *et al*., 2014). A logical starting point for this research of new agents would be the herbal medicines which have been used for centuries in rural areas by local healers. Western developed countries have harvested ethno botanical knowledge and have produced drug therapies for conventional medicines for other ailments. The activity of extracts of the active plants and their properties still require study in animal models in order to assess their future as new anti-tuberculosis agents (Lall and Meyer, 1999).

Researchers are working together in the investigation of new TB agents that can be available to patients suffering from TB. Several works have been published recording the ethno botanical use of plants in South Africa, Uganda and Mexico. In the same published papers, it is clearly stipulated that many African and Mexican plants have ethno-botanical use for the treatment of tuberculosis and related symptoms such as coughing, respiratory ailments and fever (Lall and Meyer, 1999; Camacho- Corona *et al.*, 2008; McGaw *et al.*, 2009; Ntulela *et al.*, 2009; Tabuti *et al.*, 2010).

Natural products continue to play the most significant role in the drug discovery and development process and plants are recognized as useful sources of highly active anti-microbial metabolites (Balunas and Kinghorn, 2005). Screening programmes of plant remedies are emphasized so as to validate the traditional use of herbal treatments, and for providing leads in the discovery of new active principles. Such screening programmes have to be supported by toxicity studies (McGaw *et al.*, 2009).

This study focuses on qualitative and quantitative experimental findings after the administration of a medicinal plant extract to animals. This will include daily observation of animals, recording of feed consumption, recording of animal weights, macroscopic examination of animals at necropsy, tissue harvesting, histological procedures and microscopy.

There was no available data on dosages producing toxicity for the experimental plant. The Organisation for Economic Co-operation and Development (OECD) well documents guides on testing and assessing of chemicals employing acute, sub-acute oral toxicity tests, as well as dose range finding studies. Dose range Finding Studies (also known as a 7 or 14 day sub-

acute toxicity studies) are normally carried out prior to a 28 day oral toxicity study in an attempt to establish the upper limit of the dose sequence. This sub-acute toxicity study begins with a three dose regime (e.g 1000mg/kg, 500mg/kg, 250mg/kg body weight) per day per group per sex repeatedly for 7 days. On the other hand acute toxicity testing involves a once off administration (e.g. 1000mg/kg.b.wt) of the chemical, which is then followed by animal observation for a period of 14 days. In the event whereby there is evidence of toxicity, the dose in which toxicity has occurred is used as the upper limit for the dosing sequence in the main study. If there is no such toxicity, the dose will be used as the starting dose for the main study (OECD, 2008).

1.2 PULMONARY TUBERCULOSIS PANDEMIC

Pulmonary tuberculosis is a contagious airborne disease of the lower respiratory tract. The causative agent is *Mycobacterium tuberculosis*. This disease is responsible for more deaths annually than any other disease globally. Although it was thought that TB was controlled especially in the developing countries, the disease is undergoing a dramatic resurgence throughout the world. The emergence of MDR- TB strains further complicates the treatment and control of the bacteria, influencing the need to develop new strategies for prevention and treatment beyond the current available therapies (Verrall *et al.,* 2014).

Tuberculosis (TB) is a global health burden with over a million new infections and a mortality rate of 1.5 million individuals annually (Hawn *et al.*, 2014). The emerging MDR strains of the *Mycobacterium* that is accompanied by Human Immune Deficiency Virus (HIV) in high incidence populations contribute significantly to sustaining the problem. The conventional TB treatment that is advocated by the World Health Organization (WHO) for active tuberculosis includes a long-term therapy (six months) that incorporates the use of Isoniazid, Rifampicin, Pyrazinimide and Ethambutol as front line drugs (Ntulela *et al.,* 2009). The causative agent of PTB (*Mycobacterium tuberculosis)* was first isolated by Robert Koch in 1882. The *M. tuberculosis* is a slender, straight gram positive rod with rounded ends. It is a non-motile, non-sporing organism that stains well with Ziehl-Neelsen stain. The title of the study strictly focuses on PTB since the preliminary studies carried out on ethno-botanically selected plants exhibited promising results when tested against the causative agent of PTB (Lall and Meyer, 1999).

Pulmonary tuberculosis is regarded as the disease which killed the ancient Egyptians and the Iron Age British Settlers (Mokrousov, 2008). Literature concerning scientific knowledge and

the treatment of TB in the past talks about the conventional and internationally accepted first line treatment regime as outlined by the World Health Organization (WHO). The conventional treatment includes the initial treatment with Izoniazid, Rifampicin, Pyrazinamide and Ethambutol for two months, followed by four months continuation phase of further treatment with Izoniazid and Rifampicin. The six months regime has been very successful until recently when the Human Immuno- Deficiency Syndrome came into existence (O'Mahony and McCarthy, 2010).

Multidrug resistant TB (MDR- TB) is one form of TB which is characterized by the resistance of *Mycobacterium tuberculosis* to at least Isoniazid and Rifampicin, the most effective anti-TB drugs. MDR-TB results from either primary infection with the resistant strain or may develop in the course of treatment (Mishra *et al.*, 2014).

Extensively drug-resistant TB (XDR-TB) is the second resistant form of TB that is also caused by the *Mycobacterium tuberculosis* that is resistant to Isoniazid and Rifampicin as well as any flouroquinolones and any of the second-line anti-TB injectable drugs (Amycacin, Kanamycin, or Capreomycin). These forms of TB do not respond to the standard six months treatment with first-line anti-TB drugs. Treatment can take up to two years or more with drugs that are less potent and much more expensive (WHO, 2008).

Recently, there have been reports of an increase in the numbers of drug resistant forms of TB. The WHO surveillance conducted in 2008 estimated 440 000 cases of MDR-TB and 963 cases XDR-TB globally (WHO, 2010). The surveillance documented the potential demographic risk factors as well as clinical risk factors that have contributed to increase in drug resistance. These are:

- Prior exposure to anti-TB drugs
- The association between sex and MDR-TB
- The association between HIV status and MDR-TB

Previously treated TB patients who fail to complete the prescribed conventional therapy (relapses) are likely to develop drug resistance to one or more treatment regimes. This is a major challenge and it impacts badly on the issue of drug resistance (Mishra *et al*., 2014).

In most countries the majority of TB patients who have developed the MDR-TB happen to be males. Furthermore, the WHO which reported outbreaks of drug resistance among people affected with HIV states that currently there is limited information available about the association of HIV and drug resistance TB at a population level. However, the preliminary studies conducted in Mozambique in 2007 have been found to have a significant association. Drug resistant TB maybe associated with HIV for the fact that people affected by HIV have weakened immune system; as a result they are more vulnerable to TB infection (WHO, 2010). Table 1.1 shows recent numbers of tuberculosis incidences, TB patients with HIV + status, MDR-TB in the high burden countries, South Africa inclusive.

Table 1.1 Recent Developments in the Nature of the Disease Globally

	AFGSTN	**BNGDSH**	**BRZL**	**CABODIA**	**CHINA**	**DRC**	**ETHIOP**	**INDIA**	**SA**	**INDO**	**KENYA**
POP in Million	32	150	197	14	1 348	68	85	1 241	50	242	42
Tot new case	26.8	148.2	71.3	38.6	865.1	106.4	154.5	1211.4	325.3	313.6	93.9
Tot Retreatment	1.3	7.4	10	1.5	46.8	7.9	4.6	304.4	45.9	7.7	10.1
TB Hiv +	6.4	1.9	49.1	32.5	208.6	30.6	65.1	688.5	323.4	3.5	97.1
MDR	700	1.7	560	330	49.1	2.6	1.7	21	5	5.7	2.4
NOTE: NUMBERS ARE IN THOUSANDS											
	MZBQ	**MYANMAR**	**NIGERIA**	**PAKISTAN**	**PHILLS**	**RUSSIAN FED**	**THAILAND**	**UGANDA**	**U.R TANZ**	**V.NAM**	**ZIM**
POP in Million	24	24	162	177	95	143	70	35	46	89	13
Tot new case	43.2	13.2	84.2	255.1	188.4	104.3	63.9	45	58.2	91.5	36.9
Tot Retreatment	4.3	11	8.8	11.4	136.6	55.2	37.7	4	2.9	8.6	4.3
TB Hiv +	41.8	4.5	7.6	8.3	3.9	79.5	49.8	39.4	53.5	59.1	35.3
MDR	1.3	4.4	2.5	7.1	7.5	7.5	890	560	480	2	610

(Source: Adapted from WHO Global TB Report 2010)

South Africa in particular is witnessing an explosion in the number of new cases of drug resistant tuberculosis (Klopper *et al*., 2013). It is therefore essential to have new anti-tuberculosis agents that can be produced from local sources. Herbal medicines have been dispensed by traditional healers for especially the rural population to cure a wide range of ailments including PTB (Lall and Meyer, 1999). South Africa has a record of the ethno-botanical use of medicinal plants in the country including the publications by Ntulela, Tabuti, Lall and Meyer. Furthermore, Lall and Meyer specifically emphasize running of the screening programmes of these medicinal plants. These medicinal plants can provide leads in the discovery and development of new active drugs (McGaw *et al.,* 2008). Table 1.2 shows the latest TB rates as per province in South Africa.

Table 1.2 Latest TB rates as per Province in South Africa (2001- 2005)

Province	TB rate / 100,000 2001	TB rate/10,000 2005	% Increase
E Cape	510	662	23
Free State	460	789	42
Gauteng	320	496	36
KZN	206	911	77
Limpopo	91	287	68
Mpumalanga	222	445	50
North West	383	627	39
N Cape	561	922	39
W Cape	841	1031	18
South Africa overall	424	669	37

(Source: Edginton and Naidoo, 2007)

1.2.1 THE WHO RESPONSE ON TB TREATMENT AND CONTROL IN SA

In response to treatment challenges the WHO conducted a study in which an article was published in 2008 under the heading 'Community involvement in tuberculosis care and prevention'. The main focus of the study was to involve the community in the implementation of the prevention strategies of the disease. As stated earlier, the major treatment challenge seemed to occur because TB patients do not complete the six months conventional treatment. The WHO brought up some strategies in their study conducted in South Africa at Gugulethu and Hlabisa areas. In a nutshell the main objective was to evaluate the cost-effectiveness of community-based initiatives compared with conventional approaches, involving community health workers as well as people with TB. The outcome of

the study indicated that the community-based TB care, prevention, and supervision were more cost-effective and acceptable to the patients. (WHO bulletin, 2008). In Hlabisa area the main objective was to assess the acceptability and effectiveness of traditional healers as supervisors of the conventional TB treatment over the clinics supervision. According to WHO report TB patients were satisfied with the services rendered by traditional healers, and for this reason the community-based programme was effective than the hospital care. In view of the above, it is therefore necessary to involve the traditional healers in TB treatment programmes and they can play an important role in the care and control of TB (Klopper *et al*., 2013).

1.2.2 TRADITIONAL MEDICINE AND RESEARCH IN SOUTH AFRICA

World Health Organization defines traditional medicine as the sum of the knowledge, skills and practices based on the theories, beliefs and experience of indigenous different cultures that are used to maintain health as well as to prevent, diagnose, improve or treat physical and mental illness (WHO, 2010). Herbal medicines include herbs, herbal material, herbal preparations and finished herbal products that contain parts of plants or other plant material as active ingredients. Herbal treatments are considered most popular form of traditional medicine. WHO and the member states are co-operating together in promoting the engagement of traditional medicines for health care. The core aim is to:

- Support and integrate traditional medicine into national health systems in combination with national policies and regulation for products, practices and providers to ensure safety and quality.
- Ensure the use of safe, effective and quality products and practices, based on available evidence.

- Acknowledge traditional medicine as part of primary health care, to increase access to care and preserve knowledge and resources.
- Ensure patient safety by upgrading the skills and knowledge of traditional medicine providers (WHO, 2010).

As much as WHO acknowledges traditional medicine, it is still calling for patient safety when traditional medicine is used. Researchers at the University of Pretoria in South Africa, in the department of Botany have conducted several studies on the promising medicinal plant in the fight against both the drug-sensitive (H37Rv) and drug-resistant strains of *Mycobacterium tuberculosis* (CCKO28469v). Lall and Meyer published a paper in 1999 titled 'In-vitro inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by ethno-botanically selected South African plants'. Twenty medicinal plants which are used by traditional healers to cure pulmonary related diseases were tested. Plants extracts of both water and acetone were used. In this study it is reported that *Euclea natalensis* yielded best results in both water and acetone preparations and low toxicity when compared with the rest of the plants tested. Sensitivity testing was carried on two techniques, the traditional plate method and the radiometric method.. In both the methods *Euclea natalensis* showed best results (Lall and Meyer, 1999).

Publications by Bapela have also documented the discovery of compounds, called naphthoquinones from the roots of the same plant, as well as content variation of these compounds in its seed and seedlings. It was discovered that the roots contain much high concentrations of compounds than any other part of the plant (Bapela[(a)] *et al.*, 2008).

1.3 THE LINK BETWEEN DRUG DEVELOPMENT AND TRADITIONAL MEDICINE IN SOUTH AFRICA

Research is underway in South Africa in the hope of discovering new drugs for drug-resistant TB, malaria, obesity and diabetes mellitus. The Medical Research Council of South Africa released a briefing document in 2005 to shed light on the work done by organisations that include scientific councils as well as institutes of higher learning. The National Research and Development Platform (NRDP) for novel drug development from indigenous medical plants are bringing together a multidisciplinary research and development team in a consortium. The aim is to combine technical skills, research facilities and strategies in order to structure and develop novel drugs from medicinal plants. This novel drug development platform (NDDP) project has a unique feature in that it is conducted on indigenous plants by researchers in the country in collaboration with other researchers from the developing countries. The candidate plants are being processed, extracted and screened for in-vitro activity under good laboratory practice (GLP) conditions.

The medicinal plants under investigation are indigenous to South Africa which provides opportunities for discovery specifically for the country. The following are some of the project's aims for the period of 5 years (2005-2010) since its inception.

- To develop effective and safe novel drugs for TB, malaria and diabetes.
- To make sure that researchers benefit from transfer of technology
- The creation of agricultural processing opportunities of medicinal plants in rural areas
- To patent novel plant-derived compounds

- To place South Africa in forefront of discovery and development of plant-derived medicines

The document further goes on to state that after the in-vitro screening of the medicinal plants, the in-vivo testing will follow using models of disease in animals including testing against drug-resistant TB. WHO made arrangements for the consortium to collaborate with other countries in the Sub-Saharan Africa (Folb and Bhagwandin, 2004).

1.4 *Euclea natalensis*: A MEDICINAL PLANT WITH PROMISING ANTI-TB ACTIVITY

In view of the above work being carried out in South Africa, *Euclea natalensis* plant has been identified by South African botanists to be a potential plant in the discovery of novel drugs in the fight against the drug-sensitive and drug-resistant strains of bacteria.

Cytotoxicity evaluation, particularly of plant extracts which have shown in-vitro activity against human pathogens is essential before such plant extracts could be considered for drug discovery. In an investigation of the cytotoxic effects of the crude extracts of *Euclea natalensis,* the extract showed no signs of toxicity at a concentration of 100µg/ml against primary vervet monkey kidney cells. While this might indicate a promising drug worthy of further exploration the question of cytotoxicity remains unresolved. In addition toxicity testing using animal cell cultures may not be a reliable tool to evaluate toxicological effects for humans because the metabolism of foreign compounds differs from cell to cell (Lall *et al*., 2006). Acute, sub-acute, sub-chronic and chronic toxicity studies on all organs need to be carried out in order to validate toxicological effects in humans. Cell cultures are short assays done over short periods where cells are exposed to the plants extract for short periods. TB is known to be a chronic disease with treatment administration of six months. Consequently chronic studies are needed for toxicity evaluation on the potential plants.

1.4.1THE PLANT CLASSIFICATION

The plant under investigation belongs to the *Ebenacaea* family (Figure 1.1). There are two classes within the *Ebenacaea* family called Ebony and Persimmon. Ebony has two genera namely Diospyros comprising 450-500 species and Euclea comprising 20 species. *Euclea natalensis* falls under the Euclea genera (Notton, 2010).

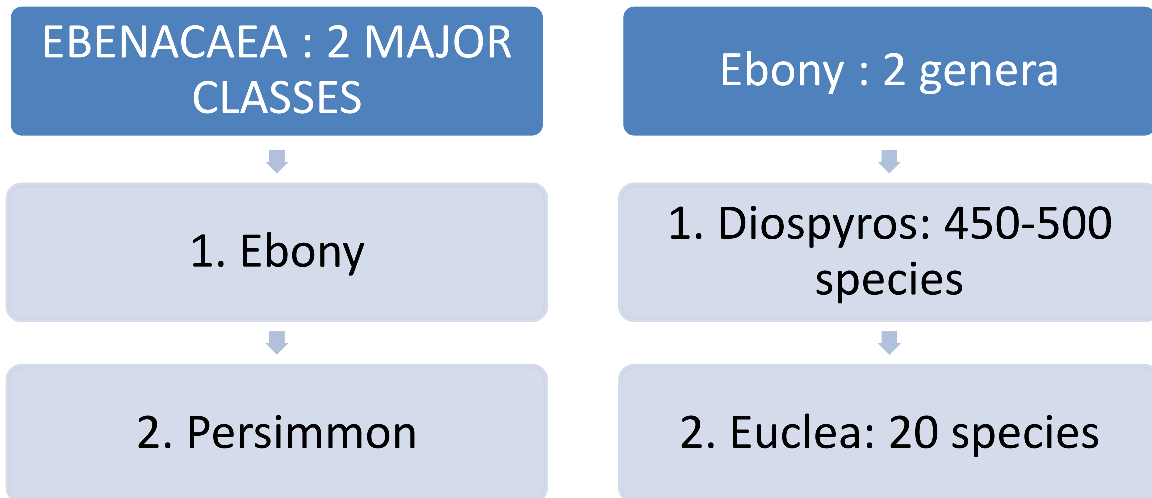


Figure 1.1 The *Ebenacaea* plant kingdom

Euclea natalensis has the following sub-species:

- Augustifolia White
- Carpensis White
- Magutensis White
- Obivata White
- Rotundifolia White and
- natalensis

1.4.2 THE DISTRIBUTION OF THE PLANT IN SOUTH AFRICA

Euclea natalensis is widely found around Southern Africa. It is found in the Western Cape along the south and the east coast through to Eastern Cape, also in Kwazulu-natal, Swaziland, Mozambique, Mpumalanga, Limpopo, Gauteng, and in Ethiopia. It grows under many different conditions in wide variety of habitats, ranging from rocky scrub to dune bush. The name *Eucle*a means of good report or famous. The species *epithet natalensis* means from Natal. The khoisan clan originally named it the guarrie. The Zulu name inKunzane means the strong enema and inKunzane the strong black enema. *Euclea natalensis* is used for tooth

brushing. The roots and the bark are used in quite many traditional remedies. It has been used in the treatment of stomach disorders, toothache, chest complaints, urinary infections (Notton, 2010).

1.4.3 THE PLANT NOMENCLAUTURE

The nomenclature of *Euclea natalensis* is variable depending on the tribe and region in South Africa. Table 1.5 is a summary of the most commonly found terms used to describe this plant in various tribal/regional areas of South Africa.

Table 1.3 Tribal and regional variations in plant nomenclature

REGION/TRIBE	NAME
Natal/Khoisan	Natal guari or Natal ebony
English	Large-leaved guari/ natal ebony
Afrikaaners	Natalghwarrie, Berggwarrie, Swartbasbom
Xhosa	umTshakisane, umKhasa
Zulus	iDungamizi, iChutamuzi, umZimane, umTshikisane, inKunzane-emnyama, umHlalanyamazane, umAnyathi
Tsonga	umHlangula

(Source: Mander, 1998)

1.4.4 PLANT DESCRIPTION

Euclea natalensis is a small bushy tree (shrub)(Figure 1.2). It is about 4-10m tall with a dense spreading crown. In warm well-watered conditions the plant can grow to be a medium-sized tree of about 12-18m tall, with spreading branches. The spread can reach to about approximately 50m. The trunk is usually straight with a thin bark that is finely cracked and grey in colour. The branches of the tree are covered in fine rusty hairs, which smoothes with

age. The leaves are hard and leathery with dark green colour. They are shiny above and pale underneath. Occasionally the upper surface is covered with hairs while the lower surface is often covered with thick velvety red hairs. The leaves vary in size, shape, and hairiness. Often the shape of the leaves is oblong with pointed or bluntly pointed, rounded tips. Leaves have prominent veins especially on the upper surface with thickened margins. In spring the new growth is very decorative with its pale, gold-tinged velvety hairs.

The plant is dioecious which means the male and the female flowers are borne on separate trees. The flowers are quite small, bell shaped and cream to yellow in colour (Figure 1.3). They have a heavy sweet scent. Usually male flowers contain sixteen stamens and a very small rudimentary ovary with two types. Female flowers do not have the staminodes. The fruits are rounded, fleshy berries of about 70-130 mm in diameter borne on hairy stalks. The berries are edible and attract birds. Flowering occurs in winter to spring, fruiting during summer and ripening in autumn. *Euclea natalensis* is an adaptable easy to grow tree with a very neat, dense rounded crown of dark green foliage, decorated with pale, gold-tinged new growth (Kei Herbarium, 2010).

Figure 1.2 Digital Photo of *Euclea natalensis*
(Source: Own digital Image Location: Xura River Lusikisiki 2011)

Figure 1.3 Digital Photo of the Flowers of *Euclea natalensis*
(Source: Notton, 2010)

1.4.5 MEDICINAL USES OF *EUCLEA NATALENSIS*

Euclea Natalensis is used for a variety of purposes such as a source of food, as firewood and to prepare dyes (Lall and Meyer, 1999; Notton, 2010). Table 1.6 outlines the common uses of this plant in rural communities in South Africa.

Table 1.4 Medicinal Uses of *Euclea Natalensis*

Food	Fruits are eaten, bark used for meat preparation, as well as milk soups
Fuel	Branches are used for fire wood
Dyes	Bark extract is used to prepare dyes
Alcohol	Fruits are used for brewing beer
Medicinal	Bark is chewed for oral hygiene and appetizer, fruits and roots used as purgative, root and bark again used for treatment of stomach disorders, chest pains, urinary infections, worms, headaches, venereal diseases as well as leprosy

(Source: Adapted from Lall and Meyer, 1999; Notton, 2010)

1.4.6 THE PHARMACOLOGICAL PROPERTIES OF *EUCLEA NATALENSIS*

The pharmacological activity of *Euclea Natalensis* has been demonstrated by the presence of naphthoquinones which are the dominant secondary metabolites of Ebenaceae family. Naphthoquinines are natural phenols and have been found to have antimicrobial properties against a broad spectrum of pathogens i.e gram positive and gram negative bacteria, mycobacterium tuberculosis, and fungi (Kishore *et al.*, 2014). Pharmacologically they are

classified as cytostatic and cytotoxic agents. It is documented that many novel synthetic naphthoquinones derivatives which have anti-carcinogenic properties have been prepared (Babula *et al.,* 2009; Kishore *et al*., 2014). It has been discovered that these metabolites are found in high concentrations in roots of *Euclea natalensis* and they are: Diospyrin, Isodiospyrin, Mamegakinone, 7-Methyljuglone, Neodiospyrin and Shinanolone (Bapela[(b)] *et al.,* 2007; Kishore *et al*., 2014).

The plant has been proven scientifically that in it novel drugs can be identified in the fight against drug-resistant TB. All the naphthoquinones of genus *Euclea* have potent anti-tuberculosis activity especially 7-Methyljuglone, Diospyrin and Shinanolone (Van der kooy *et al.,* 2006; Babula[(a)] *et al.,* 2008; Kishore *et al*., 2014).

1.5 TRADITIONAL MEDICINE AND ITS DRAWBACK

About 60-80% of population use traditional medicines commonly known as muti in South Africa, some of which are toxic. A study was conducted on patients admitted to hospitals in Johannesburg over a period of one year in 2003. The overall number of cases with a history of 'muti' administration was seventy with an overall in-hospital mortality of 33%. It is reported that identity of medication was unknown to both the patients and the relatives. Of the mentioned population, 66% were less than a year old. According to their report, the route of administration was known to only 52% and included oral (54%), rectal (23%) and a combination of both oral and rectal routes (23%). In addition side effects noted included gastro-intestinal problems (53%), dehydration (59%), symptomatic liver disease (53%), renal dysfunction (97%), malnourished (23%) and kwashiorkor (5%) (Steenkamp *et al.,* 2003).

Traditional remedies, especially those involving plant products may be considered to be pharmaceutical products. They may also have associated side effects (Krige, n.d.; Seifert *et al.*, 2011). According to Krige, toxicity of plants can vary on a seasonal basis. She further states that preparation processes have significance for toxicity in that some heating processes may eliminate toxicity or alternatively increase toxicity. Research and knowledge of pharmaceutically active compounds in traditional medicines can make a great contribution towards making the preparation methods more systematic and reliable (Krige, n.d.).

The above literature demonstrates that the use of medicinal plants does pose a health threat (Niggemann and Grüber, 2003). The WHO recommends and encourages the incorporation of traditional medicine within the health fraternity as well as proper and safe use of these medicinal plants (WHO, 2010). This study therefore intends to offer an insight of any

potential toxic effects of *Euclea natalensis* by repeatedly exposing living mammalian tissues to the plant extract and with subsequent histopathologic investigation.

1.6 THE USE OF PLANT EXTRACTS IN TOXICITY TESTING

Following a literature review search no plants with putative anti TB properties have been tested for toxic potential in a mammalian model such as rats or mice. However, literature does document toxicity testing on other plants (*Mimosaceous, Zingerber zerumbet Smith*, and *Ocimum tenuiflorum*) with anti-microbial properties over other strains of bacteria (Ahmet *et al.*, 2012, Ju Chang *et al.,* 2012 and Kalaiselvi *et al.,* 2013).

Ahmed in his study investigated the acute systemic toxicity of methanolic extracts of leaves of plants belonging to *Mimosaceous* family in Swiss albino mice (*Adenanthera pavonina L, Peltophorum roxburghii Deneger, Prosopis cineraria Druce and Prosopis juliflora* DC). These four plants are found in Pakistan as well as in India, and they possess chemicals including triterpenes, flavonoids, fatty acids, sterols, glycosides, alkaloids, amino acids, and diketones. The leaf extract was administered intraperitoneally and clinically observed for 48 hours. Table 1.5 shows the clinical observations of Swiss Albino mice exposed to methanolic extracts of leaves of plants belonging to *Mimosaceous* family.

Table 1.5 Clinical observations of Swiss Albino mice exposed to methanolic extracts of leaves of plants belonging to *Mimosaceous* family.

Treatment	Doses(mg/kg Body weight)	OBSERVATIONS						MORTALITY %
		INJECTION	AFTER					
		IMMEDIATELY	½ hr	1hr	4hrs	24hrs	48hrs	
DMSO (con	---------	Normal activity	normal	normal	normal	normal	normal	----------
A. pavonina	100	Normal	normal	normal	normal	normal		0
	150	Normal	normal	normal	normal	normal		0
	200	Breathing Problem	Breathing problem	Paralysis effect	No movement	Died	-------	100
P. roxburgii	100	Normal	normal	normal	normal	normal	normal	0
	150	Loss of sense	Paralysis on hind limbs	Paralysis Of hind limbs	3 died, 3 survived	Died	------	100
	200	Breathing problem	Breathing problem	Para, hair erection	Died	------	------	100
P.cineraria	100	Normal	normal	normal	normal	normal	normal	0
	150	Loss of senses	Paralysis of hind limbs	Paralysis of hind limbs	3 died 3 survived	Died	-----	100
	200	Breathing problem	Breathing problem	Para, hair erection	Died	-------	------	100
P. juliflora	100	Normal	normal	normal	normal	normal	normal	0
	150	Normal	Paralysis of hind limbs	Paralysis of hind limbs	3 died 3 survived	Died	------	100
	200	Breathing problem	Breathing problem	Para, hair erection	Died	-----	--------	100

(Source: Ahmet *et al*., 2012)

Zingiber zerumbet (L.) Smith (ZZEE) is a medicinal plant used widely in the tropics of Southeast Asia. It is commonly known as pine cone or shampoo ginger. It is widely used in Asia and in Arabic countries as a spice and traditional medicine to cure many ailments (Santosh Kumar *et al*., 2013). Acute and sub-acute (28 days) toxicity studies were carried out

on ethanol extracts of the plant to evaluate the toxicity following the limit test procedure as recommended by OECD guidelines. The extract was administered orally in the concentrations; 1000mg/kg, 2000mg/kg and 3000mg/kg.b.w to male and female Wistar rats. No clinical observations revealed any sign of toxicity on all treated animals. The haematological and biochemical parameters, food consumption as well as absolute organ weights and relative organ weights did not reveal any evidence of toxicity in all treated animals as compared to controls (Ju chang *et al*., 2012).

The tables below represent summary of the results obtained from this study. Wistar rats (n = 10 per group) were administered ZZEE by daily gavage for 4 weeks. Data are expressed as mean ± SD.

Table 1.6 Effects of sub-chronic oral administration of the ethanol extract of EEZZ on body weight, food and water intakes in Wistar rats.

Week (W) of Treatment	Female				Male			
	Vehicle	EEZZ (mg/ kg per day)			Vehicle	EEZZ (mg/ kg per day)		
	G1	G2(1000)	G3(2000)	G4(3000	G1	G2(1000)	G3(2000)	G4(3000)
Body weight (g)	**G1**	**G2**	**G3**	**G4**	**G1**	**G2**	**G3**	**G4**
W1	212.97 ± 15.97	214.83 ± 13.42	215.32 ± 16.41	218.92 ± 13.56	223.61 ± 16.48	225.58 ± 14.59	229.26 ± 16.41	228.12 ± 15.92
W2	229.60 ± 14.75	224.67 ± 14.87	219.83 ± 19.26	223.97 ± 16.92	241.08 ± 15.24	235.90 ± 16.11	241.81 ± 13.88	246.37 ± 19.61
W3	245.11 ± 17.36	249.32 ± 15.96	233.51 ± 13.88	241.28 ± 15.86	257.36 ± 17.91	261.78 ± 17.26	256.68 ± 19.26	265.41 ± 20.15
W4	244.72 ± 12.63	245.21 ± 17.81	237.72 ± 14.72	243.81 ± 17.41	256.96 ± 13.08	257.47 ± 18.90	261.49 ± 14.72	268.19 ± 18.44
Food intake (g /day per rat)								
W1	22.86 ± 4.04	25.95 ± 3.01	25.29 ± 2.84	24.66 ± 3.12	24.13 ± 4.13	26.23 ± 3.47	26.36 ± 4.15	26.17 ± 4.21
W2	20.31 ± 3.69	22.25 ± 2.94	21.62 ± 3.14	20.44 ± 3.68	23.16 ± 4.45	23.32 ± 4.17	23.52 ± 3.69	23.52 ± 3.68
W3	22.40 ± 3.87	20.83 ± 3.74	21.11 ± 3.49	21.45 ± 4.95	23.21 ± 4.17	22.39 ± 3.49	22.32 ± 4.18	23.58 ± 3.95
W4	19.70 ± 3.94	20.59 ± 4.78	21.70 ± 4.32	20.97 ± 3.34	20.08 ± 3.62	21.74 ± 3.35	22.58 ± 3.93	23.14 ± 4.87
Water intake (ml/ day per rat)								
W1	24.12 ± 5.62	25.63 ± 6.25	24.08 ± 6.61	24.37 ± 5.12	25.32 ± 6.18	26.38 ± 6.38	25.28 ± 7.41	25.37 ± 6.12
W2	25.09 ± 8.61	25.82 ± 5.61	24.62 ± 7.92	24.19 ± 6.83	26.34 ± 9.47	26.11 ± 6.15	25.85 ± 6.76	25.91 ± 7.38
W3	26.17 ± 5.09	25.56 ± 6.52	26.51 ± 8.12	23.82 ± 7.14	27.47 ± 5.59	26.83 ± 5.28	27.83 ± 7.31	26.27 ± 8.01
W4	24.41 ± 4.97	24.09 ± 5.18	25.41 ± 6.34	25.43 ± 7.27	25.63 ± 5.47	25.29 ± 5.18	25.41 ± 8.12	25.96 ± 6.98

(Source: Ju chang *et al*., 2012)

Table 1.7 Effects of sub-chronic oral administration of the ethanol extract of EEZZ on haematological parameters of Wistar rats.

Parameters	Female				Male			
	Vehicle	EEZZ (mg/kg per day)			Vehicle	EEZZ (mg/ kg per day)		
	G1	G2(1000)	G3(2000)	G4(3000)	G1	G2(1000)	G3(2000)	G4(3000)
WBC (10^3 L^{-1})	9.09 ± 0.59	8.69 ± 0.62	8.78 ± 0.57	8.61 ± 0.64	9.20 ± 0.68	8.90 ± 0.70	8.59 ± 0.73	8.76 ± 0.64
Lymphocytes (10^3 L^{-1})	6.76 ± 0.40	6.66 ± 0.65	6.58 ± 0.35	7.14 ± 0.65	6.92 ± 0.75	7.36 ± 0.63	7.52 ± 0.60	7.86 ± 0.71
Erythrocytes (10^6 L^{-1})	7.29 ± 0.07	7.69 ± 0.10	7.70 ± 0.08	7.76 ± 0.11	7.43 ± 0.11	7.34 ± 0.09	7.37 ± 0.08	7.46 ± 0.10
Hemoglobin (g dL^{-1})	16.28 ± 0.18	16.36 ± 0.17	16.29 ± 0.14	16.47 ± 0.17	17.97 ± 0.24	17.72 ± 0.18	17.48 ± 0.16	17.59 ± 0.23
Hematocrit (%)	41.17 ± 0.25	42.93 ± 0.21	43.29 ± 0.19	43.14 ± 0.21	42.53 ± 0.26	40.28 ± 0.31	40.51 ± 0.29	40.94 ± 0.23
MCV (fL)	52.14 ± 0.32	52.26 ± 0.28	50.13 ± 0.29	51.97 ± 0.32	50.35 ± 0.29	51.91 ± 0.25	52.76 ± 0.27	49.55 ± 0.23
MCH (pg)	18.86 ± 0.13	19.49 ± 0.19	20.56 ± 0.12	19.43 ± 0.12	19.85 ± 0.19	19.81 ± 0.18	19.78 ± 0.16	19.31 ± 0.17
MCHC (g dL^{-1})	35.43 ± 0.31	35.62 ± 0.42	35.57 ± 0.38	35.76 ± 0.29	35.30 ± 0.25	36.07 ± 0.33	36.04 ± 0.27	36.01 ± 0.27
RDW (%)	12.12 ± 0.15	12.15± 0.16	12.16 ± 0.14	12.15 ± 0.17	17.04 ± 0.13	17.10 ± 0.12	17.55 ± 0.13	17.57 ± 0.17
Platelets (10^3 L^{-1})	647.54 ± 26.98	626.31 ± 25.23	665.08 ± 28.80	642.08 ± 32.44	679.54 ± 37.73	611.85 ± 39.58	619.09 ± 25.29	606.15 ± 28.31
MPV (fL)	6.08 ± 0.18	5.90 ± 0.08	5.89 ± 0.06	5.88 ± 0.08	5.48 ± 0.09	5.21 ± 0.08	5.29 ± 0.10	5.29 ± 0.07

(Source: Ju chang *et al*., 2012)

Table 1.8 Effects of sub-chronic oral administration of the ethanol extract of EEZZ on biochemical parameters of Wistar rats.

Parameter	Female				Male			
	Vehicle	EEZZ (mg/ kg per day)			Vehicle	EEZZ (mg /g per day)		
	G1	G2(1000)	G3(2000)	G4(3000)	G1	G2(1000)	G3(2000)	G4(3000)
Glucose (mg dL^{-1})	81.30 ± 7.73	84.51 ± 5.35	82.10 ± 6.19	85.88 ± 7.34	87.62 ± 5.29	89.02 ± 6.14	92.38 ± 5.21	98.62 ± 4.52
BUN (mg dL^{-1})	44.83± 2.14	45.76 ± 2.16	45.82 ± 1.98	43.51 ± 1.79	45.24 ± 1.63	46.26 ± 1.93	46.76 ± 1.72	43.52 ± 2.11
Creatinine (mg dL^{-1})	0.56 ± 0.01	0.56 ± 0.02	0.58 ± 0.01	0.55 ± 0.01	0.56 ± 0.01	0.57 ± 0.01	0.58 ± 0.01	0.56 ± 0.01
AST (U L^{-1})	149.51 ± 10.38	149.81 ± 11.51	146.24 ± 10.94	147.64 ± 12.87	143.54 ± 12.48	144.62 ± 11.29	145.83 ± 13.54	144.85 ± 10.76
ALT (U L^{-1})	71.46 ± 3.21	73.85 ± 3.19	72.25 ± 3.62	74.38 ± 2.85	70.77 ± 2.54	79.00 ± 3.47	75.58 ± 3.26	72.54 ± 2.78
Total cholesterol (mg dL^{-1})	96.50 ± 4.84	98.28 ± 5.70	100.41 ± 4.66	98.21 ± 5.50	97.77 ± 4.20	97.58 ± 5.28	93.05 ± 4.24	94.12 ± 5.15
Triglycerides (mg dL^{-1})	76.85 ± 7.65	74.23 ± 7.78	74.31 ± 8.10	73.41 ± 7.53	80.52 ± 5.67	82.45 ± 6.90	83.41 ± 8.41	82.10 ± 8.13
HDL (mg dL^{-1})	65.59 ± 3.77	61.83 ± 3.65	63.84 ± 3.24	65.72 ± 3.57	43.10 ± 2.51	40.52 ± 1.17	42.03 ± 1.89	41.30 ± 1.68
Amylase (U L^{-1})	515.26 ± 31.56	485.62 ± 37.97	482.50 ± 38.96	498.64 ± 31.67	702.64 ± 59.21	682.43 ± 29.07	714.65 ± 35.41	664.32 ± 56.11
GGT (U L^{-1})	2.47 ± 0.02	2.49 ± 0.01	2.48 ± 0.02	2.50 ± 0.01	2.53 ± 0.01	2.54 ± 0.02	2.51 ± 0.03	2.50 ± 0.02
Total bilirubin (mg dL^{-1})	0.18 ± 0.01	0.17± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.16 ± 0.01
Direct bilirubin (mg dL^{-1})	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Indirect bilirubin (mg dL^{-1})	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.13 ± 0.02	0.11 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.11 ± 0.01

(Source: Ju chang *et al*., 2012)

Table 1.9 Effects of subchronic oral administration of the ethanol extract of EEZZ on organ weights of female Wistar rats.

Organs	Organ weight (g)				Relative organ weight (g 100 g of body weight)			
	Vehicle	EEZZ (mg/ kg per day)			Vehicle	EEZZ (mg/kg per day)		
	G1	G2(1000)	G3(2000)	G4(3000)	G1	G2(1000)	G3(2000)	G4(3000)
Spleen	0.445 ± 0.028	0.436 ± 0.031	0.456 ± 0.027	0.454 ± 0.027	0.182 ± 0.011	0.178 ± 0.018	0.184 ± 0.013	0.186 ± 0.012
Brain	1.111 ± 0.026	1.124 ± 0.030	1.097 ± 0.027	1.116 ± 0.031	0.454 ± 0.019	0.458 ± 0.015	0.443 ± 0.014	0.458 ± 0.016
Heart	0.853 ± 0.018	0.847 ± 0.023	0.825 ± 0.025	0.860 ± 0.019	0.349 ± 0.014	0.345 ± 0.017	0.333 ± 0.011	0.353 ± 0.009
Esophagus	0.093 ± 0.002	0.088 ± 0.003	0.093 ± 0.003	0.093 ± 0.002	0.038 ± 0.003	0.036 ± 0.002	0.038 ± 0.002	0.039 ± 0.004
Stomach	1.385 ± 0.027	1.369 ± 0.026	1.371 ± 0.031	1.379 ± 0.029	0.566 ± 0.013	0.558 ± 0.017	0.553 ± 0.015	0.566 ± 0.016
Liver	8.631 ± 0.048	8.633 ± 0.052	8.523 ± 0.046	8.651 ± 0.061	3.527 ± 0.017	3.520 ± 0.022	3.441 ± 0.019	3.548 ± 0.021
Adrenal gland	0.023 ± 0.002	0.025 ± 0.001	0.026 ± 0.002	0.029 ± 0.002	0.009 ± 0.001	0.010 ± 0.002	0.010 ± 0.003	0.011 ± 0.002
Hypophysis	0.004 ± 0.002	0.004 ± 0.001	0.004 ± 0.001	0.004 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001
Hypothalamus	0.027 ± 0.002	0.028 ± 0.001	0.029 ± 0.001	0.027 ± 0.002	0.011 ± 0.003	0.011 ± 0.002	0.011 ± 0.001	0.012 ± 0.003
Small intestine	0.516 ± 0.021	0.525 ± 0.018	0.515 ± 0.019	0.521 ± 0.022	0.211 ± 0.009	0.214 ± 0.008	0.208 ± 0.007	0.214 ± 0.009
Lungs	1.309 ± 0.053	1.278 ± 0.046	1.308 ± 0.055	1.320 ± 0.043	0.535 ± 0.017	0.521 ± 0.014	0.528 ± 0.019	0.541 ± 0.022
Kidney	0.859 ± 0.029	0.867 ± 0.034	0.874 ± 0.045	0.864 ± 0.037	0.351 ± 0.018	0.354 ± 0.019	0.353 ± 0.017	0.355 ± 0.020
Ovary	0.044 ± 0.003	0.043 ± 0.003	0.042 ± 0.002	0.042 ± 0.002	0.018 ± 0.002	0.017 ± 0.002	0.017 ± 0.003	0.017 ± 0.002
Uterus	0.546 ± 0.026	0.566 ± 0.029	0.574 ± 0.031	0.551 ± 0.039	0.223 ± 0.022	0.230 ± 0.026	0.231 ± 0.025	0.226 ± 0.025

(Source: Ju chang *et al*., 2012)

Table 1.10 Effect of sub-chronic oral administration of the ethanol extract of EEZZ on organ weights of male Wistar rats.

Organs	Organ weight (g)				Relative organ weight (g 100 g of body weight)			
	Vehicle	EEZZ (mg /kg per day)			Vehicle	EEZZ (mg /kg per day)		
	G1	G2(1000)	G3(2000)	G4(3000)	G1	G2(1000)	G3(2000)	G4(3000)
Spleen	0.591 ± 0.038	0.571 ± 0.041	0.599 ± 0.039	0.585 ± 0.043	0.230 ± 0.012	0.222 ± 0.009	0.229 ± 0.011	0.218 ± 0.013
Brain	1.188 ± 0.015	1.197 ± 0.017	1.198 ± 0.018	1.171 ± 0.021	0.464 ± 0.022	0.466 ± 0.027	0.459 ± 0.019	0.437 ± 0.023
Heart	1.083 ± 0.048	1.072 ± 0.039	1.074 ± 0.043	1.086 ± 0.047	0.427 ± 0.009	0.330 ± 0.016	0.319 ± 0.013	0.282 ± 0.018
Esophagus	0.133 ± 0.010	0.127 ± 0.009	0.122 ± 0.011	0.130 ± 0.008	0.423 ± 0.008	0.049 ± 0.011	0.046 ± 0.009	0.049 ± 0.013
Stomach	1.438 ± 0.038	1.482 ± 0.045	1.453± 0.047	1.461 ± 0.041	0.561 ± 0.021	0.577 ± 0.019	0.556 ± 0.023	0.546 ± 0.021
Liver	9.971 ± 0.035	9.952 ± 0.043	9.986 ± 0.048	9.994 ± 0.052	3.894 ± 0.118	3.872 ± 0.153	3.826 ± 0.175	3.729 ± 0.143
Adrenal gland	0.024 ± 0.002	0.025 ± 0.001	0.021 ± 0.003	0.023 ± 0.003	0.009 ± 0.003	0.009 ± 0.002	0.008 ± 0.002	0.009 ± 0.003
Hypophysis	0.004 ± 0.001	0.004 ± 0.001	0.004 ± 0.001	0.004 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001
Hypothalamus	0.036 ± 0.001	0.035 ± 0.001	0.035 ± 0.001	0.034 ± 0.001	0.014 ± 0.002	0.014 ± 0.001	0.013 ± 0.001	0.013 ± 0.002
Small intestine	0.572 ± 0.045	0.586 ± 0.057	0.582 ± 0.063	0.597 ± 0.068	0.223 ± 0.021	0.228 ± 0.019	0.223 ± 0.017	0.223 ± 0.025
Lungs	1.435 ± 0.066	1.441 ± 0.059	1.463 ± 0.065	1.470 ± 0.078	0.381 ± 0.025	0.561 ± 0.028	0.559 ± 0.031	0.549 ± 0.032
Kidney	0.975 ± 0.053	0.967 ± 0.059	0.968 ± 0.062	0.948 ± 0.058	0.336 ± 0.015	0.346 ± 0.017	0.351 ± 0.016	0.313 ± 0.015
Epididymis	0.615 ± 0.056	0.603 ± 0.045	0.625 ± 0.038	0.620 ± 0.047	0.239 ± 0.013	0.235 ± 0.009	0.239 ± 0.010	0.232 ± 0.012
Vas deferens	0.125 ± 0.011	0.129 ± 0.009	0.131 ± 0.008	0.132 ± 0.009	0.049 ± 0.003	0.050 ± 0.005	0.050 ± 0.004	0.049 ± 0.006
Prostate	0.553 ± 0.071	0.547 ± 0.053	0.569 ± 0.064	0.565 ± 0.056	0.215 ± 0.011	0.213 ± 0.009	0.218 ± 0.013	0.211 ± 0.012
Testicle	1.618 ± 0.072	1.627 ± 0.068	1.614 ± 0.078	1.637 ± 0.059	0.629 ± 0.032	0.632 ± 0.027	0.617 ± 0.031	0.610 ± 0.025
Seminal vesicle	0.467 ± 0.022	0.471 ± 0.017	0.478 ± 0.021	0.469 ± 0.019	0.182 ± 0.007	0.183 ± 0.010	0.183 ± 0.009	0.175 ± 0.013

(Source: Ju chang *et al*., 2012)

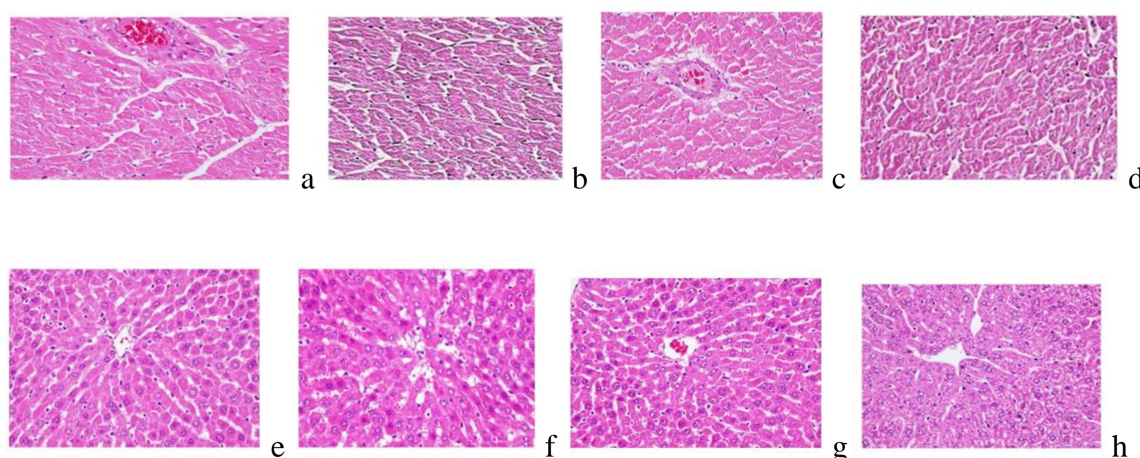


Figure 1.4 Photo Micrographs of heart and liver of EEZZ-treated rats. Heart of control (a) and 1000, 2000, and 3000 mg /kg EEZZ-treated rat (b, c, d), and liver of control (e) and 1000, 2000, and 3000 mg/kg EEZZ-treated rat (f, g, h) stained with haematoxylin and eosin (100x). (Source: Ju chang *et al*., 2012)

Similarly, another toxicity study was conducted on leave extract of *Ocimum tenuiflorum* over 30 days (chronic toxicity study) (Kalaiselvi et al., 2013. The plant is located in Sembakkam, Tambaram, TamilNadu India, and its phytochemical analysis revealed the presence of chemicals such as phenols, terpenoids, tannins, flavonoids and reducing sugars. The study documented on anti-bacterial properties of the plant including properties such a hypoglycemic, anti-inflammatory and wound healing. The aqueous leave extract of the plant was administered by oral gavage to wistar albino rats to assess its toxicity for 30 days. Both biochemical parameters and the body weight well fell within the normal ranges. Histopathological examination of major vital organs (heart, liver, kidney, lung and brain) revealed no significant pathological alterations in the treated animals. The table below shows the results obtained.

Table 1.11 Summary of Body weight changes in experimental rats treated with *Ocimum tennuiflorum* extract.

Groupings	Initial weight	Final weight
Control Group 1	143±14.75	226±20.10
Group 2	152±15.97	240±21.19
Group 3	153±15.43	253±20.97
Group 4	151±15.38	276±20.50

(Source: Kalaiselvi *et al*., 2013)

Table 1.12 Summary of Biochemical parameters analyzed in serum of controls and *Ocimum* treated rats

parameters	Group 1	Group 2	Group 3	Group 4
Urea	8.87±0.89	8.97±0.77	9.13±0.81	9.33±
Creatinine	0.40±0.03	0.47±0.05	0.50±0.05	0.53±
Uric acid	1.35±0.14	1.23±0.16	1.32±0.17	1.54±
Protein	6.33±0.59	6.38±0.51	6.32±0.72	6.40±
AST	13.43±1.37	13.53±1.29	13.7±1.3	12.01±
ALT	8.34±0.83	8.99±0.90	9.52±1.05	9.95±

Note: Values expressed as Mean ± SD of 6 animals/group G1- control, G2-100mg/kg, G3-500mg/kg, &G4-1000mg/kg b.w.

(Source: Kalaiselvi et al., 2013)

1.7 THE AIM AND OBJECTIVES OF THE STUDY

Investigate the histopathology that may be induced by the plant extract that has shown in-vitro inhibition against both drug- resistant and drug-sensitive strains of *Mycobacterium tuberculosis*.

The central objectives of this study are two-fold:

1. Carry out a 7 day pilot repeated oral toxicity study on albino Swiss mice to:
    - establish the dosages to be administered in the 28 day study,
    - standardise methodologies for assessing animal weight, feed consumption, clinical observations and histopathology.
2. Carry out a 28 day repeated oral toxicity study using the dosages obtained from the 7 day study on Swiss albino mice.

CHAPTER TWO

REPEATED DOSE (7 DAY) ORAL TOXICITY STUDY OF *EUCLEA NATALENSIS* IN SWISS ALBINO MICE

2.1 INTRODUCTION

Short-term studies (both acute and sub-acute) are conducted to establish the mechanism of action of the test compounds, to give some ideas of the toxicity, also to identify possible target organs. The studies utilise standard histopathological and clinical pathology techniques. The main goal of toxicological pathology is to establish the exact degree of risk posed to the treated animals under controlled conditions. The very similar process is applicable to diagnostic pathology in lesion evaluation, but differing in the manner in which data are compiled. In toxicological studies the focus is on representative response of the treated group verses individual patients in diagnostic pathology. The objective being to quantify the average response of the treated groups compared to untreated control group (Haschek, Rousseaux, and Walling, 2002).

The Naphthoquinones have been the subject of interest in this study. These are a group of compounds identified in the root material of the *Euclea natalensis* plant. The compounds have shown (in-vitro) microbial activity against both drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* (Lall and Meyer, 1999). The recent study has shown that these compounds (e.g. diospyrin and 7-methyljuglone) have been shown to have therapeutic potential, particularly against *Mycobacterium tuberculosis*. It is found that these compounds are inhibitors of the supercoiling reaction catalyzed by *Mycobacterium tuberculosis* gyrase and other gyrases (Karkare *et al*., 2013).

The evaluation and the assessment of chemical substances for their possible toxic characteristics are very important. This assessment is very useful in the determination of toxicity of substances where such substances are going to be tested in animal studies following a repeated dosing regimen. The initial information on toxicity of such substances could be obtained by acute toxicity testing prior to conducting repeat toxicity studies enabling selection of the right dose (OECD 407, 2008). However, in case the test compound is considered safe (e.g., a herbal formulation/extract used traditionally), a sub-acute toxicity study could be carried out following a 7 or 14 day dosing regimen with the limit dose (1000 mg/kg) as the high dose and at least two graded lower doses with the view of identifying the NOEL/NOAEL or Maximum Tolerated Dose (MTD) as well as target organs, if any.

Sub-acute toxicity tests are usually conducted as they provide the information on the relative toxicity that is likely to arise from repeated exposure of the test substance. There are basic parameters that are taken into consideration in the event of carrying out sub-acute toxicity tests. These include the species of animals to be engaged in the study, the age, number of animals, randomization of animals, the dosages as well as the in-life and terminal observations (Simmons and Gennings, 1996; Lilienblum *et al*., 2008; Gupta and Bhardwaj, 2012).

A 28 day repeat dose study will be carried out after the dosage levels have been established from the outcome of sub-acute toxicity study. The determined dose levels will then be orally administered to the study animals in the main study in an attempt to investigate the histopathology of the target organs, if any, that may be induced by the crude extract of *Euclea Natalensis*, which has shown anti-microbial activity against the drug sensitive and drug resistant strains of *Mycobacterium tuberculosis*.

The primary objective of 7 day study was to evaluate and determine the dose levels that could display the gross behavior, NOEL/NOAEL or MTD and target organs in mice given a repeated dose of the crude extract by oral administration for seven consecutive days.

2.1.1 *EUCLEA NATALENSIS* PLANT – A BRIEF REVIEW

This is a plant that is indigenous to South Africa, which is mostly found along the coast of the country. It's of the *Ebenacaeae* family, *natalensis* species. The plant is used traditionally to treat many ailments including pre-dominantly pulmonary related diseases. Prior studies have documented the anti-microbial activity of the plant against both drug sensitive and drug resistant strains of *Mycobacterium tuberculosis* (Lall and Meyer, 1999). Furthermore in the same study it is documented that the root material of the plant contains high concentration of compounds (naphthoquinones), as compared to the rest of other parts of the plant. Hence the root material is the substance of interest in this study.

TB is an Infectious disease caused by a microorganism posing a serious and growing problem the world over. It is the most deadly infectious disease in the world with approximately a billion people infected and over a million deaths every year. Current treatments involve therapy over a long period (6 months), and there are serious problems with drug-resistant strains (multidrug-resistant TB and extensively drug resistant-TB). One strategy for developing new antibacterial agents is to seek new targets (Verrall *et al.*, 2014). Discovery of anti-mycobacterium properties of naphthoquinones present in *Euclea natalensis* is of great importance towards the development of new TB drugs (Babula *et al.*, 2009). Until this study, no previous toxicity studies have been done on these species to rule out the toxicity potential of the plant on mammalian tissues.

2.1.2 ORCHID CHEMICALS AND PHARMACUETICAL COMPANY LTD. RESEARCH LABORATORIES

This study was conducted as part of an experiential training programme at Orchid Chemicals and Pharmaceutical Company Ltd Research Laboratories, in TamilNadu State in the city of Chennai, (Madras) in India. Orchid Pharmaceutical Company is located in Chennai India. The company manufactures both oral and sterile cephalosporin anti-biotic for the world market which started in 1999 after being issued with certification for commencement with business in 1992. As India’s largest cephalosporin manufacturer, the company ranks as Top-5 in the world. It has sophisticated manufacturing facilities that are GMP (Good Manufacturing Practice) approved by WHO. It received the GMP certification in 1995. Orchid is India’s only Pharmaceutical Company with both ISO9001-2000 and ISO14001 Certification for its quality and environmental management systems respectively. The company runs 3 research campuses, 3 formulation manufacturing sites and 3 Application Programming Interface (API) manufacturing sites in India. The experiential training programme was initiated and sponsored by India Institute of Advanced Scientific Research through the Department of Science and Technology in collaboration with Centre for International Co-operation in Science (CICS) India. Orchid Pharmaceutical Company collaborates well with other scientific research institutions in India. It came to be the centre of my study through the research proposal that was submitted which required the research facilities applicable to my field of study (animal house facility, histopathology laboratory, personnel supervision- expertise and skill including statistical analysis and methodologies required for toxicity studies). The company was identified to meet the research field by the Director of Medical Research council (MRC), TB unit in Madras (Dr Somiya).

2.2 MATERIALS AND METHODS

2.2.1 Animal selection and quantity

A total 40 healthy adult Swiss albino mice were selected for the study. The animals used in the experiment were obtained from the Breeding Facility, Orchid Chemicals & Pharmaceuticals Ltd. The animals were 6-7 weeks old with the average weight of 25-30g at the time of treatment. Animals were grouped into four groups of five per sex. Animals were selected in such a way that the mean body weight of all animals in a group fell between +/- 20% of the mean body weight range in order to minimize biological variation. Female mice were nulliparous and non-pregnant.

2.2.2 Room Sanitation

Before the animal could occupy the experimental room, the room was decontaminated and room sterility was confirmed by performing a surface viable count. The floor of experimental room was swept daily. All worktops and floors were mopped daily with a disinfectant (Suprasol LC 5%).

2.2.3 Animal Welfare

All animals were handled similarly and with due regard for their welfare. Humane treatment and care of animals were complied with the Regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Protocol N° 14-IAEC:02/TOX/2010).

2.2.4 Acclimatization Period

Acclimatization of animals was not entertained as the animals were taken from within the Animal House at Orchid Chemicals & Pharmaceuticals Ltd. And maintained under similar conditions.

2.2.5 Grouping

The animals were randomly allocated and were equally distributed to four groups; vehicle control G1 (0 mg/kg body weight), low dose G2 (125 mg/kg body weight), mid dose G3 (250 mg/kg body weight) and high dose G4 (500 mg/kg body weight). Each group comprised of 5 males and 5 females.

2.2.6 Animal Identification

Animals were given temporary numbers as their identity during randomization. After allocation of groups, permanent numbers were issued to all animals and each animal was given a tag bearing its number on the cage, with unique color for the specific group (white tag for controls, pink tag for low dose, yellow tag for mid dose and red color for high dose).

2.2.7 Animal Husbandry

Animals were housed individually in polypropylene mice cages (290 mm x 220 mm x 140 mm) using autoclaved paddy husk as bedding material. Each cage was fitted with a stainless steel top grill having provision to keep pellet feed and a polypropylene water bottle with stainless steel nozzle. Cages were placed on 5 tier racks. Cages were changed once in three days a week and water bottles once a week.

The experimental animals were fed *ad libitum* mice pellet feed (Nutrilab brand, Tetragon Chemie Pvt. Ltd, Bangalore) with unlimited supply of clean drinking water in polypropylene bottles (capacity 300mL) filtered through Aquaguard® water filtration system. The quality of feed was checked and the batch was accompanied with a certificate of analysis of nutrient content. The quality of water was monitored for any known chemical and microbial contaminants. There were no contaminants in feed and water at the level which can affect the results of the experiment. Animals were maintained in a controlled environment with a temperature of 21 - 25°C; humidity of 54 - 69 %; light/dark cycle of ~12 hours and 14 fresh air changes per hour.

2.2.8 Study Design

Summary of group designations is given below (Table 2.1) for the repeated dose (7 day) sub-acute toxicity study.

Table 2.1 Summary of Animal Grouping

Group N°	**Treatment**	**Dose (mg/kg b.wt.)**	**N° of animals**		**Animal ID. N°**	
			Male	**Female**	**Male**	**Female**
G1	Control	0	5	5	1 - 5	6-10
G2	Low Dose	125	5	5	11-15	16-20
G3	Mid Dose	250	5	5	21-25	26-30
G4	High Dose	500	5	5	31-35	36-40

Four groups of Swiss albino mice comprising 5 males and 5 females per group were administered the test formulation at the dose levels of 0, 125, 250 and 500 mg/kg body weight through oral route for 7 consecutive days. The test substance was formulated in 20% DMSO and 80 % of 0.25% Carboxy Methyl Cellulose (CMC). The control groups received the vehicle alone.

2.2.9 Dose Preparation and Administration

The crude extract was repeatedly administered orally once daily using graduated syringe and stainless steel intubation cannula for the 7 days of the study. The required quantity of the test substance was weighed using a calibrated analytical balance and was suspended in DMSO followed by CMC in the stipulated ratios. Due to the thickness of the suspension, Dose volume was maintained as 20 ml/kg body weight for all animals. The test suspension was prepared every day and was administered immediately. Individual dosage volume was calculated based on the initial body weight. The crude extract of *Euclea natalensis* was suspended in DMSO/CMC to attain the required concentrations i.e., 6.25, 12.5, 25mg/ml for the low, mid and high dose groups respectively. Fresh suspension of *Euclea natalensis* was prepared every morning prior to dosing. The dose volume administered to each mouse was calculated on the basis of a constant factor of 20 ml/kg body weight throughout the study.

2.2.10 Repeated Dose Toxicity Evaluations

2.2.10.1 Mortality

All the animals were checked at least twice a day for mortality and signs of morbidity, in the morning after oral dosing and in the afternoon.

2.2.10.2 Clinical Signs

Animals were observed for all visible signs of reaction to the treatment such as skin and fur changes, eye and mucous membrane changes, respiratory, circulatory, and autonomic and central nervous system, somatomotor activity, behaviour pattern and general changes twice daily.

2.2.10.3 Body weight

The body weight of individual animal was recorded prior to first dosing, at 4th day, 7th day and before scheduled sacrifice.

2.2.10.4 Feed consumption

Feed consumption was recorded cage wise for all animals at the 4th day of commencement of exposure and at day 7. The average daily feed consumption of individual animals was calculated as follows:

$$\text{Feed Consumption (g/mouse/day)} = \frac{\text{Feed input} - \text{Feed left over}}{\text{Number of mice in the cage X No. of days}}$$

2.2.10.5 Clinical pathology observation

At the end of the 7-day period, the animals were fasted overnight. The following morning (prior to sacrifice), blood was collected from all the animals by puncturing the orbital sinus with a heparinised capillary under mild isoflourane anaesthesia. Blood was collected into a 7.5 cm (length) capillary tube and the clotting time was recorded manually. Approximately 0.1 ml of blood was collected in vials containing Ethyl-Diamine Tetra Acetic Acid (EDTA) for haematology analysis.

The following haematological parameters were measured; total erythrocyte count (RBC), haemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count and total differential leucocyte count (WBC). All parameters were determined using haematological analyzer (Sysmex KX 21). The procedure specified in the

Sysmex Operation's Manual (1988) for analysis was followed. Table 2.2 outlines the haematology investigations performed.

Table 2.2 Summary of Haematological Investigations

Parameter	Method
White blood count	DC Detection Method
Differential leukocyte count	DC Detection Method
Red blood count	DC Detection Method
Haemoglobin	Non-cyanide Haemoglobin Analysis Method
Packed cell volume	RBC Pulse High Detection Method
Mean Corpuscular Volume	Calculated from Haematocrit and RBC values
Mean Corpuscular Haemoglobin	Calculated from Haemoglobin and RBC values
Mean Corpuscular Haemoglobin Concentration	Calculated from Haemoglobin and Haematocrit values
Platelet Count	DC Detection Method
Clotting Time	Capillary Method-Manual

Zero point five to 0.8 ml of blood was collected from each animal in heparinized centrifuge tubes and then centrifuged using cooling centrifuge (Eppendorf Centrifuge 5804 R) at 5000 RPM for 10 minutes and plasma was separated. Plasma biochemical parameters were analysed using a fully automated clinical chemistry analyser (ERBA XL 300, Transasia Biomedicals Ltd., Mumbai) (Table 2.3). Table 2.3 Summarises the biochemical investigations performed.

Table 2.3 Summary of Biochemical Investigations

Parameter	Method
Fasting Glucose	GOD-POD Method
Total Bilirubin	Diazo Method
Triglycerides	GPO Trinder Method
Urea	Urea analysed using GLDH-UREASE Method.
Creatinine	Jaffe's Method
Alanine Aminotransferase (ALT)	IFCC, Kinetic without pyridoxal phosphate
Alanine Aminotransferase (ALT)	IFCC, Kinetic without pyridoxal phosphate
Total Protein	Biuret Method
Phosphorous	Ammonium Molybdate Method
Chloride	Ferric Thiocynate Method

2.2.10.6 Gross Examination and Histopathology

All the animals were euthanized with a dose of isoflurane and subjected to a complete necropsy under the direct supervision of a veterinary pathologist at the end of the treatment period. Gross pathological examination included examination of the external surface of the body, all orifices, thoracic and abdominal cavities and their contents. The appearance of macroscopic abnormalities was recorded. The thoracic, abdominal and cranial cavities were then cut open and thorough examinations of organs were carried out to detect changes or abnormalities. Figure 2.1 below outlines the necropsy procedure used in this study.

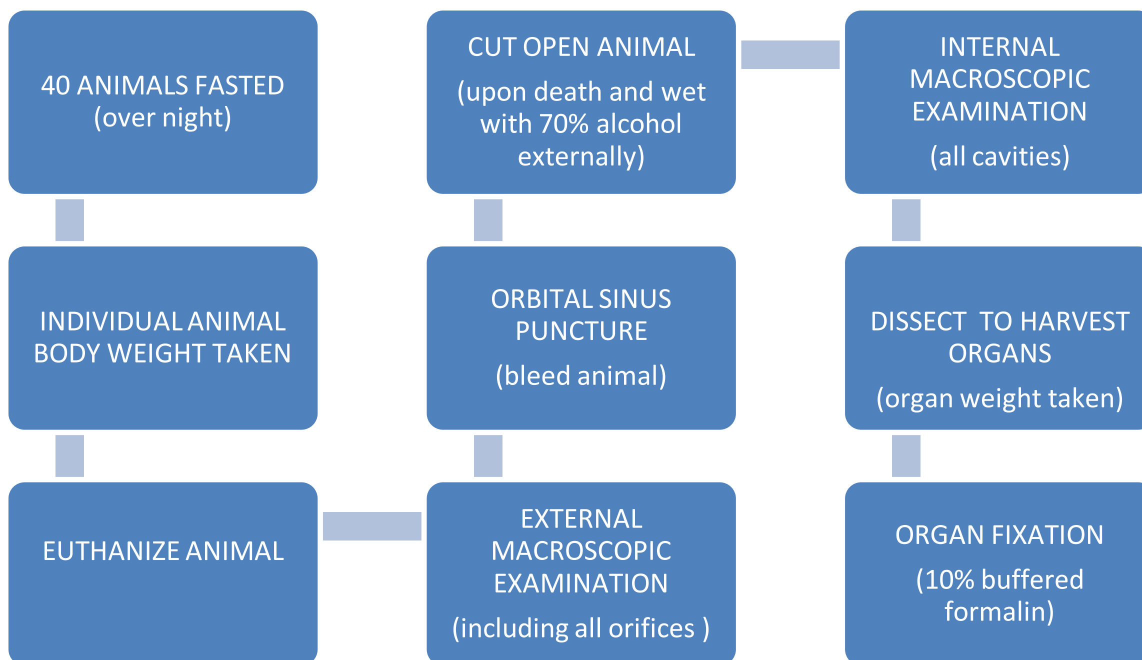


Figure 2.1 Necropsy procedure.
Source: (Adapted from Orchid Pharmaceutical SOPs, 2013)

2.2.10.6.1 Tissue Fixation

Tissue fixation preserves and saves (stabilize proteins) the tissues from decaying (autolysis) and maintains the texture to accommodate and to set a good platform for other procedures to follow. Good tissue sections and staining start with good properly fixed tissues. The minimum time for tissue fixation is about 1-4 hours, but the specimens maybe kept for longer times (Suvarna *et al*., 2013).

The chemical composition of the 10% neutral buffered formalin fixative:

Formalin (37-40%)	100ml
Distilled water	900ml
NaH_2PO_4	4g
Na_2HPO_4	6.5g

Chemicals were mixed to dissolve. (PH 6.8 was maintained)

2.2.10.6.2 Tissue Processing

Tissue processing is the second step in the processing of tissues for histology analysis. In this procedure, tissues go through a series of steps to give (them) some structural format that prepares the tissues for subsequent sectioning and staining (both for diagnostic and research).

The process primarily functions to remove water in the tissues in a very slow manner through ascending grades of alcohol (ethanol as is the most commonly used dehydrant). It is done slowly to avoid rapid harsh removal of water which could result in tissue shrinkages. On completion of the process, the alcohol is removed (clearing). Xylene is the chemical used to clear tissues off the dehydrating solvent. It is the transitional step between dehydration and infiltration with the embedding medium. Infiltration (interpenetration) process of tissues is the actual filling of tissue cavities and cells created by water removed with supporting substance, which in this step will be filled with the medium to be used for embedding (molten wax) (Suvarna *et al*., 2013).

The table below illustrates the stages and chemicals useful in the automatic processing of tissues with time frames (Leica, TP1020 operational manual 2009; Histology Lab, Walter Sisulu University).

Table 2.4 The stages and chemicals used in the automatic processing of tissues

Process	Reagents	Action	Time frames
Fixation	10% neutral BF	Stabilize proteins	1hr
(2 stations)	10% neutral BF		1hr
Dehydration: ascending	70% ethanol		1:30hr
grades of alcohol	80% ethanol		1:30hr
	95% ethanol	Removal of water	1:30hr
(5 stations)	100% ethanol		1:30hr
	100% ethanol		1:30hr
Clearing	Xylene		1:30hr
(3 stations)	Xylene	Removal of alcohol	1:30hr
	Xylene		1:30hr
Infiltration	Molten wax		2hr
(2 stations)	Molten wax	Interpenetration	2hr

Source: Leica TP20 Histology laboratory operational manual

2.2.10.6.3 Tissue Embedding

Tissue embedding is one of the techniques used in histology laboratory that primarily prepares wax infiltrated tissues for section cutting (microtomy). It is the immediate step after tissue processing. The processed and infiltrated tissues are at this stage supported in a medium which allows the tissues to be cut without crushing and damaging. This is the outlined embedding procedure:

- Place the paraffin infiltrated tissue cassettes in the tissue embedding centre's tank, that has been heated to the temperature of about 60-65°C
- Warm the metal block containers in the warm storage area of the machine
- Place the empty metal container over the hot plate of the embedder
- Dispense a little hot paraffin wax from the tank into the metal container
- Take the paraffin infiltrated tissue from the tissue cassette using a forceps and place it in the metal container
- Using heated forceps orientate the tissue specimen as desired and pore small volume hot wax
- Put back the labeled empty plastic tissue cassette on top of the wax pore more hot paraffin wax to cover the plastic cassette
- Now shift the metal container from the hot plate to the cold plate (-15°C) of the tissue embedder
- When the wax is hard, take out the paraffin block from the metal case; the paraffin block is now ready for section cutting (Suvarna *et al*., 2013).

2.2.10.6.4 Tissue/Section Cutting (Microtomy)

Figure 2.2 Rotary Microtome HM 355 S. Histology Laboratory, Walter Sisulu University

After the long processes of tissue dehydration, infiltration, clearing and embedding all tissues are subjected to cutting using either a bench microtome or a rotary microtome. Section cutting involves a number of steps before getting the sections on the glass slide ready for staining. A rotary microtome (figure 2.2) is a heavy duty microtome especially for paraffin and hard sectioning techniques in biology, medicine, industry and research. The machine cuts sections in the range of 0.5µm -100µm. This piece of equipment will normally have both trimming (30µm) and cutting (5µm) function modes. Tissue trimming permits fine adjustments up to first cuts and this result in larger section thickness during trimming. When trimming is complete the fine mode cutting is selected to produce ribbons of sections that are floated in a 60°C water bath to align sections properly on the slide (Micron International GmbH, 2007).

Procedure for Sectioning (HM 355 S Instruments Instructional Manual no 387841, WSU):

- Make sure that the clamping screws and clamping levers on the blade holder and specimen holder are well tightened.
- Make sure that the specimen and specimen holder are well oriented
- Always use sharp blades to produce neat thin sections (avoid compressions)
- Sectioning should always be carried out in ambient temperatures (excluding frozen sections)
- Cutting speed must be programmed in accordance with texture of the material
- Trimming thickness must be set at 30μm and cutting thickness at 5μm to produce good sections
- Upon production of tissue ribbons, the sections are flooded in a water bath (58-60°C) to flatten and iron out any folds
- Good, neat sections are picked from the ribbon in the water bath using a labeled slide for the exact tissue just cut
- Sections are left to dry before staining

2.2.10.6.5 Tissue Staining/ Staining Mechanism

Tissues that are examined microscopically immediately after sectioning without staining appear very dull and uninteresting. They lack contrast of all fixed tissue materials and all have a similar refractive index and colour. In order to bring out the microscopic structure of these tissues, it is essential to stain the cells and connective tissue to expose the different parts in contrasting colours. Staining is therefore not simply colouring the sections but relies on using differences in chemistry of the tissues to show the different components in different colours.

These are achieved by using dyes that can bind to tissues selectively. The most common stain in use in histology staining is haematoxylin and eosin (H & E) stain (Suvarna *et al*., 2013). The binding of dyes to tissues is similar to other chemical bonding and the mechanisms is the same in that they all rely on the same binding forces occurring in all other organic compounds? Eosin is a negatively charged stain (anionic dye) and will stain positively charged (cations) tissue ions (cytosol of the cell and connective tissue). Haematoxylin is a versatile stain and is used in combination with mordants (alum salts) that alters the specificity and colour of the stain. It will stain the acidic environment of the cell (nucleus) dark blue of purple (Suvarna *et al*., 2013).

After drying the slides are ready to stain, but paraffin sections are still impregnated with paraffin wax which forms a water proofing coat that can prevent the stain to access proteins within the tissues. Therefore the wax must melt in the oven (60°C) and then be removed before sections could be rehydrated (Orchid histopathology staining protocol, 2013). Below is a point-form summary of the histopathology staining procedure used in this study:

- Xylene 5 minutes (2-3 changes)
- Absolute alcohol 5 minutes
- 95% alcohol 5 minutes
- 70% alcohol 5 minutes
- Distilled water 3 minutes
- Harris Haematoxylin 5 minutes
- Differentiate in running tap water 2 minutes
- Eosin-Phloxin 1 minute
- 80% alcohol Dips

- 95% alcohol Dips
- Absolute alcohol Dips (2 changes)
- Xylene 5 minutes (3 changes)
- Mount in DPX

2.2.10.6.6 Section Mounting

Staining is subsequently followed by mounting procedure. Normally the mounting medium must have a high refractive index (RI). Tissues mostly have an RI of 1.5-1.55 and the mounting medium of the same index will offer maximum clarity. The common mountant in histology procedures is DPX. It's a synthetic polystyrene resin that has been dissolved in xylene with some plasticizer added to it. It is a clear waterless solution that hardens in about 24 hours. (D= Distrene 80, that is a commercial polystyrene; P=Plasticizer, dibutyl phthalate and X=Xylene) (Suvarna *et al.*, 2013). Mounting is done under a cover slip and it gives best and clear view of the specimen. The cover slip should have the thickness of about 0.17mm to give best results.

- Mounting is done with slides immersed in xylene.
- General safety rules must always be adhered to (GLP)
- Align cover slipper on a flat surface
- Dispense a small drop of DPX over the cover slip
- Remove the slide from the xylene bath and right face the slide over the slip
- Press very gently tapping at the same time to release the bubbles
- Leave flat on the surface to dry
- Sort and file in slide boxes ready for microscopy (Orchid histopathology Research Laboratory mounting procedure, 2012).

2.2.10.7 Organ Weight

Absolute wet weights of adrenals, brain, uterus, ovaries, testes, epididymides, heart, kidneys, liver, spleen and thymus were recorded immediately after dissection for all animals. Relative weights of these organs were calculated later. Organs were harvested and cleared from the adhering fat and blotted free from blood (Orchid Histopathology Research Laboratory, 2013). The absolute weight was recorded to the nearest milligram using a calibrated balance.

$$\text{Relative organ weight} = \frac{\text{organ weight}}{\text{Animal weight}} \times 100$$

The following organs of all the animals were collected at necropsy and preserved in 10% buffered neutral formalin for processing and microscopic examination; stomach, duodenum, jejunum, ileum, caecum, colon, rectum, liver, brain (representative regions including forebrain, cerebrum, cerebellum), adrenals, lungs, heart, aorta, spleen, thymus, kidneys, testes, epididymides, ovaries, cervix and the uterus.

All the preserved organs from control and high dose groups (G1 and G4) of the animals were processed following suitable techniques of washing by tap water, dehydration in ascending grades of ethyl alcohol, clearing in xylene, embedding and blocking in paraffin wax. Paraffin sections (5µm) were cut, stained with hematoxylin and eosin (H&E), and mounted on slides as outline previously. Veterinary pathologist assisted histopathology screening was carried out on all preserved organs and tissues of all the animals in control and high dose groups.

2.2.10.8 Statistical Analysis of Results

Raw data were processed statistically. The means and standard deviations were calculated with significance between the control and treated groups and all data were summarized in tabular form. All the parameters characterized by continuous data such as body weight, feed consumption, organ weight, relative organ weight, haematology, clinical chemistry were subjected to Bartlett's test to meet the homogeneity of variance. All homogenous data were analyzed using ANOVA and data showing significance in their variances were subjected to Dunnett's t-test.

Significance was calculated at 1% as well as 5% level, and indicated in the summary table as follows:

↑ -Significantly higher than control ($p > 0.05$), ↑↑ -Significantly higher than control ($p > 0.01$), ↓- Significantly lower than control ($p < 0.05$), ↓↓- significantly lower than control ($p < 0.01$)

2.3 RESULTS

2.3.1 Mortalities

No mortalities were observed in the control group (G1) as well as any of the treated group animals (G2, G3 and G4) exposed to crude extract of *Euclea natalensis.*

2.3.2 Clinical Observations

No treatment related visible signs of toxicity were observed in animals in all treated groups. Table 2.5 is an outline of the clinical observations noted in the 7 day repeat oral toxicity study.

Table 2.5 Summary of Clinical Observations for Repeated Dose Toxicity Study

Group N° & Dose (mg/kg b.wt.)	**Gender**	**Clinical sign**	**N° of Animals Showing Clinical Signs***							
			Experiment Day							
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
G1 (0)	Male	Normal	5	5	5	5	5	5	5	5
	Female	Normal	5	5	5	5	5	5	5	5
G2 (125)	Male	Normal	5	5	5	5	5	5	5	5
	Female	Normal	5	5	5	5	5	5	5	5
G3 (250)	Male	Normal	5	5	5	5	5	5	5	5
	Female	Normal	5	5	5	5	5	5	5	5
G4 (500)	Male	Normal	5	5	5	5	5	5	5	5
	Female	Normal	5	5	5	5	5	5	5	5

Key: * = Animals which exhibited clinical signs at least once during the week

2.3.3 Effect of Body Weight

No significant deviation of weights in mean of all the treated groups was observed as compared to the control group, both at day 4, day 7, and at terminal point.

Table 2.6 Summary of Body Weight (g)

Gender: Male

Day N°	G1 (0 mg/kg /Bwt.)	G2 (125mg/kg Bwt.)	G3 (250mg/kg/Bwt))	G4 (500mg/kg/Bwt.)
	Mean	Mean	Mean	Mean
#	27.240 ± 3.06	26.620 ± 1.731	27.160±3.244	26.6408±2.438
Day 4	25.940± 2.092	25.600± 1.672	28.140±4.166	24.040± 1.726
Day 7	24.000± 1.625	25.180± 2.105	28.460±5.879	22.720±2.523
Ter. B.wt	24.080± 1.639	24.980± 1.424	28.460±5.748	22.680±2.326

#-pre exposure

Gender: Female

Week N°	G1 (0 mg/kg B.wt.)	G2 (125mg/kg B.wt.)	G3 250mg/kgB.wt.)	G4 (500 mg/kg B.wt.)
	Mean	Mean	Mean	Mean
#	25.920±1.279	24.960± 1.686	25.280± 1.318	25.860± 1.777
Day 4	26.160±1.607	25.500± 2.373	25.040± 1.410	25.320± 2.069
Day 7	26.060±1.702	26.260±2.324	25.760±1.736	26.140±1.244
Ter. B.wt	26.020±1.163	25.920±2.331	25.440±1.641	25.500±1.751

 # - Pre exposure

2.3.4 Feed Consumption

No significant changes were observed in the feed consumption of the treated groups of animals as compared to the control group.

Table 2.7 Summary of Average Daily Feed Consumption (g/mice/day)

Gender: Male

Day N°	G1 (0 mg/kg Bwt.)	G2 (125 mg/kg Bwt.)	G3 (250 mg/kg Bwt.)	G4 (500 mg/kg Bwt.)
	Mean	**Mean**	**Mean**	**Mean**
Day4	1.520± 0.614	1.200± 0.689	1.100± 0.951	1.006± 0.922
Day 7	0.140± 0.055	0.132± 0.064	0.508± 0.428	0.530± 1.101

Gender: Female

Day N°	G1 (0 mg/kg Bwt.)	G2 (125 mg/kg Bwt.)	G3 (250 mg/kg Bwt.)	G4 (500 mg/kg Bwt.)
	Mean	**Mean**	**Mean**	**Mean**
Day4	1.740± 1.062	1.062± 0.907	0.960± 0.684	1.620±1.203
Day 7	1.060±0.089	1.320±0.792	0.800±0.721	0.680±0.349

Key: # = Pre-exposure

2.3.5 Haematology

No statistically significant changes were noticed in all animals (in all sexes) treated with *Euclea natalensis* as compared to non-treated control group.

Table 2.8 Summary of Haematology Values

Gender: Male

Parameter	G1 (0 mg/kg b.wt.)	G2 (125 mg/kg b.wt.)	G3 (250 mg/kg b.wt.)	G4 (500 mg/kg b.wt.)
	Mean	**Mean**	**Mean**	**Mean**
RBC (10^6/µL)	9.050± 0.970	10.018± 0.710	9.378± 0.888	9.958± 0.670
HGB (g/dL)	14.760± 0.385	15.140± 0.737	14.700± 0.752	15.260± 0.929
HCT (%)	49.700± 4.836	54.100± 2.791	51.780± 4.225	53.920± 4.428
MCV (fL)	54.980± 1.363	54.100± 2.747	55.300± 1.612	54.120± 1.566
MCH (pg)	16.520± 2.229	15.160± 0.902	15.720± 1.028	15.320± 0.522
MCHC (g/dL)	30.000± 3.924	27.980± 0.277	28.420± 1.165	28.340± 0.826
PLT (10^3/µL)	1359.600± 441.726	1333.200± 130.051	1067.800± 374.905	1401.600± 249.077
CL.Time (Sec)	138.000± 12.550	135.000± 15.000	135.000± 15.000	129.000± 13.416
WBC (10^3/µL)	5.340±0.945	5.180±1.28	6.280±1.499	5.840±2.442
LYM (%)	67.000± 11.363	69.520± 12.983	67.840± 8.871	63.580± 17.320
NEU (%)	28.380± 9.949	26.880± 11.954	28.300± 7.703	31.960± 15.820
MIX (%)	4.620± 1.992	3.600± 1.170	3.860± 1.316	4.460± 1.581

Gender: Female

Parameter	G1 (0 mg/kg b.wt.)	G2 (125mg/kg b.wt.)	G3 (250 mg/kg b.wt.)	G4 (500 mg/kg b.wt.)
	Mean	**Mean**	**Mean**	**Mean**
RBC (10^6/µL)	9.220± 0.647	9.372±0.284	9.502±0.328	9.586±0.483
HGB (g/dL)	14.600± 0.453	14.580±0.606	14.900±0.596	15.020±0.396
HCT (%)	50.960±2.092	51.640±1.877	52.500±3.145	52.360±1.250
MCV (fL)	55.400±2.661	55.100±1.531	55.220±2.032	54.680±1.908
MCH (pg)	15.880±0.811	15.560±0.623	15.680±0.259	15.700±0.797
MCHC (g/dL)	28.640± 0.321	28.260±0.467	28.400±0.570	28.720±0.522
PLT (10^3/µL)	1109.800±131.089	1015.600±122.692	1197.200±172.967	1131.800±159.588
CL.Time (Sec)	138.000±12.550	135.000±10.607	147.00±22.24	139.000±10.840
WBC (10^3/µL)	4.640±1.527	4.700±1.965	6.425±2.326	5.340±1.278
LYM (%)	80.840± 5.801	77.32 ±8.255	68.450±11.794	76.100±14.015
NEU (%)	16.480± 4.927	19.920±7.425	27.625±9.981	20.280±11.681
MIX(%)	2.680± 1.161	2.760±0.974	3.925±1.916	3.620±2.347

Key: ↓ = significantly low at P ≤ 0.05, ↑ = significantly high at P ≥ 0.05, ↓↓ = significantly low at P ≤ 0.01
↑↑ - Significantly higher than control (p≥ 0.01)

2.3.6 Clinical Chemistry

No statistical significance was observed in all parameters in the male treated animals, except for G3 group (250mg/kg b.wt.) which showed a decrease in total protein as compared to the untreated animals at 1% significance level (p<0.01). In females, all treated animals showed a decrease in total cholesterol as compared to control animals although statistically not significant. G3 (500mg/kg.b.wt.) showed an increase in phosphorus at 5% (p<0.05) as compared to control group. G2 (125mg/kg b.wt) showed a statistical significance in triglyceride level at 5% (p<0.05).

Table 2.9 Summary of Clinical Biochemistry Values

Gender: Male

Parameter	G1 (0 mg/kg b.wt.)	G2 (125mg/kg b.wt.)	G3 (250 mg/kg b.wt.)	G4 (500 mg/kg b.wt.)
	Mean	**Mean**	**Mean**	**Mean**
GLU (mg/dL)	128.780± 34.866	126.660± 26.750	147.520± 67.368	111.680± 21.881
URE(mg/dL)	27.800± 8.044	26.400± 7.369	29.200± 1.304	31.000± 5.568
CRE (mg/dL)	0.400± 0.024	0.432± 0.049	0.468± 0.110	0.400±0.060
T.PRO (g/dL)	7.034± 1.116	6.842±0.829	↓5.726±0.578	6.450±0.648
ALB (g/dL)	3.768± 1.451	3.906±1.110	2.282±0.641	4.450±1.255
CLO (mEq/L)	98.020± 4.110	101.100±2.064	102.840±2.341	96.940±5.505
PHO (mg/dL)	12.250±4.153	12.662±0.608	11.936±2.873	7.348±1.298
CHOL (mg/dL)	143.800± 42.204	154.600±24.358	118.600±14.381	182.200±25.352
TGL (mg/dL)	173.200± 41.294	181.600±51.786	168.400±61.211	194.000±41.851
T.BIL (mg/dL)	2.286± 1.244	2.876±0.560	2.374±0.998	1.832±0.850
GOT (IU/L)	149.660± 41.219	162.820±49.563	133.120±44.371	169.900±17.476
GPT (IU/L)	103.960± 39.435	83.560±14.933	97.440±30.156	99.100±19.047
ALP (IU/L)	118.200±41.590	145.000±32.381	135.800±42.056	84.000±20.893

Table 2.9 (Continued)
Gender: Female

Parameter	G1 (0 mg/kg b.wt.)	G2 (125 mg/kg b.wt.)	G3 (250 mg/kg b.wt.)	G4 (500 mg/kg b.wt.)
	Mean	Mean	Mean	Mean
GLU (mg/dL)	145.140±12.885	127.320±11.253	140.680±11.989	138.780±21.176
URE(mg/dL)	20.000± 2.121	18.200±2.387	20.000±1.414	21.800±2.387
CRE (mg/dL)	0.384± 0.028	0.388±0.027	0.396±0.021	0.380±0.034
T.PRO (g/dL)	5.948±1.610	5.598±0.412	6.240±0.608	5.592±0.537
ALB (g/dL)	3.404± 0.940	2.392±0.337	3.188±1.089	2.282±0.213
CLO (mEq/L)	98.280±1.455	98.860±3.449	101.180±3.507	100.480±2.406
PHO (mg/dL)	8.876±1.984	8.876±1.984	↑12.046±1.544	10.596±1.383
CHOL (mg/dL)	146.600± 42.495	110.600±9.290	121.400±34.595	120.000±14.124
TGL mg/dL)	162.400±21.916	↓131.200±17.079	156.400±68.755	169.200±69.694
T.BIL (mg/dL)	1.530± 0.584	1.606±0.598	1.486±0.514	2.332±1.240
GOT (IU/L)	146.880± 44.743	107.720±33.345	163.620±56.303	142.780±85.821
GPT (IU/L)	60.360±24.822	53.640±16.822	64.960±21.036	43.840±6.738
ALP (IU/L)	166.600± 23.480	189.400±34.290	146.800±44.110	148.800±31.412

Key: ↓ = significantly low at $P \leq 0.05$, ↓↓ = significantly low at $P \leq 0.01$
↑ = significantly high at $P \geq 0.05$, ↑↑ = significantly high at $P \geq 0.01$

2.3.7 Absolute Organ Weight

No significant change in males was seen in all organ weights when compared to the control group, except for the weight of the testes in G2 group (250mg/kg.b.wt.) which was significantly higher than the control at 1% level. ($p>0.01$). In female animals, no deviation of weights is observed in all organs. However, the heart weight in both G3 and G4 showed a reduction with no statistical significance. This observed change in the absolute and relative heart weight could be of biological concern. Table 2.9 is a summary of the absolute organ weights recorded.

Table 2.10 Summary of Absolute Organ Weight (g)

Gender: Male

Parameter	Group N° & Dose (mg/kg b.wt.)			
	G1 (0)	**G2 (125)**	**G3 (250)**	**G4 (500)**
	Mean	**Mean**	**Mean**	**Mean**
Terminal Body Weight	24.080±1.639	24.980±1.639	28.460±5.748	22.680±2.326
Adrenals	0.012±0.003	0.010±0.003	0.012±0.002	0.009±0.004
Testes	0.183±0.012	↑↑0.211±0.007	0.178±0.012	0.201±0.021
Thymus	0.048±0.017	0.045±0.008	0.054±0.015	0.040±0.016
Epididymis	0.071±0.009	0.075±0.008	0.079±0.011	0.071±0.012
Spleen	0.115±0.024	0.119±0.027	0.127±0.016	0.115±0.026
Heart	0.158±0.011	0.163±0.016	0.178±0.034	0.152±0.029
Brain	0.446±0.022	0.468±0.032	0.469±0.025	0.450±0.021
Kidneys	0.436±0.061	0.452±0.084	0.554±0.171	0.394±0.072
Liver	1.546±0.245	1.537±0.093	1.678±0.285	1.386±0.113

Gender: Female

Parameter	Group N° & Dose (mg/kg b.wt.)			
	G1 (0)	**G2 (125)**	**G3 (250)**	**G4 (500)**
	Mean	**Mean**	**Mean**	**Mean**
Terminal Body Weight	26.020±1.163	25.920±2.331	25.440±1.641	25.500±1.751
Adrenals	0.021±0.004	0.017±0.003	0.017±0.002	0.022±0.005
ovaries	0.389±0.041	0.369±0.035	0.363±0.019	0.379±0.034
Thymus	0.017±0.005	0.014±0.002	0.016±0.005	0.018±0.002
uterus	0.102±0.022	0.115±0.034	0.098±0.013	0.098±0.036
Spleen	0.175±0.087	0.193±0.080	0.160±0.031	0.189±0.056
Heart	0.142±0.032	0.143±0.034	0.136±0.026	0.131±0.016
Brain	0.165±0.019	0.165±0.023	0.158±0.009	0.164±0.016
Kidneys	0.485±0.013	0.482±0.021	0.483±0.012	0.472±0.027
Liver	1.447±0.160	1.398±0.223	1.413±0.200	1.418±0.120

Key: ↑↑ = significantly high at $P \geq 0.01$.↓↓ = significantly low at $P \leq 0.01$

2.3.8 Relative Organ Weight

No treatment related changes were observed in relative organ weights of animals treated with *Euclea natalensis*, except for changes seen in female animals in both G3 and G4. Table 2.10 summarises the relative organ weights recorded.

Table 2.11 Summary of Relative Organ Weight (g)

Gender: Male

Parameter	Group N° & Dose (mg/kg b.wt.)			
	G1 (0)	**G2 (125)**	**G3 (250)**	**G4 (500)**
	Mean	**Mean**	**Mean**	**Mean**
Adrenals	0.048±0.012	0.042±0.014	0.045±0.015	0.041±0.012
Testes	0.765±0.077	0.845±0.060	0.644±0.127	0.894±0.146
Thymus	0.202±0.072	0.178±0.029	0.197±0.059	0.174±0.058
Epididymis	0.293±0.027	0.301±0.017	0.283±0.052	0.314±0.051
Spleen	0.478±0.099	0.481±0.137	0.458±0.092	0.512±0.141
Heart	0.658±0.022	0.651±0.047	0.628±0.050	0.668±0.094
Brain	1.858±0.137	1.874±0.086	1.692±0.274	1.996±0.162
Kidneys	1.808±0.176	1.801±0.260	1.909±0.247	1.732±0.223
Liver	6.406±0.825	6.160±0.337	5.943±0.584	6.132±0.483

Gender: Female

Parameter	Group N° & Dose (mg/kg b.wt.)			
	G1 (0)	**G2 (125)**	**G3 (250)**	**G4 (500)**
	Mean	**Mean**	**Mean**	**Mean**
Adrenals	0.081±0.016	0.065±0.014	0.066±0.011	0.086±0.020
ovary	1.491±0.097	1.424±0.110	1.429±0.058	1.489±0.130
thymus	0.065±0.024	0.055±0.011	0.063±0.025	0.070±0.011
uterus	0.394±0.092	0.336±0.170	0.389±0.063	0.379±0.129
Spleen	0.679±0.356	0.737±0.256	0.634±0.155	0.734±0.186
Heart	0.549±0.133	0.574±0.087	0.534±0.092	0.516±0.057
Brain	0.635±0.057	0.636±0.038	0.622±0.042	0.646±0.063
Kidneys	1.867±0.062	1.872±0.185	1.905±0.147	1.857±0.161
Liver	5.555±0.485	5.373±0.400	5.548±0.655	5.564±0.302

Key: ↑↑ = significantly high at $P \leq 0.01$.↓↓ = significantly low at $P \leq 0.01$

2.3.9 Gross Pathological Findings

2.3.9.1 External Findings

Gross pathological observations did not reveal any abnormality externally in any of the animals belonging to both the control and treated group animals.

2.3.9.2 Internal Findings

No treatment related gross lesions were observed in any of the animals belonging to either sex treated with *Euclea natalensis* at the dose levels of 500, 250, and 125 mg/kg body weight as compared to the control.

Table 2.12 Summary of Gross Pathological Findings

Gender: Male

Group and Dose (mg/kg b. wt)	Gross Pathological Findings	
	External	**Internal**
G1(0 mg/kg b. wt)	No abnormalities detected in 5/5	No abnormalities detected in 5/5
G2 (125 mg/kg b. wt.)	No abnormalities detected in 5/5	No abnormalities detected in 5/5
G3 (250 mg/kg b. wt)	No abnormalities detected in 5/5	No abnormalities detected in 5/5
G4 (500 mg/kg b. wt)	No abnormalities detected in 5/5	No abnormalities detected in 5/5

Gender: Female

Group and Dose (mg/kg b. wt)	Gross Pathological Findings	
	External	**Internal**
G1(0 mg/kg b. wt)	No abnormalities detected in 5/5	No abnormalities detected in 5/5
G2 (125 mg/kg b. wt.)	No abnormalities detected in 5/5	No abnormalities detected in 5/5
G3 (250 mg/kg b. wt)	No abnormalities detected in 5/5	No abnormalities detected in 5/5
G4 (500 mg/kg b. wt)	No abnormalities detected in 5/5	No abnormalities detected in 5/5

2.3.10 Histopathological Observations

Table 2.13 Summary of Histopathological Findings

Group	Animal N°	Sex	Histopathological Observation
Control (0 mg/kg)	SA45/1/2	Male	**Heart:** Degenerative cardiac myocytes
	SA/45/6/2	Female	**Heart:** Degenerative cardiac myocytes
	SA45/7/2	Female	**Adrenal:** Involution of the X-Zone
Treated (500 mg/kg)	SA45/31/1	Male	**Lung:** Inflammation
	SA45/34/1	Male	**Lung:** Inflammation
	SA45/39/2	Female	**Heart:** Degenerative cardiac myocytes
	SA45/37/2	Female	**Adrenal:** Involution of the X-Zone
	SA45/40/2	Female	**Adrenal:** Involution of the X-Zone

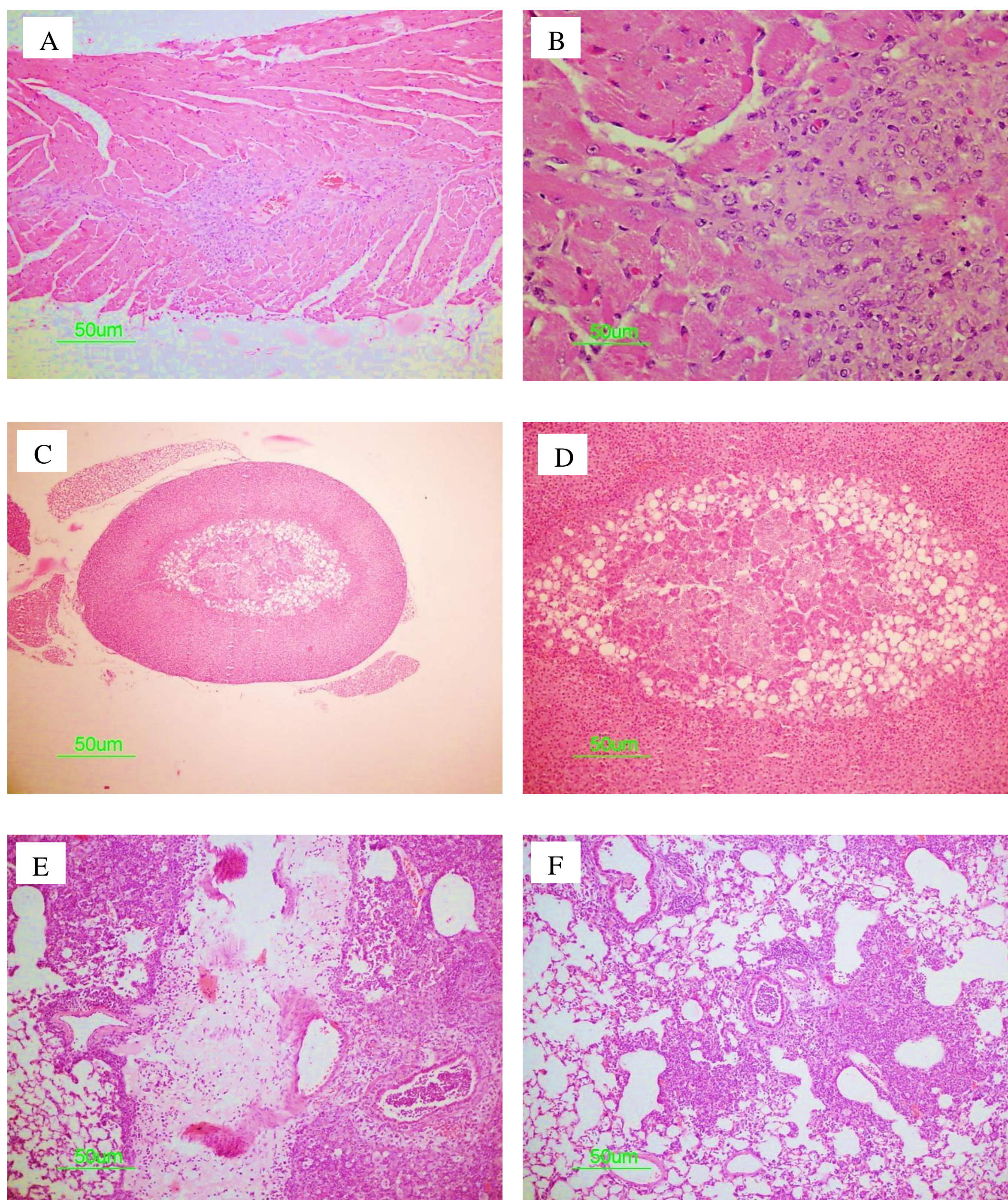


Figure 2.3 Histopathology Found in the 7 day Repeat Dose Oral Toxicity Study. [A] Degenerated cardiac myocytes (H&E, 100x, scale bar = 50µm, Control Mouse, Male). [B] Degenerated cardiac myocytes, H&E, 200x, scale bar = 50µm, Control Mouse, Male. [C] Adrenal- involution of X-xone, H&E, 100x, scale bar = 50µm, Mouse treated with *Euclea natalensis* at 500mg/kg. [D] Adrenal, Involution of X-zone, H&E, 200x, scale bar = 50µm, Mouse treated with *Euclea natalensis* at 500mg/kg. [E] Airway of Lungs, Inflammation, Mononuclear cell Infiltration, H&E, 100x, scale bar = 50µm Mouse treated with *Euclea natalensis* at 500mg/kg. [F] Lungs, Inflammation, dilatation of alveoli, H&E, 100x, scale bar = 50µm, Mouse treated with *Euclea natalensis* at 500mg/kg.

CHAPTER THREE

REPEATED DOSE (28 DAY) ORAL TOXICITY STUDY OF *EUCLEA NATALENSIS* IN SWISS ALBINO MICE

A 28-Day toxicity study was carried out to assess the toxicological profile of the crude extract of *Euclea natalensis* following a repeated dose regime. The study provided information on major toxicological effects to target organs and estimated a No Observed Adverse Effects Level or No Observed Effects Level (NOAEL/NOEL) (OECD, 407).

Based on the findings from the 7 day repeat dose toxicity study the 28 day repeat dose toxicity study was optimised. The following section outlines the methodology used in conducting this investigation.

3.1 MATERIAL AND METHODS

3.1.1 Production of the Extracts

3.1.1.1 Plant Collection

Effort was made to locate the places where the medicinal plant could be collected in the country. National Biodiversity Institute of South Africa (NBISA) was contacted and it released a guide with various places where the medicinal plant could be found. Research proposal of the study was requested and sent to the institution. Furthermore application form was sent to the institute through the authorization of the office of the Head of Department in the School of Biomedical technology, Nelson Mandela Metropolitan University (NMMU), Professor N Smith. Among the places listed was Lusikisiki which is much nearer to Walter Sisulu University where the research work was run. The exact location of the medicinal plant in Lusikisiki was obtained in the Kei Herbarium of Walter Sisulu University (WSU). The

medicinal plant was collected along Xura River which is about one kilometre from the main road.

Figure 3.1 Roots of *Euclea natalensis*

3.1.1.2 Plant Authentication

The sourcing of the medicinal plant was achieved with the assistance of the Botany technician (Mr Wopula). The specimen was authenticated by Dr Kathy in the Department of Botany Walter Sisulu University, and voucher specimen number one was preserved in the Kei Herbarium. Roots were obtained as illustrated by the figure above. A week later they were chopped into smaller pieces and dried at 40°c overnight. (Kambezi, 2011, Oral Communication). After drying the roots were ground using a grinding apparatus.

3.1.1.3 The Extract Production

The extraction procedure was carried out according to the procedure recommended by Draper (Draper, 1976). In this procedure 100g of milled root was dissolved in 1000ml of acetone. The mixture was left on the shaker for three days to allow for maximum extraction of compounds at 25°c at 180r/min. The mixture was then sieved and filtered. The residue was washed twice with acetone to complete the extraction. The filtrate was then dried on a rota vapour machine at medium speed temperature ranging 40- 56°c to obtain the crystallized black crude extract. The crude extract was then stored in capped sterile plastic bottle at room temperature.

Figure 3.2 Dry Crude Extract

3.1.2 Albino Mice

3.1.2.1 Animal Selection and Quantity

A total of 40 healthy adult albino mice were selected for the study. The animals used in the experiment were obtained from Bloemfontein through Shalom Laboratories Suppliers. The animals were 6-7 weeks old with the average weight of 25-30g at the time of treatment. Animals were grouped into four groups of five per sex. In order to minimize biological variation, individual body weight of all animals in a group was recorded and the range of all

weights fell between +/- 20% of the mean body weight range. Female mice were nulliparous and non-pregnant.

3.1.2.2 Room Sanitation and Animal Welfare

Before the animals could occupy the experimental room, the room was decontaminated. The floor of the experimental room was swept daily and all worktops and floors were mopped daily with a disinfectant (5% Bleach). All animals were handled similarly and with due regard for their welfare.

3.1.2.3 Acclimatization Period

All animals were allowed to acclimatize for at least five days before treatment.

3.1.2.4 Grouping

The animals were randomly allocated to different groups. The animals were equally distributed to four groups; vehicle control G1 (0 mg/kg body weight), low dose G2 (125 mg/kg body weight), mid dose G3 (250 mg/kg body weight) and high dose G4 (500 mg/kg body weight). Each group comprised of 5 males and 5 females.

3.1.2.5 Animal Identification

Animals were given temporary numbers as their identity during randomization. After allocation of groups, permanent numbers were issued to all animals and each animal was given a tag bearing its number on the cage, with a unique colour for the specific group. (white tag for controls, pink tag for low dose, yellow tag for mid dose and purple colour for high dose).

3.1.2.6 Animal Husbandry

Animals were housed individually in polypropylene mice cages (410 mm x 282 mm x 180 mm) using soar dust as bedding material. Each cage was fitted with a stainless steel top grill having provision to keep pellet feed and a polypropylene water bottle with stainless steel nozzle. Cages were placed on 5 tier racks. The bedding in cages was changed once a week and water bottles once a week.

The experimental animals were fed *ad libitum* mice pellet feed (Nutrilab brand, shalom laboratories Durban) with unlimited supply of clean and boiled drinking water in polypropylene bottles (capacity 300mL). The quality of feed was checked and the batch was accompanied with a certificate of analysis of nutrient content. The quality of water was monitored for any known chemical and microbial contaminants. There were no contaminants in feed and water at the level, which can affect the results of the experiment. Animals were maintained in a controlled environment with a temperature of 21 - 25°C; light/dark cycle of ~12 hours.

3.1.3 Study Design

Summary of group designations is given below for the repeated dose sub acute toxicity study

Table 3.1 Summary of Animal Grouping

Group N°	**Treatment**	**Dose (mg/kg b.wt.)**	**N° of animals**		**Animal ID. N°**	
			Male	**Female**	**Male**	**Female**
G1	Control	0	5	5	1 – 5	6-10
G2	Low Dose	125	5	5	11-15	16-20
G3	Mid Dose	250	5	5	21-25	26-30
G4	High Dose	500	5	5	31-35	36-40

Four groups of Swiss albino mice comprising 5 males and 5 females per group were administered the test formulation at the dose levels of 0, 125, 250 and 500 mg/kg body weight through oral route for 28 consecutive days. The control groups received Dimethyl Sulfur Oxide (DMSO) and Carboxyl Methyl Cellulose (CMC) in the ratio of 20%; 80% of 0.25% respectively.

3.1.3.1 Dose Preparation and Administration

The crude extract was repeatedly administered orally once daily using graduated syringe and stainless steel intubation cannula for 28 days of study. Required quantity of the test substance was weighed using a calibrated analytical balance and was suspended in DMSO and CMC in the stipulated ratios. Due to the thickness of the suspension, dose volume was maintained as 20 ml/kg body weight for all animals. The test suspension was prepared every day and was administered immediately. Individual dosage volume was calculated based on the initial body weight. The crude extract of *Euclea natalensis* was suspended in DMSO/CMC to attain the required concentrations i.e., 6.25, 12.5, 25mg/ml for the low, mid and high dose groups respectively. Fresh suspension of *Euclea natalensis* was prepared every morning prior to dosing. The dose volume administered to each mouse was calculated on the basis of a constant factor of 20 ml/kg body weight throughout the study.

3.1.4 Repeated Dose Toxicity Evaluation

3.1.4.1 Mortality

All the animals were checked at least twice a day for mortality and signs of morbidity, in the morning after oral dosing and in the afternoon.

3.1.4.2 Clinical Signs/Observations

Animals were observed for all visible signs of reaction to the treatment such as skin and fur changes, eye and mucous membrane changes, respiratory, circulatory, and autonomic and central nervous system, somatomotor activity, behaviour pattern and general changes twice daily

3.1.4.3 Body Weight

The body weight of individual animals was recorded prior to first dosing, at 7th day, $14th^{th}$ 21st day and at necropsy (28th day).

3.1.4.4 Feed Consumption

Feed consumption was recorded cage wise for all animals at every 7th day of commencement of exposure and at 28th day. The average daily feed consumption of individual animal was calculated as follows:

$$\text{Feed Consumption (g/mouse/day)} = \frac{\text{Feed input} - \text{Feed left over}}{\text{Number of mice in the cage X No. of days}}$$

3.1.4.5 Gross Examination

All the animals were euthanized with a dose of ether and subjected to a complete necropsy. Gross pathological examination included examination of the external surface of the body, all orifices, thoracic and abdominal cavities and their contents. The appearance of macroscopic abnormalities was recorded, if any. The thoracic, abdominal and cranial cavities were then cut open and thorough examinations of organs were carried out to detect changes or abnormalities, if any. The necropsy procedure is outlined in figure 3.3 below.

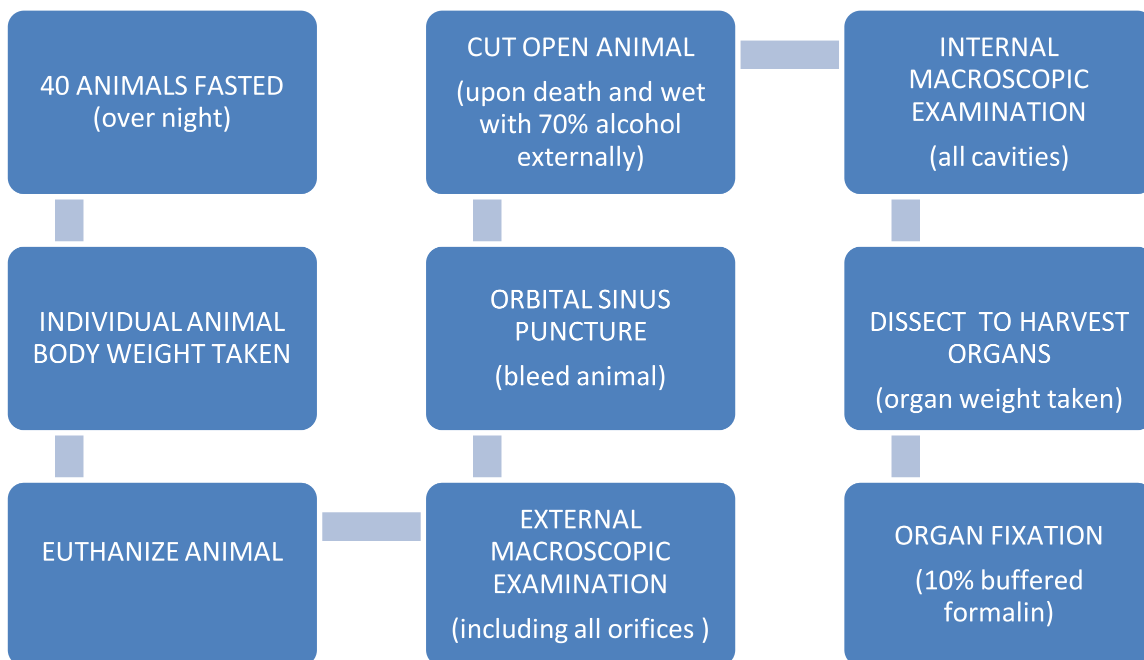


Figure 3.3 Necropsy procedure

Source: (Adapted from Orchid Pharmaceutical SOPs, 2013)

3.1.4.6 Organ Weight

Absolute wet weights of adrenals, brain, uterus, ovaries, testes, epididymides, heart, kidneys, liver, spleen and thymus were recorded immediately after dissection for all animals. Relative weights of these organs were calculated later. Organs were harvested and cleared from the

adhering fat and blotted free from blood (Raghul, 2013). The absolute weight was recorded to the nearest milligram using a calibrated balance. The relative organ weigh was determined using this formular:

$$\text{Relative organ weight (\%)} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight (g)}} \times 100$$

The following organs of all the animals were collected at necropsy and preserved in 10% buffered neutral formalin for processing and microscopic examination; stomach, duodenum, jejunum, ileum, caecum, colon, rectum, liver, brain (representative regions including forebrain, cerebrum, cerebellum), adrenals, lungs, heart, aorta, spleen, thymus, kidneys, testes, epididymides, ovaries, cervix and the uterus.

3.1.4.7 Histopathology

All the preserved organs from control and high dose groups (G1 and G4) of the animals were processed following suitable techniques of washing by tap water, dehydration in ascending grades of ethyl alcohol, clearing in xylene, embedding and blocking in paraffin wax. Paraffin sections (5µm) were cut, stained with hematoxylin and eosin, and mounted on slides (Stevens and Bancroft, 1996).

Histopathology screening was carried out on all preserved organs and tissues of all the animals in control and high dose groups. Complete histopathology was carried out on all gross lesions, the preserved organs and tissues of all the animals in the control and high dose group. If treatment related effects were noted in any of the tissues, the tissues of animals from the next lower dose level were be examined. Successive examination of the next lower dose

level continued until no effects were noted. In addition, all tissues from animals, which died during the course of the study, were examined microscopically to assess any potential toxic effects. The preparatory steps for histopathology evaluation are outlined in sections 3.1.4.7.1 – 3.1.4.7.6.

3.1.4.7.1 Tissue Fixation

Tissue fixation preserves and saves (stabilize proteins) the tissues from decaying (autolysis) and maintains the texture to accommodate and to set a good platform for other procedures to follow. Good tissue sections and staining start with good properly fixed tissues. The minimum time for tissue fixation is about 1-4 hours, but the specimens may be kept for longer times (Survana *et al.,* 2013).

In this study a Formalin solution of 10% buffered neutral buffered formaldehyde was used. The chemical composition of the fixative was:

Formalin (37-40%)	100ml
Distilled water	900ml
NaH_2PO_4	4g
Na_2HPO_4	6.5g

All solid chemicals were adequately dissolved and a pH of 6.8 was maintained and monitored with a pH meter.

3.1.4.7.2 Tissue Processing

Tissue processing is the second step in the processing of tissues for histology analysis. In this procedure, tissues go through a series of steps to give (them) some structural format that prepares the tissues for subsequent sectioning and staining (both for diagnostic and research) (David Carson, nd.) The process primarily functions to remove water in the tissues in a very slow manner through ascending grades of ethanol (ethanol as is the most commonly used dehydrant). It is done so slowly to avoid rapid harsh removal of water which could result in tissue shrinkages. On completion of the process, the alcohol is removed (clearing). Xylene is

the chemical used to clear tissues of the dehydrating solvent. It is the transitional step between dehydration and infiltration with the embedding medium. Infiltration (interpenetration) process of tissues is the actual filling of tissue cavities and cells created by water removed with supporting substance, which in this step will be filled with the medium to be used for embedding (molten wax) (Suvarna *et al.*, 2013).

The table below illustrates the stages and chemicals useful in the automatic processing of tissues and appropriate time frames.

Table 3.2 Chemical Constituents of the Automated Tissue Processor Leica, TP1020 Used in this Study

Process	Reagents	Action	Time frames
Fixation	10% neutral BF	Stabilize proteins	1hr
(2 stations)	10% neutral BF		1hr
Dehydration: ascending	70% ethanol		1:30hr
grades of alcohol	80% ethanol		1:30hr
	95% ethanol	Removal of water	1:30hr
(5 stations)	100% ethanol		1:30hr
	100% ethanol		1:30hr
Clearing	Xylene		1:30hr
(3 stations)	Xylene	Removal of alcohol	1:30hr
	Xylene		1:30hr
Infiltration	Molten wax		2hr
(2 stations)	Molten wax	Interpenetration	2hr

Source: (Adapted from (Leica, TP1020 operational manual 2009; Histology Lab, Walter Sisulu University).

3.1.4.7.3 Tissue Embedding

Tissue embedding is one of the techniques used in histology laboratory that primarily prepares wax infiltrated tissues for section cutting (microtomy). It is the immediate step after tissue processing. The processed and infiltrated tissues are at this stage supported in a medium which allows the tissues to be cut without crushing and damaging. This is the outlined embedding procedure:

- Place the paraffin infiltrated tissue cassettes in the tissue embedding centre's tank, that has been heated to the temperature of about 60-65°C
- Warm the metal block containers in the warm storage area of the machine
- Place the empty metal container over the hot plate of the embedder
- Dispense a little hot paraffin wax from the tank into the metal container
- Take the paraffin infiltrated tissue from the tissue cassette using a forceps and place it in the metal container
- Using heated forceps orientate the tissue specimen as desired and pore small volume of hot wax
- Put back the labelled empty plastic tissue cassette on top of the wax pore more hot paraffin wax to cover the plastic cassette
- Now shift the metal container from the hot plate to the cold plate (-15°C) of the tissue embedder
- When the wax is hard, take out the paraffin block from the metal case. The paraffin block is now ready for section cutting (Suvarna *et al.*, 2013).

3.1.4.7.4 Tissue/Section Cutting (Microtomy)

Figure 3.4 Rotary Microtome HM 355 S. Histology Laboratory, Walter Sisulu University.

After the long processes of tissue dehydration, infiltration, clearing and embedding all tissues are subjected to cutting using either a bench microtome or a rotary microtome. Section cutting involves a number of steps before getting the sections on the glass slide ready for staining. A rotary microtome (figure 3.4) is a heavy duty microtome especially for paraffin and hard sectioning techniques in biology, medicine, industry and research. The machine cuts sections in the range of 0.5µm -100µm. This piece of equipment will normally have both trimming (30µm) and cutting (5µm) function modes. Tissue trimming permits fine adjustments up to first cuts and this result in larger section thickness during trimming. When trimming is complete the fine mode cutting is selected to produce ribbons of sections that are floated in a 60°C water bath to align sections properly on the slide (Micron International GmbH, 2007).

Below is a point-form summary of the histopathology microtome procedure used in the this study (Procedure for Sectioning (HM 355 S Instruments Instructional Manual no 387841, WSU):

- Make sure that the clamping screws and clamping levers on the blade holder and specimen holder are well tightened.
- Make sure that the specimen and specimen holder are well oriented
- Always use sharp blades to produce neat thin sections (avoid compressions)
- Sectioning should always be carried out in ambient temperatures (excluding frozen sections)
- Cutting speed must be programmed in accordance with texture of the material
- Trimming thickness must be set at 30µm and cutting thickness at 5µm to produce good sections
- Upon production of tissue ribbons, the sections are flooded in a water bath (58-60°C) to flatten and iron out any folds
- Good, neat sections are picked from the ribbon in the water bath using a labeled slide for the exact tissue just cut
- Sections are left to dry before staining

3.1.4.7.5 Tissue Staining and the Staining Mechanism

Tissues that are examined microscopically immediately after sectioning without staining appear very dull and uninteresting. They lack contrast of all fixed tissue materials and all have a similar refractive index and colour. In order to bring out the microscopic structure of these tissues, it is essential to stain the cells and connective tissue to expose the different parts in contrasting colours. Staining is therefore not simply colouring the sections but relies on using differences in chemistry of the tissues to show the different components in different colours. This is achieved by using dyes that can bind to tissues selectively. The most common stain in use in histology staining is haematoxylin and eosin (H & E) stain (Suvarna *et al*., 2013).

The binding of dyes to tissues are similar to other chemical bonding and the mechanisms is the same in that they all rely on the same binding forces occurring in all other organic compounds? Eosin is a negatively charged stain (anionic dye) and will stain positively charged (cations) tissue ions (cytosol of the cell and connective tissue). Haematoxylin is a versatile stain and is used in combination with mordants (alum salts) that alters the specificity and colour of the stain. It will stain the acidic environment of the cell (nucleus) dark blue or purple (Suvarna *et al*., 2013).

After drying, the slides are ready to stain, but paraffin sections are still impregnated with paraffin wax which forms a water proofing coat that can prevent the stain to access proteins within the tissues. Therefore the wax must melt in the oven (60°C) and then be removed before sections could be rehydrated (Orchid histopathology staining protocol, 2013). Below is a point-form summary of the histopathology staining procedure used in this study:

- Xylene 5 minutes (2-3 changes)
- Absolute alcohol 5 minutes
- 95% alcohol 5 minutes
- 70% alcohol 5 minutes
- Distilled water 3 minutes
- Harris Haematoxylin 5 minutes
- Differentiate in running tap water 2 minutes
- Eosin-Phloxin 1minute
- 80% alcohol Dips
- 95% alcohol Dips
- Absolute alcohol Dips (2 changes)
- Xylene 5 minutes (3 changes)
- Mount in DPX

3.1.4.7.6 Section Mounting

Staining is subsequently followed by mounting procedure. Normally the mounting medium must have a high RI. Tissues mostly have an RI of 1.5-1.55 and the mounting medium of the same index will offer maximum clarity. The common mountant in histology procedures is DPX. It's a synthetic polystyrene resin that has been dissolved in xylene with some plasticizer added to it. It is a clear waterless solution that hardens in about 24 hours. (D= Distrene 80, that is a commercial polystyrene; P=Plasticizer, dibutyl phthalate and X=Xylene) (Suvarna *et al.*, 2013). Mounting is done under a cover slip and it gives best and clear view of the specimen. The cover slip should have the thickness of about 0.17mm to give best results.

- Mounting is done with slides immersed in xylene.
- General safety rules must always be adhered to (GLP)
- Align cover slip on a flat surface
- Dispense a small drop of DPX onto the cover slip
- Remove the slide from the xylene bath and right face the slide over the slip
- Press very gently tapping at the same time to release any bubbles
- Leave flat on the surface to dry
- Sort and file in slide boxes ready for microscopy (Orchid histopathology Research Laboratory mounting procedure, 2012).

3.1.5 Statistical Analysis of Results

Raw data were processed statistically. The means and standard deviations were calculated with significance between the control and treated groups and all data were summarized in tabular form. All the parameters characterized by continuous data such as body weight, feed consumption, organ weight, relative organ weight, haematology, clinical chemistry were subjected to Bartlett's test to meet the homogeneity of variance. All homogenous data were analyzed using ANOVA and data showing significance in their variances were subjected to Dunnett's t-test.

Significance was calculated at 1% as well as 5% level, and indicated in the summary table as follows:

↑ -Significantly higher than control ($p > 0.05$), ↑↑ -Significantly higher than control ($p > 0.01$), ↓- Significantly lower than control ($p < 0.05$), ↓↓- significantly lower than control ($p < 0.01$)

3.2 RESULTS

3.2.1 Mortalities

Seven animals died in all. In the control group (G1), female animal #6 died on the 11th day of treatment. On the 10th day the animal looked a bit weak showing sluggish movement on examination and the faecal material was soft and mucoid. Animal #14 (male) and Animal #19 (female) both belonging to low dose group (G2) died on 5th and 8th days of treatment respectively. There were no signs of illness during earlier observations. Female animal #27 in the mid dose group (G3) died on the 10th day of treatment after a short period of deteriorating health which was observed by clinical examination. In addition, animal #28, a female in mid dose group (G3) also died on the 10th day. For the two previous days the animal's stomach was empty, due to poor feeding. In high dose (G4) two male animals (#33 and #35) and one female animal (#37) also died in the third week of treatment. Table 3.2 summarises the mortalities found in this study.

Table 3.2 Survival of Mice and Clinical Observations for 28 days Repeated Dose Toxicity Study

Group Number		Group 1 / Control	Group 2	Group 3	Group 4
Dose		0 mg/kg	125 mg/kg	250 mg/kg	500 mg/kg
Clinical Observations		Normal	Normal	Normal	Normal
Animals Initially in Study	Male (M)	5	5	5	5
	Female (F)	5	5	5	5
Natural Deaths	Number	1 (F)	1 (M)	2 (F)	2 (M) & 1 (F)
	Day of Experiment	11	8	10	7 & 15 (M) 17 (F)
Total Deaths per Group	7	1	1	2	3
Animals Surviving to Study Termination	Male	5	4	5	3
	Female	4	5	3	4

3.2.2 Body Weight Changes

No significant deviation of weights from the mean of all the treated groups was observed as compared to the control group at week 1, week 2, week 3,week 4 and at termination of the study. Table 3.3 summarises the changes in body for the duration of the study.

Table 3.3 Summary of Body Weight (g)/week

Gender: Male

Week N°	G1 (0 mg/kg /Bwt.)	G2 (125mg/kg Bwt.)	G3 (250mg/kg/Bwt))	G4 (500mg/kg/Bwt.)
	Mean	Mean	Mean	Mean
Pre- exp	25.240±2.059	24.940±2.564	24.940±2.224	24.960±2.526
Week 1	26.020±2.640	27.460±1.399	25.280±2.247	25.125±0.818
Week 2	25.240±3.009	26.625±1.952	24.880±3.093	25.167±1.124
Week 3	27.340±2.390	27.375±1.850	26.880±2.860	25.667±1.002
Week 4	28.180±2.607	28.675±1.841	27.360±3.044	24.667±2.281
Terminal point	25.960±2.626	26.350±0.785	25.240±2.535	22.533±1.172

Gender: Female

Week N°	G1 (0 mg/kg /Bwt.)	G2 (125mg/kg Bwt.)	G3 (250mg/kg/Bwt))	G4 (500mg/kg/Bwt.)
	Mean	Mean	Mean	Mean
Pre- exp	23.440±1.609	23.700±2.269	23.420±2.044	23.780±2.264
Week 1	24.420±2.262	22.725±3.694	23.340±2.548	23.720±2.401
Week 2	23.025±1.477	22.625±3.357	22.933±2.303	21.960±4.230
Week 3	23.875±1.021	22.175±3.106	25.033±2.871	23.350±1.453
Week 4	24.250±0.933	24.025±4.088	25.333±2.676	24.475±1.808
Terminal B.Wt	22.600±0.983	22.525±3.87	23.633±2.303	23.300±1.643

3.2.3 Feed Consumption

No significant changes were observed in the feed consumption of the treated groups of animals as compared to the control group in four weeks of treatment.

3.2.4 Absolute Organ Weight

No significant change in females was seen in all organ weights when compared to the control group, except for the weight of the adrenal gland in all doses (125, 250, 500mg/kg.b.wt.) which was significantly higher than the control ($p \geq 0.05$). Absolute weight of the heart in female animals was significantly high in the low dose group ($p \geq 0.01$). No significant changes were observed in weights in all organs (125, 250, 500 mg/kg.b/wt). Table 3.4 is a summary of the absolute mice organ weights at the time of necropsy.

Table 3.4 Summary of Absolute Organ Weight (g)

Gender: Male

Parameter	Group N° & Dose (mg/kg b.wt.)			
	G1 (0)	G2 (125)	G3 (250)	G4 (500)
	Mean	Mean	Mean	Mean
Terminal Body Weight	25.240±2.059	24.940±2.564	24.940±2.224	24.960±2.526
Adrenals	0.011±0.005	0.036±0.024	0.027±0.021	0.042±0.025
Thymus	0.072±0.044	0.064±0.035	0.068±0.043	0.057±0.036
Epididymis	0.027±0.057	0.027±0.029	0.119±0.030	0.093±0.017
Spleen	0.155±0.055	0.240±0.130	0.148±0.036	0.149±0.018
Heart	0.135±0.016	0.156±0.033	0.171±0.054	0.129±0.010
Brain	0.385±0.016	0.454±0.047	0.437±0.086	0.441±0.042
Kidneys	0.436±0.055	0.460±0.027	0.522±0.238	0.359±0.033
Testes	0.265±0.079	0.227±0.042	0.331±0.129	0.145±0.077
Liver	1.532±0.170	1.670±0.085	1.541±0.233	1.346±0.136

Table 3.4 Cont. Summary of Absolute Organ Weight (g)

Gender: Female

Parameter	Group N° & Dose (mg/kg b.wt.)			
	G1 (0)	**G2 (125)**	**G3 (250)**	**G4 (500)**
	Mean	**Mean**	**Mean**	**Mean**
Terminal Body Weight	22.600±0.983	22.525±3.87	23.633±2.303	23.300±1.643
Adrenals	0.006±0.002	0.013±0.006	0.072±0.062	0.013±0.004
Thymus	0.084±0.054	0.065±0.004	0.062±0.023	0.069±0.011
Uterus	0.198±	0.102±0.081	0.954±1.146	0.133±0.034
Spleen	0.191±0.112	0.146±0.052	0.102±0.041	0.160±0.028
Heart	0.117±0.006	0.131±0.006	0.153±0.040	0.118±0.017
Brain	0.394±0.082	0.449±0.057	0.428±0.049	0.458±0.059
Kidneys	0.297±0.050	0.393±0.034	0.331±0.062	0.327±0.034
Ovaries	0.115±0.131	0.024±0.014	0.084±0.026	0.049±0.036
Liver	1.383±0.137	1.409±0.186	1.387±0.078	1.358±0.223

3.2.5 Relative Organ Weight

No treatment related changes were observed in relative organ weights of animals treated with *Euclea natalensis*, except for changes seen in the testes in males of a relative weight that is significantly low in the high dose group (500mg/kg.b.wt) ($p \leq 0.01$). Table 3.5 summarises the relative organ weights for the animals treated with *Euclea natalensis*.

Table 3.5 Summary of Relative Organ Weight (g)

Gender: Male

Parameter	Group N° & Dose (mg/kg b.wt.)			
	G1 (0)	G2 (125)	G3 (250)	G4 (500)
	Mean	Mean	Mean	Mean
Adrenals	0.034±0.027	0.081±0.098	0.087±0.096	0.076±0.121
Thymus	0.284±0.204	0.145±0.161	0.260±0.143	0.155±0.187
Epididymis	0.107±0.240	0.062±0.099	0.301±0.290	0.246±0.229
Spleen	0.587±0.178	0.730±0.596	0.582±0.097	0.395±0.363
Heart	0.524±0.074	0.472±0.279	0.669±0.151	0.344±0.319
Brain	1.500±0.231	1.375±0.779	1.727±0.271	0.885±0.987
Kidneys	1.680±0.134	1.397±0.783	2.061±0.906	0.957±0.878
Testes	1.022±0.274	0.691±0.412	1.301±0.460	0.226±0.327
Liver	5.900±0.224	5.075±2.863	6.095±0.641	3.591±3.314

Table 3.5 Cont. Summary of Relative Organ Weight (g)

Gender: Female

Parameter	Group N° & Dose (mg/kg b.wt.)			
	G1 (0)	G2 (125)	G3 (250)	G4 (500)
	Mean	Mean	Mean	Mean
Adrenals	0.019±0.013	0.045±0.033	0.130±0.235	0.044±0.029
Thymus	0.290±0.248	0.239±0.145	0.102±0.143	0.239±0.144
Uterus	0.165±0.369	0.203±0.363	1.479±2.978	0.460±0.294
Spleen	0.672±0.542	0.545±0.417	0.283±0.265	0.548±0.320
Heart	0.414±0.233	0.476±0.280	0.396±0.398	0.409±0.246
Brain	1.395±0.838	1.625±0.971	1.094±1.022	1.579±0.925
Kidneys	1.048±0.609	1.414±0.812	0.836±0.769	1.123±0.636
Ovaries	0.293±0.465	0.046±0.076	0.134±0.188	0.164±0.154
Liver	4.890±2.756	5.038±2.845	3.536±3.244	4.641±2.646

3.2.6 Gross Pathological Findings

3.2.6.1 External Findings

Gross pathological observations did not reveal any abnormality externally in any of the animals belonging to both the control and treated group animals. Figure 3.3 depicts the necropsy process undertaken during the 28 day repeated oral toxicity study.

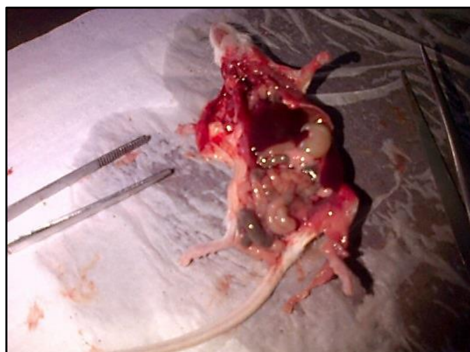


Figure 3.5 Digital Images of the Necropsy on Albino mice

3.2.6.2 Internal Findings

No treatment related gross lesions were observed in any of the animals belonging to either sex treated with *Euclea natalensis* at the dose levels of 125, 250, 500mg/kg body weight as compared to the control. Below (Table 3.6) is a summary of the gross pathological investigation done at necropsy after 28 days exposure to *Euclea natalensis*.

Table 3.6 Summary of Gross Pathological Findings

Group Number		Group 1 / Control	Group 2	Group 3	Group 4
Dose		0 mg/kg	125 mg/kg	250 mg/kg	500 mg/kg
Animals Initially in Study	Male	5	5	5	5
	Female	5	5	5	5
Gross Pathology (External)	Male	0	0	0	0
	Female	0	0	0	0
Gross Pathology (Internal)	Male	0	0	0	0
	Female	0	0	0	0

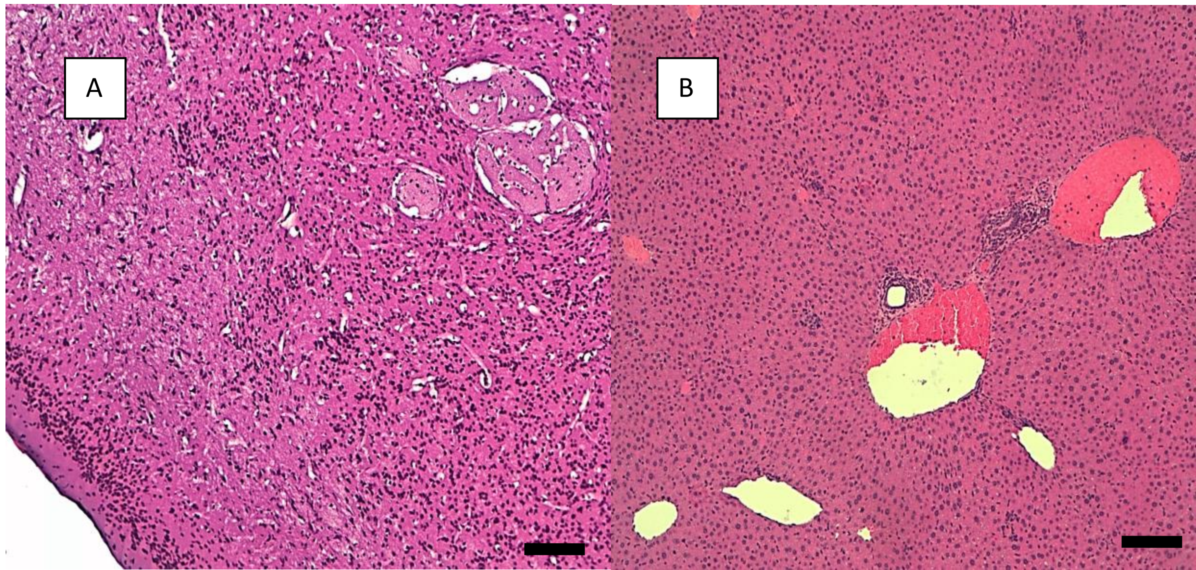
3.2.7 Histopathological Observations

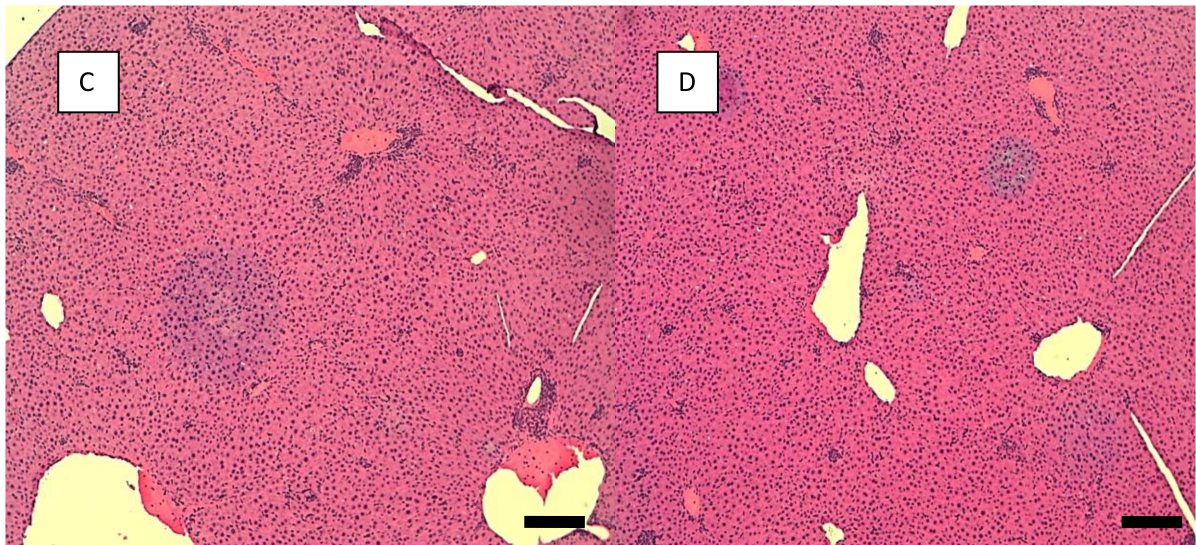
The histopathology is outlined in table 3.7 below.

Table 3.7 Summary of Histopathological Findings

Group	Animal N°	Sex	Histopathological Observation
Control (0 mg/kg)	SA45/9/3	Female	**Brain**: Medial mineralization
	SA/45/5/1	Male	**Liver:** Granulopoetic hyperplasia
	SA45/5/1	Male	**Liver:** Hepatocellular basophilic cell focus
	SA45/5/1	Male	**Lung:** Perivascular and Peribronchial lymphoid aggregates
Treated (500 mg/kg)	SA45/39/1	Female	**Liver:** Hepatocellular basophilic cell focus
	SA45/39/1	Female	**Lung:** Perivascular and Peribronchial lymphoid aggregates
	SA45/31/1	Female	**Lung:** Lymphoid aggregates

Medial mineralization of the brain was found in a female control mouse (Figure 3.6[A]). In addition granulopoetic hyperplasia of the liver from a male mouse in the control group was noted (Figure 3.6[B]). Hepatocellular basophilic cell foci were recorded in the liver of a male control mouse (Figure 3.6[C]) and a female mouse (Figure 3.6[D]) treated with *Euclea natalensis* (500mg/kg). Perivascular and peribronchial lymphoid aggregates of the lung of a male control mouse (Figure 3.6[E]) and female mouse (Figure3.6 [F]) treated with *Euclea natalensis* (500mg/kg) was also noted.


A
B


C
D

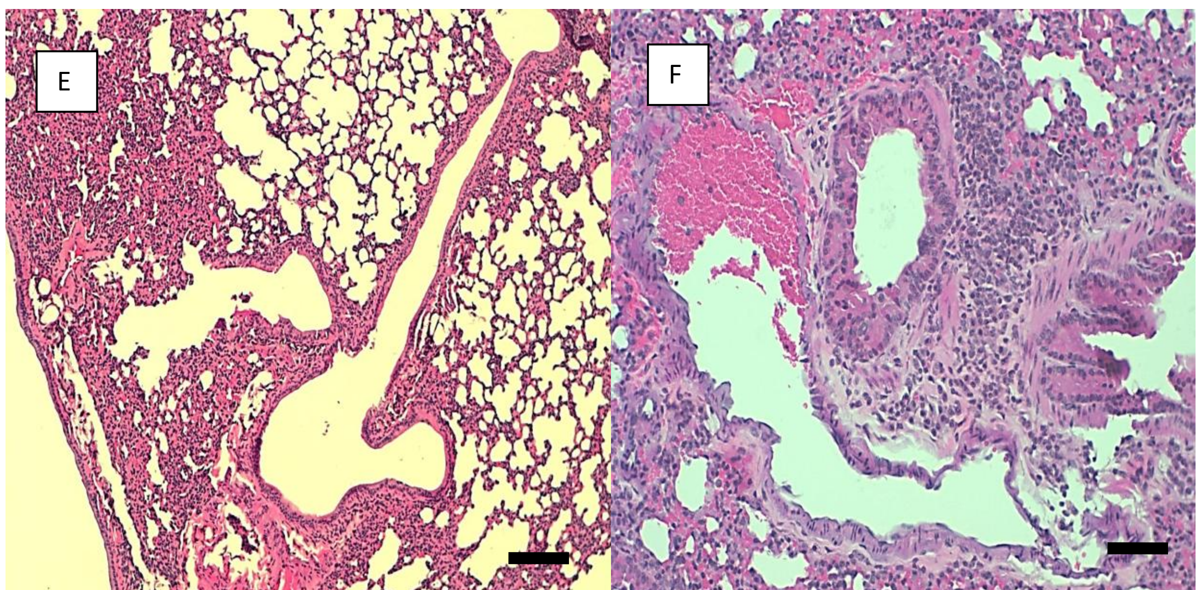

E
F

Figure 3.6 Photomicrographs of Histopathology Found in the 28 day Repeat Dose Oral Toxicity Study. [A] Medial mineralization of the brain found in a female control mouse (*H&E, x10 magnification, scale bar = 120µm*). [B] Granulopoetic hyperplasia of the liver from a male mouse in the control group (*H&E, x10 magnification, scale bar = 120µm*) [C] and [D] Hepatocellular basophilic cell focus in the liver of a male control mouse and female mouse [D] treated with *Euclea natalensis* (500mg/kg) (*H&E, x10 magnification, scale bar = 120µm).* [E] and [F] Perivascular and peribronchial lymphoid aggregates of the lung of a male control mouse and female mouse [F] treated with *Euclea natalensis* (500mg/kg) (*H&E, x10 magnification, scale bar = 120µm).*

CHAPTER 4

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 INTRODUCTION

The primary objective of the this study was to conduct a 7 Day Repeat Oral Toxicty Study (ROTS) on Swiss albino mice as a pilot study to prepare for the 28 Day ROTS on the same species of animals, in an effort to achieve the main aim of the study which remains a histopathological investigation. In order to meet this objective, methodologies were put in place to assess and evaluate the possible toxicity of compounds of interest that have been extracted from the root material of the medicinal plant (*Euclea natalensis*). These compounds have been identified and tested against the drug resistant and drug sensitive strains of the *Mycobacterium tuberculosis*. The parameters of interest in the two studies were; the body weight, the organ weight, the relative organ weight, feed consumption, clinical observations, both external and internal gross examinations including the cavities and orifices of all animals and lastly the histopathological screening of processed tissues (Lall and Meyer, 1999).

The overall welfare of all animals, the quantity, the in house allocation and housekeeping, the dose levels, and gender distribution were addressed in a similar manner in the two studies. The only difference is that the pilot was conducted in India at Orchid Chemicals and Pharmaceuticals Ltd, in the biology Laboratories, Toxicology Department. The main study was conducted at Walter Sisulu University, Anatomy, Histology and Embryology Department. Effort was made in order to adhere to and maintain good laboratory standards and protocols mandated by the toxicology studies guidelines (OECD 407, 2008).

4.2 REPEATED DOSE (7 DAY) ORAL TOXICITY STUDY OF *EUCLEA NATALENSIS* IN SWISS ALBINO MICE

In this dose range finding study forty (twenty per sex) Swiss albino mice were grouped and treated with *Euclea natalensis* crude extract dissolved in 20% DMSO, which was then suspended in 80% of 0.25% CMC. The dosages were administered as stipulated in the methodology in chapters two and three. Food consumption as well as the clinical observations was recorded during the period of dosing. The treated animals did not show any change when compared to the control animals. Both externally and internally, gross pathological observations in treated animals did not show any abnormality when compared to control animals. Animal absolute body weight in treated animals showed no deviation from untreated animals. This is expectedly so since the animals consumed food quite well, and were healthy which is reflected by weight records. Although absolute weight was maintained, treated female animals (G3 & G4) in mid dose (250mg/kg b.wt) and high dose (500mg/kg b.wt) showed a decrease in weight of the heart when compared to control animals. This was not a significant change statistically but it called for biological concern. As a result the relative weight of the heart was decreased. The decreased weight in these animals could be attributed to the cardiac mass loss due to degenerative changes noticed in high dose animal, although controls did not lose any weight while having a similar histopathological picture as that seen in high dose group.

The absolute weight of the testes in treated male animals, G2 (125mg/kg b.wt) was statistically elevated at 1% significance level when compared with control animals ($p \geq 0.01$). Slegtenhorst and colleagues (1998) found that normal developmental increases in testes size in growing mice was the result of increased Sertoli cell proliferation. It is plausible that the

testicular weight gain can be attributed to increased Sertoli cell stimulation in the G2 animals but remains dubious given the absence of similar changes in the higher dosage groups. Haematology results of all treated animals in both the sexes did not show any abnormality when compared to their untreated counterparts. In clinical pathology, G3 animals (250mg/kg b.wt) showed a significant decline in total protein at 5% level ($p \leq 0.05$). Protein levels in females did not decrease when compared with control animal.

Total cholesterol level in all treated female groups (G2; 125mg/kg b.wt, G3; 250mg/kg b.wt, G4; 500mg/kg b.wt) was decreased when compared with control animals. This is not a statistically significant change, but since all treated groups show the same declining picture, further investigation might be warranted.

Triglycerides level was significantly low at 5% in female G2 (250mg/kg b.wt) as compared to controls group ($p \leq 0.05$). It cannot be concluded that mice in this group suffered malabsorption of fats, as the fecal material did not show any signs of undigested fats during the period of study. The outstanding fact could be that mice have a very high metabolic rate (Speakerman *et al.*, 2004).

Phosphorus level was also significantly higher at 5% level in G3 (250mg/kg b.wt). ($p \geq 0.05$). High levels are correlated with atherosclerosis in both animals and humans especially in kidney diseases (Foley *et al*., 2008). This is not the case with mice in this group since renal histopathology was absent. A treatment related phenomenon isn't likely given the absence of this clinical finding in the remaining treatment groups.

Histopathological observations revealed degenerative changes in two control animals (SA45/1, SA45/6) and one high dose (500mg/kg b.wt) male animal (SA45/39) in the right ventricular wall of the heart. The degenerative lesions in mice carry a spectrum of spontaneous and progressive age-related changes in the heart. These various changes have been reported with a variety of species of laboratory mice (Maronpot *et al.*, 1999). The morphologic changes include myocyte degeneration, as seen in the three animals. As these degenerative changes develop, the interstitial tissue in affected areas become fibrotic. There may be myocyte atrophy and inflammatory cell infiltration. In these animals the affected areas are infiltrated with fibrocytes, implying that there could be laying down of collagen. But since the stain used cannot demonstrate collagen, at this stage it cannot be concluded that fibrosis did take place. However, this degeneration of muscle tissue could lead to cardiomyopathy. Since the two control animals show similar changes seen in treated animals, it cannot be concluded that these lesions observed in treated animal are dose dependent.

The X-zone of the adrenal gland is a band of basophilic cells seen just around the medulla of the adrenal gland. In female animals, this zone grows until animals are nine weeks old. It starts regressing gradually in virgin animals and rapidly upon first pregnancy (Maronpot *et al.*, 1999). Initial evidence of regression in females is the lipid vacuolization as seen in the three animals, and this should not be taken as a pathological process. The adrenal gland in one control animal (SA45/7) and two high dose animals (SA45/37 and SA45/40) exhibited the involution of the X-zone with extensive lipid vacuolization and is consequently not considered a dose dependent change.

The lung tissue of treated male animals SA45/31 and SA45/34 (500mg/kg b.wt) showed massive infiltration of mononuclear cells within the lung parenchyma and peri-bronchiolar when compared with controls. The alveolar, alveolar sacs, alveolar ducts and the alveolar septum have completely lost the normal morphology. There is dilatation of the alveoli. Inflammation suggests lung infection, and the dilated alveoli are trying to put in place a compensatory mechanism so that the lung is able to continue with normal function. Despite the severity of the lung pathology the contextual significance implies a limited extent devoid of dose dependence.

In conclusion, all lesions observed in the heart and adrenal glands were spontaneous, age-related to laboratory animals and are not dose dependent. Therefore, the NOAL/NOAEL/MTD of *Euclea natalensis* in Swiss albino mice, at termination of the 7 day study, was 500mg/kg b.wt .

4.3 REPEATED DOSE (28 DAY) ORAL TOXICITY STUDY OF EUCLEA NATALENSIS IN SWISS ALBINO MICE

In this 28 day sub-acute repeated oral toxicity study, forty healthy animals were used, twenty males and twenty females. Seven animals died in the 28-Days of treatment in four groups. All these deaths are not dose dependent as one control animal also died. The autopsy was performed on all animals that died and no abnormalities were found. The internal cavities, orifices and gross external and internal examination revealed no abnormality of organs. The evidence of poor feed consumption was noted in one animal which showed emptiness of the stomach for about three days before dying.

Animals were obtained in Bloemfontein through Shalom Laboratories Suppliers in Durban. All parameters (absolute weight, terminal weight, organ weight, feed consumption) did not show any statistical significance in all treated groups (500mg/kg, 250mg/kg, and 125mg/kg) in both sexes when compared to the control animals. The exception was the relative organ weight of testes which is significantly low in high dose (500mg/kg) at 1% level ($p \leq 0.01$). In females the absolute weight of adrenal gland was significantly high in all treated groups (500mg/kg, 250mg/kg, and 125mg/kg) at 5% level ($p \geq 0.05$). Low dose (125mg/kg) female animals showed significantly high absolute weight of the heart at 1% level ($p \geq 0.01$).

The low relative weight of the testes in high dose could be due to poor or compromised differentiation and maturation of the sertoli cells of the semineferous tubules. Sertoli cells contribute the bulk of mass within the testes. Low relative weight of testes has been observed in mice. Mice treated with triidothyronine resulted in low relative weight of testes. This is not the case in this study (Auharek *et al*., 2010). Animals were treated with root extract of *Euclea natalensis* rich in Napthoquinones. The assumption on the observed low relative weight could

be that the Napthoquinones have somehow properties and characteristics similar to triidothyronine that delays the differentiation and maturation of the sertoli cells which will in turn affect the maturation of the germination epithelium in the testes. Further investigation might be warranted.

The absolute weight of the adrenal gland was significantly high in all female treated groups (500mg/kg, 250mg/kg and 125mg/kg), ($p \geq 0.05$). This phenomenon has been observed (Bielohuby *et al.,* 2007). Absolute weight of the adrenal gland in female animals has been shown to be heavier than in male animals. The increase in absolute weight was observed between the age of 3 weeks and 11 weeks. In male animals the weight increased from week 3 up to week 7 and there was a decline between the age of 9 weeks and 11 weeks. In female animals the weight escalated from week 3 up to week 7 and remained at that level until week 11. All animals in the study were between the age of 6 weeks and 7 weeks at the onset of treatment (to include all that showed increase in absolute weight of the adrenal gland). This age coincides well with animal age in the Bielohuby study (2007). Female adrenal glands are heavier than male adrenal glands in mice. The point being made is that this is normal according to his research (Bielohuby *et al.,* 2007). In this study however, the significance found was between control animals not gender differences.

The absolute weight of heart in treated female animals in the low dose (125mg/kg) group was significantly high at 1% level ($p \geq 0.01$). This might be due to hypertrophied striated myocytes of the heart. However, microscopically the heart did not show any signs of hypertrophied muscle cells. This weight increase cannot be regarded as dose dependent since animals in high dose (500mg/kg) have not shown this picture. In human's alcohol-induced cardiomyopathy, the heart shows areas of hypertrophy with an increase of mass usually of

600g or even more in adult heart (Case Files, Pathology, 2008). This was not seen microscopically in the heart of these animals.

In conclusion, all observed lesions are not dose dependent, but are spontaneous lesions associated with age in laboratory mice. Therefore, the NOAL/NOAEL/MTD of *Euclea natalensis* in albino mice is 500mg/kg.b.wt for this study

4.4 COMPARISON OF REPEATED DOSE (7 DAY) ORAL TOXICITY STUDY OF *EUCLEA NATALENSIS* IN SWISS ALBINO MICE *AND* REPEATED DOSE (28 DAY) ORAL TOXICITY STUDY OF *EUCLEA NATALENSIS* IN SWISS ALBINO MICE

No treatment related changes were observed in relative organ weights of animals treated with *Euclea natalensis*, except for changes seen in the testes in males of a relative weight that is significantly low in the high dose group (500mg/kg.b.wt) ($p \leq 0.01$). It is interesting to note that the weight of the testes in the 7 Day study was increased and decreased in 28 Day study. From this finding it can be deliberated that the shorter the animals were exposed to plant extract the more the testes developed and the longer they got exposed to the plant extract the more the delay in differentiation and maturation of the germination epithelium within the tubules. There was no evidence of atrophy of the seminiferous tubules in the organ as seen below in both control animal and high dose animal.

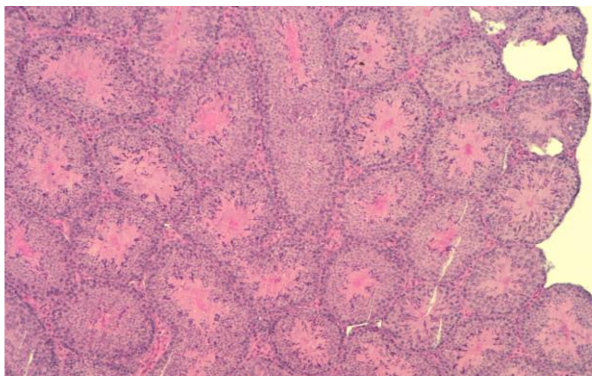


Figure 4.1 Photomicrograph of the Control Testes

H&E stain. 10x

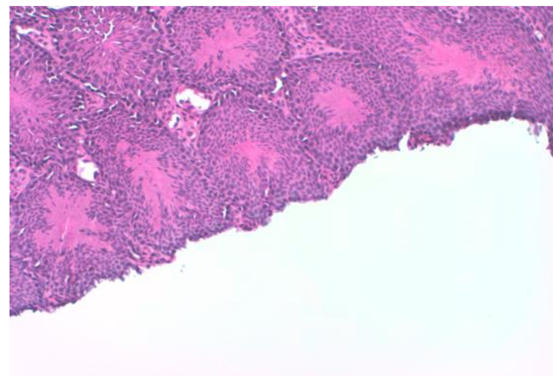


Figure 4.2 Photomicrograph of the Testes in the High Dose Group

H&E stain 20x

In both studies the same statistical package was employed to analyse all collected data (body weights, absolute organ weights, relative organ weights, and feed consumed) (Toxistat version, 2005.2). Three tests were followed namely Bartlett's test, ANOVA and Dunnett's t-test. The t-test correlated and compared all mentioned parameters of three treated groups

against the non-treated groups (controls) in the two sexes. Analysis of blood for haematology and clinical biochemistry was done in 7 Day study and not done in 28 Day study since this was beyond the scope of the study. The results that emanated from the blood analysis revealed no significant decrease and increases in animals treated with *Euclea natalensis* as compared to the control counterparts in both sexes. The exception was with just a few such as a decrease in phosphorus in G4 (500mg/kg.b.wt) (Table 2.3).

The statistical analysis in the two studies reveals mass variations in the absolute weight of the heart. In low dose (125mg/kg/b.wt) animals and in control 1, male at 7 Days the weight decreased whereas in the 28 Day study the mass of the same organ increased in 28 Day in the same low dose group (125mg/kg.b.wt). The reduction in mass was supported by loss of myocytes in the affected animals where there was evidence of myocyte degeneration in the right ventricular wall of the heart. (Figure 3.6 micrograph A). This lesion was not dose dependent but regarded as age related alterations associated with laboratory mice since the control group also underwent the similar alteration. This has not been the case with neither controls nor treated animals in the 28 Day study. Although the weight of the heart increased, there was no evidence in the histopathology screening that suggest hypertrophy of the heart muscle. This increase might be induced by some unknown factors at present and requires further investigation.

There is a common lesion that has been seen in the two studies. The control animals as well as the treated animals in 7 Day study exhibited massive white blood cell infiltration within the lung parenchyma. In the 28 Day study the lung tissue exhibited aggregates of lymphocyte around the blood vessels and the bronchioles (perivascular and peribronchiolar lymphocytic aggregations) (Figure 4.3 and Figure 4.4). These lesions were not dose dependent as

discussed earlier. The figure bellow shows massive lymphocytic infiltration and perivascular and peribronchiolar lymphocytic aggregation in 7 Days and 28 Days respectively.

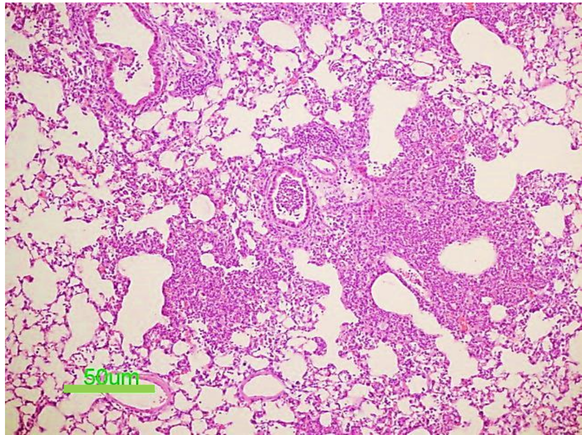


Figure 4.3Photomicrograph of Treatment Lung from 7 Day Study H&E statin

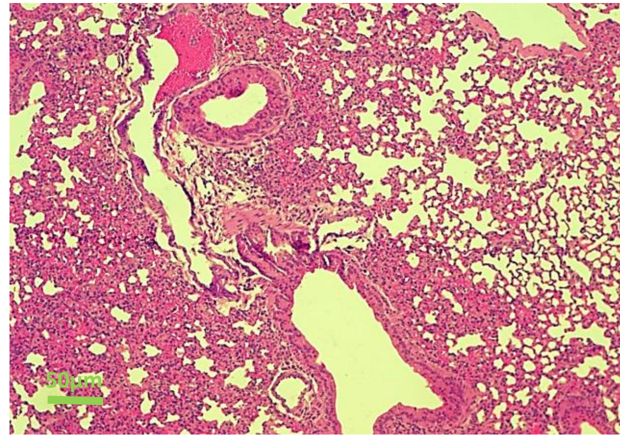


Figure 4.4 Photomicrograph of Treatment Lung from 28 Day Study H&E stain

No lesions were seen in the liver and brain of animals treated for seven days in the pilot study. These lesions were only seen in the 28 days treatment (medial brain mineralization and hepatocellular basophilic focus) in both control animals as well as treated animals in both sexes. The involution of the X-Zone of the adrenal gland in females control animal was observed at 7 Days treatment but was not observed in the 28 Days treatment.

In the 7-Days of treatment, the control and treated animals did not show any age related lesions of the liver or the brain. Histopathological observations in the 28-Days of treatment also revealed lesions that are spontaneous and age related in mice. Female control animal #45/9 showed medial mineralization of the brain. Control animal #45/4 male showed liver basophilic hepatocellular cell focus (due to increased amounts of polyribosomes or rough endoplasmic reticulum), as well as liver granulo-poetic hyperplasia.

4.5 CONCLUSIONS AND RECOMMENDATIONS

Medicinal plants have been used by humans as far back as 60 000 years. Ethno medicine is broadly defined as the use of plants by humans as medicines. Traditionally this is a non-Western medicinal practice. According to World Health Organization (WHO), 65% of the world's population has incorporated the traditional medicines into their primary health care (Daniel and Norman, 2000).

WHO has documented TB treatment guidelines in effort to control the spread of the disease globally (Treatment of Tuberculosis Guidelines, 2009). TB surveillance is ongoing in some parts of the world so that the programmes and initiatives instituted by WHO in the fight against the disease are met (European Centre for disease prevention, 2008).

Generally, toxicity studies form the basis of many of the standard repeated dose safety evaluation studies such as sub-acute, sub-chronic and chronic toxicity studies. These studies are designed to characterize the effect of test chemicals over repeated exposure to experimental animals. They evaluate health hazards that may result from repeated exposure during limited part of test animal's lifetime, and also evaluate dose related effect on survival, body weight, feed consumption, metabolism; gross and microscopic organ changes (Stephen, 2013).

In South Africa, if we are to reverse the escalating numbers in drug resistance, the list is long of questions to be addressed and posed as to the fate of the TB pandemic. But looking at the past, present and the future strategies and the state of the disease itself (Edginton, 2000, Edginton and Naidoo, 2007), and the enthusiasm exhibited by researchers of the country in indigenous plants, there is hope for new inventions of drugs against TB.

For South Africa to be able to compete in the global debate on TB prevention and eradication, the indigenous plants (*Euclea natalensis*) available in the country have to be considered, appreciated as well as marketed by programmes such as Natural Resources Management Programmes together with Department of Environmental Affairs (Mander, 1998). *Euclea natalensis* has been shown to have a wide spectrum on microbial activity not only in TB but also as having anti-fungal properties (Lall *et al.,* 2006). It's a promising medicinal plant together with other plants selected that have shown anti-TB properties (Green *et al*., 2010).

Euclea natalensis plant therefore has shown no toxic effects in animals in these two studies conducted. Animals that died in the course of treatment in the 28 Days repeated oral toxicity study died due to natural courses and was not dose dependent. In conclusion I therefore, recommend further exploration of the leading compounds identified in this plant at molecular level to completely declare the extracts non toxic. Some compounds that have been discovered to be of great importance are 7-Methyl-Juglone and Diospyron (Lall *et al.,* 2006). Further research should be done to clarify the underlying mechanisms that account for the adrenal and testicular changes noted in this study.
To elaborate more on future studies on genotoxity profiling of the compounds in this plant, studies are underway where the identified active compounds are discovered to inhibit the super-coiling reactions of Deoxy-ribose nucleic acid (DNA) in *Mycobacterium tuberculosis*. Enzymes associated with transcription processes of the molecule were inhibited. This new chapter in this plant clearly points to a new TB drug that could be produced in the country and made available at low cost in the fight against the TB pandemic in South Africa. Therefore genotoxicity profiling studies of the leading compounds must be conducted.

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APPENDIX 1

RAW DATA

CAGEWISE FEED INPUT, LEFTOVER AND FEED CONSUMPTION RECORD

G1 0mg/kg

A. NO	DAY 1	FDL	AFC		DAY 4	FDL	AFC
1	100	93	2.3		50	49.7	0.1
2	100	96	1.3		50	49.5	0.2
3	100	96.5	1.2		50	49.6	0.1
4	100	94.2	2		50	49.5	0.2
5	100	97.6	0.8		50	49.7	0.1
6	100	89.7	3.4		50	46.7	1.1
7	100	96.3	1.2		50	46.6	1.1
8	100	97.2	0.9		50	47.4	0.9
9	100	93.5	2.2		50	46.7	1.1
10	100	96.9	1		50	46.6	1.1
				G2	**125mg/kg**		
11	100	95.7	1.4		50	49.7	0.1
12	100	99	0.3		50	49.8	0.06
13	100	94	2		50	49.6	0.1
14	100	97.9	0.7		50	49.5	0.2
15	100	95.3	1.6		50	49.3	0.2
16	100	96.2	1.3		50	49.1	0.3
17	100	90.2	3.3		50	45	1.6
18	100	96.7	1.1		50	44.7	1.8
19	100	95.1	1.6		50	43.3	2.2
20	100	92.8	2.4		50	47.8	0.7
				G3	**250mg/kg**		
21	100	93.3	2.2		50	47.8	0.7
22	100	99.8	0.1		50		
23	100	95.4	1.5		50	49.1	0.3
24	100	95.1	1.6		50	47	1
25	100	99.8	0.1		50	49.9	0.03
26	100	94.9	1.7		50	44.4	1.9
27	100	95	1.7		50	48.3	0.6
28	100	98.1	0.6		50	46.8	1.1
29	100	99	0.3		50	49.7	0.1
30	100	98.5	0.5		50	49	0.3
				G4	**500mg/kg**		
31	100	99.7	0.1		50	49.8	0.06
32	100	96.8	1.1		50	49.9	0.03
33	100	94.6	1.8		50	42.5	2.5
34	100	94.1	2		50	49.9	0.03
35	100	99.9	0.03		50	49.9	0.03
36	100	96.8	1.1		50	46.7	1.1
37	100	99.8	0.1		50	47.4	0.9
38	100	94.5	1.8		50	49.5	0.2
39	100	94.7	1.7		50	48.5	0.5
40	100	89.8	3.4		50	47.9	0.7

Raw data 1; G1-G4 Males

31.6	27.0	22.2	23.1	0.007	0.023	0.061	0.082	0.144	0.458	0.443	0.191	1.676	0.030	0.100	0.264	0.355	0.623
27.3	25.8	24.4	24.6	0.010	0.053	0.076	0.113	0.164	0.416	0.419	0.163	1.399	0.041	0.215	0.309	0.459	0.667
28.5	27.7	24.6	24.6	0.013	0.066	0.065	0.105	0.164	0.440	0.397	0.186	1.743	0.053	0.268	0.264	0.427	0.667
25.0	26.8	26.2	26.2	0.015	0.042	0.084	0.142	0.171	0.475	0.538	0.190	1.727	0.057	0.160	0.321	0.542	0.653
23.8	22.4	22.6	21.9	0.013	0.058	0.067	0.133	0.149	0.441	0.384	0.187	1.185	0.059	0.265	0.306	0.607	0.680
28.6	28.0	28.5	26.4	0.011	0.039	0.085	0.085	0.166	0.519	0.567	0.213	1.626	0.042	0.148	0.322	0.322	0.629
28.3	26.2	24.9	25.2	0.013	0.050	0.077	0.128	0.185	0.455	0.463	0.216	1.600	0.052	0.198	0.306	0.508	0.734
25.9	24.4	23.5	24.3	0.011	0.051	0.075	0.116	0.157	0.471	0.345	0.201	1.582	0.045	0.210	0.309	0.477	0.646
25.6	23.7	23.3	22.9	0.012	0.034	0.064	0.158	0.141	0.434	0.402	0.216	1.412	0.052	0.148	0.279	0.690	0.616
24.7	25.7	25.7	26.1	0.005	0.049	0.076	0.107	0.165	0.461	0.482	0.207	1.466	0.019	0.188	0.291	0.410	0.632
31.9	31.7	31.6	30.9	0.015	0.064	0.081	0.142	0.174	0.496	0.632	0.192	1.685	0.049	0.207	0.262	0.460	0.563
25.0	31.5	36.0	36.3	0.011	0.036	0.096	0.118	0.224	0.485	0.774	0.187	1.967	0.030	0.099	0.264	0.325	0.617
28.4	28.0	26.8	27.4	0.010	0.070	0.076	0.146	0.192	0.472	0.513	0.175	1.880	0.036	0.255	0.277	0.533	0.701
27.0	28.1	27.7	27.1	0.012	0.061	0.065	0.113	0.168	0.460	0.541	0.160	1.624	0.044	0.225	0.240	0.417	0.620
23.5	21.4	20.2	20.6	0.014	0.041	0.077	0.114	0.132	0.432	0.309	0.176	1.234	0.068	0.199	0.374	0.553	0.641
29.0	24.3	21.8	21.3	0.008	0.034	0.079	0.110	0.153	0.458	0.384	0.216	1.350	0.038	0.160	0.371	0.516	0.718
28.4	25.3	24.6	24.4	0.015	0.026	0.087	0.121	0.197	0.469	0.473	0.215	1.451	0.061	0.107	0.357	0.496	0.807
26.9	25.3	26.1	25.9	0.011	0.066	0.068	0.093	0.155	0.461	0.439	0.175	1.473	0.042	0.255	0.263	0.359	0.598
26.1	24.2	20.9	21.1	0.007	0.030	0.055	0.156	0.123	0.414	0.392	0.181	1.451	0.033	0.142	0.261	0.739	0.583
22.8	21.1	20.2	20.7	0.006	0.043	0.066	0.093	0.131	0.448	0.283	0.216	1.203	0.029	0.208	0.319	0.449	0.633

Raw data 1; G1-G4 Females

27.6	28.5	28.2	27.5	0.022	0.014	0.103	0.161	0.163	0.172	0.486	0.446	1.675	0.080	0.051	0.375	0.585	0.593
26.6	26.8	26.7	26.5	0.016	0.016	0.084	0.081	0.126	0.177	0.496	0.410	1.516	0.060	0.060	0.317	0.306	0.475
26.1	26.1	26.4	25.9	0.026	0.015	0.078	0.103	0.095	0.154	0.482	0.384	1.400	0.100	0.058	0.301	0.398	0.367
24.7	25.0	23.6	24.3	0.017	0.026	0.112	0.251	0.174	0.139	0.465	0.347	1.398	0.070	0.107	0.461	1.033	0.716
24.6	24.4	25.4	25.9	0.024	0.013	0.133	0.278	0.154	0.185	0.498	0.356	1.244	0.093	0.050	0.514	1.073	0.595

25.4	25.2	25.5	25.4	0.014	0.015	0.090	0.111	0.148	0.151	0.514	0.410	1.346	0.055	0.059	0.354	0.437	0.583
27.3	29.1	29.8	29.4	0.018	0.012	0.167	0.324	0.199	0.198	0.481	0.401	1.782	0.061	0.041	0.057	1.102	0.677
25.3	26.0	27.3	27.0	0.018	0.013	0.119	0.151	0.126	0.181	0.462	0.361	1.373	0.067	0.048	0.441	0.559	0.467
24.0	22.6	24.6	24.0	0.013	0.014	0.079	0.196	0.114	0.144	0.488	0.338	1.224	0.054	0.058	0.329	0.817	0.475
22.8	24.6	24.1	23.8	0.021	0.017	0.119	0.183	0.127	0.153	0.465	0.333	1.266	0.088	0.071	0.500	0.769	0.534
26.8	26.6	28.0	28.0	0.016	0.009	0.079	0.132	0.159	0.159	0.478	0.392	1.538	0.057	0.032	0.282	0.471	0.568
25.7	25.4	25.4	24.8	0.017	0.020	0.095	0.167	0.149	0.145	0.497	0.371	1.256	0.069	0.081	0.383	0.673	0.601
26.0	26.0	27.0	25.6	0.018	0.013	0.109	0.171	0.100	0.167	0.484	0.347	1.372	0.070	0.051	0.426	0.668	0.391
23.5	24.0	23.7	23.5	0.019	0.022	0.098	0.201	0.117	0.154	0.490	0.347	1.209	0.081	0.094	0.417	0.855	0.498
24.4	23.2	24.7	25.3	0.014	0.014	0.110	0.127	0.155	0.165	0.465	0.358	1.690	0.055	0.055	0.435	0.502	0.613
28.2	28.1	28.1	27.9	0.015	0.018	0.132	0.230	0.146	0.187	0.465	0.406	1.608	0.054	0.065	0.473	0.824	0.523
27.0	22.6	25.7	24.2	0.024	0.016	0.045	0.124	0.106	0.158	0.511	0.341	1.410	0.099	0.066	0.186	0.512	0.438
25.6	26.3	26.3	26.4	0.028	0.015	0.093	0.226	0.139	0.156	0.481	0.411	1.440	0.106	0.057	0.352	0.856	0.527
24.8	25.3	25.9	25.5	0.021	0.020	0.132	0.235	0.126	0.148	0.465	0.344	1.297	0.082	0.078	0.518	0.922	0.494
23.7	24.3	24.7	23.5	0.021	0.020	0.086	0.131	0.140	0.173	0.437	0.394	1.337	0.089	0.085	0.366	0.557	0.596

Raw data 2; Males

5.3	7.51	15.3	41.3	55	20.4	37	663	49.1	6	44.9	150	2.3	0.1
5.9	9.3	14.5	51.4	55.3	15.6	28.2	1510	73	3.2	23.8	135	1.3	0.2
6.5	9.31	14.8	51.8	55.6	15.9	28.6	1819	62.3	7.3	30.4	135	1.2	0.1
4	8.96	14.3	50.4	56.3	16	28.4	1233	75.6	2.5	21.9	150	2	0.2
5	10.17	14.9	53.6	52.7	14.7	27.8	1573	75	4.1	20.9	120	0.8	0.1
6.7	10.27	16.2	57.7	56.2	15.8	28.1	1333	47.6	5.3	47.1	150	1.4	0.1
4.5	11.13	15.5	56.2	50.5	13.9	27.6	1213	69.4	3.3	27.3	120	0.3	0.06
3.8	9.34	15	53	56.7	16.1	28.3	1521	73.9	3.3	22.8	135	2	0.1
6.4	9.78	14.3	50.8	51.9	14.6	28.1	1388	75.3	4	20.7	150	0.7	0.2
4.5	9.57	14.7	52.8	55.2	15.4	27.8	1211	81.4	2.1	16.5	120	1.6	0.2

7.8	8.77	15.1	50.6	57.7	17.2	29.8	617	66.4	4.9	28.7	120	2.2	0.7
7.8	9.26	14.2	49.5	53.5	15.3	28.7	915	79.9	2.4	17.7	150	0.1	
6.1	9.78	14.9	53.9	55.1	15.2	27.6	1220	57.3	5.3	37.4	120	1.5	0.3
4.5	8.41	13.7	47	55.9	16.3	29.1	969	73.1	2.6	24.3	135	1.6	1
5.2	10.67	15.6	57.9	54.3	14.6	26.9	1618	62.5	4.1	33.4	150	0.1	0.03
6.2	9.98	15.5	54.1	54.2	15.5	28.7	1699	34.8	6.8	58.4	120	0.1	0.06
3.8	10.17	15.2	53.3	52.4	14.9	28.5	1234	65	4.4	30.6	150	1.1	0.03
2.9	8.94	13.7	47.2	52.8	15.3	29	1288	69.7	4.7	25.6	120	1.8	2.5
7.8	9.9	15.9	55.6	56.2	16.1	28.6	1637	66.9	4	29.1	120	2	0.03
8.5	10.8	16	59.4	55	14.8	26.9	1150	81.5	2.4	16.1	135	0.03	0.03

Raw data 2; Females

3.5	9.18	14.2	48.8	53.2	15.5	29.1	973	77.8	4.1	18.1	135	3.4	1.1
5.7	9.65	14.7	51.7	53.6	15.2	28.4	1096	90.7	1	8.3	150	1.2	1.1
2.6	10.01	15.2	53.7	53.6	15.2	28.3	1284	81.4	2.9	15.7	120	0.9	0.9
5.2	8.92	14.8	51.7	58	16.6	28.6	1196	76.6	3.2	20.2	135	2.2	1.1
6.2	8.34	14.1	48.9	58.6	16.9	28.8	1000	77.7	2.2	20.1	150	1	1.1
7.2	9.54	14.1	50.4	52.8	14.8	28	1114	85	2.6	12.4	150	1.3	0.3
2.8	9.02	14.2	49.7	55.1	15.7	28.6	1020	67.2	4.2	28.6	135	3.3	1.6
6.1	9.17	14.2	50.8	55.4	15.5	28	1129	82.4	1.8	15.8	135	1.1	1.8
2.8	9.73	14.9	53.6	55.1	15.3	27.8	823	69.6	3.2	27.2	120	1.6	2.2
4.6	9.4	15.5	53.7	57.1	16.5	28.9	992	82.4	2	15.6	135	2.4	0.7
5.9	9.12	14.5	50.9	55.8	15.9	28.5	1267	57.6	5.9	36.5	180	1.7	1.9
9	9.77	15	52.2	53.4	15.4	28.7	1240	66.4	3.4	30.2	150	1.7	0.6
7.3	9.91	15.9	58	58.5	16	27.4	1422	64.6	4.9	30.5	120	0.6	1.1
3.5	9.32	14.5	50.4	54.1	15.6	28.8	976	85.2	1.5	13.3	150	0.3	0.1
	9.39	14.6	51	54.3	15.5	28.6	1081				135	0.5	0.3

4.6	8.92	14.5	50.6	56.7	16.3	28.7	1222	83.7	2.6	13.7	150	1.1	1.1
5.7	10.24	15	53.8	52.5	14.6	27.9	1292	51.2	7.8	41	150	0.1	0.9
7.1	9.76	14.9	52	53.3	15.3	28.7	892	83.7	2.5	13.8	125	1.8	0.2
3.7	9.41	15.6	53.3	56.6	16.6	29.3	1054	82.2	2.3	15.5	135	1.7	0.5
5.6	9.6	15.1	52.1	54.3	15.7	29	1199	79.7	2.9	17.4	135	3.4	0.7

TOXICSTAT, VERSION 2005.2
DEPARTMENT OF TOXICOLOGY, OCPL

STUDY N°	SA45	SPECIES	MOUSE
DATA	B.WT., ABS. WT., REL.WT.	SEX	MALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	15-04-2013
STUDY DIRECTOR	MATHLO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	BARTLETT'S TEST			
	CALCULATED VALUE	P = 0.05	P = 0.01	SIGNIFICANCE
PRE-EXP	1.557	7.815	11.345	N.S
DAY 4	4.574	7.815	11.345	N.S
DAY 7	7.559	7.815	11.345	N.S
TER. B.WT.	9.542	7.815	11.345	S *
ADR	1.119	7.815	11.345	N.S
THY	2.251	7.815	11.345	N.S
EPI	0.991	7.815	11.345	N.S
SPL	1.061	7.815	11.345	N.S
HEA	4.874	7.815	11.345	N.S
BRN	0.715	7.815	11.345	N.S
KID	5.072	7.815	11.345	N.S
TES	4.518	7.815	11.345	N.S
LIV	5.899	7.815	11.345	N.S
ADR	0.148	7.815	11.345	N.S
THY	2.709	7.815	11.345	N.S
EPI	5.405	7.815	11.345	N.S
SPL	1.010	7.815	11.345	N.S
HEA	6.755	7.815	11.345	N.S
BRN	4.829	7.815	11.345	N.S
KID	0.606	7.815	11.345	N.S
TES	3.477	7.815	11.345	N.S
LIV	2.911	7.815	11.345	N.S

TOXICSTAT, VERSION 2005.2
DEPARTMENT OF TOXICOLOGY, OCPL

STUDY N°	SA45	SPECIES	MOUSE
DATA	B.WT., ABS. WT., REL.WT.	SEX	MALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	15-04-2013
STUDY DIRECTOR	MATHLO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	ANOVA				DUNNETT'S T-TEST							
	CALCULATED F-VALUE	TABLE F-VALUE 0.05	TABLE F-VALUE 0.01	SIGNIFICANCE	T-VALUE 0.05	T-VALUE 0.01	G2	SIGNIFICANCE	G3	SIGNIFICANCE	G4	SIGNIFICANCE
PRE-EXP	0.076	3.240	5.290	N.S	2.340	3.170	0.365	N.S	0.047	N.S	0.353	N.S
DAY 4	2.076	3.240	5.290	N.S	2.340	3.170	0.205	N.S	1.327	N.S	1.146	N.S
DAY 7	2.523	3.240	5.290	N.S	2.340	3.170	0.539	N.S	2.036	N.S	0.584	N.S
TER. B.WT.	2.809	3.240	5.290	N.S	2.340	3.170	0.433	N.S	2.108	N.S	0.674	N.S
ADR	0.937	3.240	5.290	N.S	2.340	3.170	0.622	N.S	0.415	N.S	1.141	N.S
THY	0.935	3.240	5.290	N.S	2.340	3.170	0.421	N.S	0.665	N.S	0.953	N.S
EPI	0.753	3.240	5.290	N.S	2.340	3.170	0.740	N.S	1.295	N.S	0.062	N.S
SPL	0.278	3.240	5.290	N.S	2.340	3.170	0.255	N.S	0.778	N.S	0.027	N.S
HEA	1.054	3.240	5.290	N.S	2.340	3.170	0.287	N.S	1.279	N.S	0.431	N.S
BRN	1.119	3.240	5.290	N.S	2.340	3.170	1.376	N.S	1.439	N.S	0.250	N.S
KID	2.036	3.240	5.290	N.S	2.340	3.170	0.232	N.S	1.752	N.S	0.626	N.S
TES	6.002	3.240	5.290	S **	2.340	3.170	3.119	S *	0.619	N.S	1.972	N.S
LIV	1.759	3.240	5.290	N.S	2.340	3.170	0.069	N.S	1.035	N.S	1.258	N.S
ADR	0.319	3.240	5.290	N.S	2.340	3.170	0.716	N.S	0.310	N.S	0.883	N.S
THY	0.282	3.240	5.290	N.S	2.340	3.170	0.648	N.S	0.128	N.S	0.760	N.S
EPI	0.538	3.240	5.290	N.S	2.340	3.170	0.341	N.S	0.372	N.S	0.847	N.S
SPL	0.176	3.240	5.290	N.S	2.340	3.170	0.045	N.S	0.271	N.S	0.449	N.S
HEA	0.401	3.240	5.290	N.S	2.340	3.170	0.176	N.S	0.791	N.S	0.262	N.S
BRN	2.457	3.240	5.290	N.S	2.340	3.170	0.144	N.S	1.476	N.S	1.220	N.S

KID	0.505	3.240	5.290	N.S	2.340	3.170	0.047	N.S	0.697	N.S	0.524	N.S
TES	5.101	3.240	5.290	S *	2.340	3.170	1.173	N.S	1.768	N.S	1.888	N.S
LIV	0.528	3.240	5.290	N.S	2.340	3.170	0.665	N.S	1.251	N.S	0.740	N.S

DATA	**B.WT., ABS. WT., REL.WT.**	**SEX**	**MALE**
T.S.CODE	**NQ**	**N° OF GROUPS**	**4**
STUDY TITLE	**REPEATED DOSE (7 DAYS) TOXICITY STUDY**	**DATE OF ANALYSIS**	**15-04-2013**
STUDY DIRECTOR	**MATHLO SHAULI**	**P VALUE**	**0.05 , 0.01**

T TEST

PARAMETER	G1		G2			G3			G4		
	MEAN	SD	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE
PRE-EXP	27.240	3.060	26.620	1.731	N.S	27.160	3.244	N.S	26.640	2.438	N.S
DAY 4	25.940	2.092	25.600	1.672	N.S	28.140	4.166	N.S	24.040	1.726	N.S
DAY 7	24.000	1.625	25.180	2.105	N.S	28.460	5.879	N.S	22.720	2.523	N.S
TER. B.WT.	24.080	1.639	24.980	1.424	N.S	28.460	5.748	N.S	22.680	2.326	N.S
ADR	0.012	0.003	0.010	0.003	N.S	0.012	0.002	N.S	0.009	0.004	N.S
THY	0.048	0.017	0.045	0.008	N.S	0.054	0.015	N.S	0.040	0.016	N.S
EPI	0.071	0.009	0.075	0.008	N.S	0.079	0.011	N.S	0.071	0.012	N.S
SPL	0.115	0.024	0.119	0.027	N.S	0.127	0.016	N.S	0.115	0.026	N.S
HEA	0.158	0.011	0.163	0.016	N.S	0.178	0.034	N.S	0.152	0.029	N.S
BRN	0.446	0.022	0.468	0.032	N.S	0.469	0.025	N.S	0.450	0.021	N.S
KID	0.436	0.061	0.452	0.084	N.S	0.554	0.171	N.S	0.394	0.072	N.S
TES	0.183	0.012	0.211	0.007	S **	0.178	0.012	N.S	0.201	0.021	N.S
LIV	1.546	0.245	1.537	0.093	N.S	1.678	0.285	N.S	1.386	0.113	N.S
ADR	0.048	0.012	0.042	0.014	N.S	0.045	0.015	N.S	0.041	0.012	N.S
THY	0.202	0.072	0.178	0.029	N.S	0.197	0.059	N.S	0.174	0.058	N.S
EPI	0.293	0.027	0.301	0.017	N.S	0.283	0.052	N.S	0.314	0.051	N.S
SPL	0.478	0.099	0.481	0.137	N.S	0.458	0.092	N.S	0.512	0.141	N.S
HEA	0.658	0.022	0.651	0.047	N.S	0.628	0.050	N.S	0.668	0.094	N.S
BRN	1.858	0.137	1.874	0.086	N.S	1.692	0.274	N.S	1.996	0.162	N.S
KID	1.808	0.176	1.801	0.260	N.S	1.909	0.247	N.S	1.732	0.223	N.S
TES	0.765	0.077	0.845	0.060	N.S	0.644	0.127	N.S	0.894	0.146	N.S
LIV	6.406	0.825	6.160	0.337	N.S	5.943	0.584	N.S	6.132	0.483	N.S

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STUDY N°	SA45	SPECIES	MOUSE
DATA	B.WT., ABS. WT., REL.WT.	SEX	FEMALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	15-04-2013
STUDY DIRECTOR	MATHLO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	BARTLETT'S TEST			
	CALCULATED VALUE	P = 0.05	P = 0.01	SIGNIFICANCE
PRE-EXP	0.606	7.815	11.345	N.S
DAY 4	1.189	7.815	11.345	N.S
DAY 7	1.380	7.815	11.345	N.S
TER. B.WT.	1.690	7.815	11.345	N.S
ADR	2.963	7.815	11.345	N.S
THY	5.524	7.815	11.345	N.S
EPI	4.158	7.815	11.345	N.S
SPL	3.903	7.815	11.345	N.S
HEA	2.080	7.815	11.345	N.S
BRN	3.008	7.815	11.345	N.S
KID	3.063	7.815	11.345	N.S
TES	1.978	7.815	11.345	N.S
LIV	1.478	7.815	11.345	N.S
ADR	1.508	7.815	11.345	N.S
THY	3.873	7.815	11.345	N.S
EPI	3.676	7.815	11.345	N.S
SPL	2.936	7.815	11.345	N.S
HEA	2.452	7.815	11.345	N.S
BRN	1.282	7.815	11.345	N.S
KID	3.789	7.815	11.345	N.S
TES	2.212	7.815	11.345	N.S
LIV	2.258	7.815	11.345	N.S

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STUDY N°	SA45	SPECIES	MOUSE
DATA	B.WT., ABS. WT., REL.WT.	SEX	FEMALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	15-04-2013
STUDY DIRECTOR	MATHLO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	ANOVA				DUNNETT'S T-TEST							
	CALCULATED F-VALUE	TABLE F-VALUE 0.05	TABLE F-VALUE 0.01	SIGNIFICANCE	T-VALUE 0.05	T-VALUE 0.01	G2	SIGNIFICANCE	G3	SIGNIFICANCE	G4	SIGNIFICANCE
PRE-EXP	0.459	3.240	5.290	N.S	2.340	3.170	0.991	N.S	0.661	N.S	0.062	N.S
DAY 4	0.313	3.240	5.290	N.S	2.340	3.170	0.548	N.S	0.931	N.S	0.698	N.S
DAY 7	0.071	3.240	5.290	N.S	2.340	3.170	0.176	N.S	0.265	N.S	0.071	N.S
TER. B.WT.	0.137	3.240	5.290	N.S	2.340	3.170	0.089	N.S	0.518	N.S	0.464	N.S
ADR	2.553	3.240	5.290	N.S	2.340	3.170	1.773	N.S	1.773	N.S	0.338	N.S
THY	0.744	3.240	5.290	N.S	2.340	3.170	1.021	N.S	0.471	N.S	0.393	N.S
EPI	0.409	3.240	5.290	N.S	2.340	3.170	0.723	N.S	0.215	N.S	0.248	N.S
SPL	0.254	3.240	5.290	N.S	2.340	3.170	0.427	N.S	0.356	N.S	0.337	N.S
HEA	0.194	3.240	5.290	N.S	2.340	3.170	0.023	N.S	0.364	N.S	0.626	N.S
BRN	0.212	3.240	5.290	N.S	2.340	3.170	0.000	N.S	0.676	N.S	0.091	N.S
KID	0.479	3.240	5.290	N.S	2.340	3.170	0.278	N.S	0.213	N.S	1.113	N.S
TES	0.584	3.240	5.290	N.S	2.340	3.170	0.951	N.S	1.218	N.S	0.447	N.S
LIV	0.063	3.240	5.290	N.S	2.340	3.170	0.425	N.S	0.295	N.S	0.248	N.S
ADR	2.221	3.240	5.290	N.S	2.340	3.170	1.579	N.S	1.437	N.S	0.546	N.S
THY	0.530	3.240	5.290	N.S	2.340	3.170	0.818	N.S	0.217	N.S	0.418	N.S
EPI	0.236	3.240	5.290	N.S	2.340	3.170	0.754	N.S	0.066	N.S	0.192	N.S
SPL	0.193	3.240	5.290	N.S	2.340	3.170	0.365	N.S	0.285	N.S	0.348	N.S
HEA	0.130	3.240	5.290	N.S	2.340	3.170	0.033	N.S	0.247	N.S	0.554	N.S
BRN	0.179	3.240	5.290	N.S	2.340	3.170	0.037	N.S	0.383	N.S	0.346	N.S
KID	0.101	3.240	5.290	N.S	2.340	3.170	0.045	N.S	0.406	N.S	0.112	N.S
TES	0.652	3.240	5.290	N.S	2.340	3.170	1.033	N.S	0.968	N.S	0.025	N.S
LIV	0.183	3.240	5.290	N.S	2.340	3.170	0.601	N.S	0.020	N.S	0.030	N.S

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STUDY N°	SA45	SPECIES	MOUSE
DATA	B.WT., ABS. WT., REL.WT.	SEX	FEMALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	15-04-2013
STUDY DIRECTOR	MATHLO SHAULI	P VALUE	0.05 , 0.01

T TEST

PARAMETER	G1		G2			G3			G4		
	MEAN	SD	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE
PRE-EXP	25.920	1.279	24.960	1.686	N.S	25.280	1.318	N.S	25.860	1.777	N.S
DAY 4	26.160	1.607	25.500	2.373	N.S	25.040	1.410	N.S	25.320	2.069	N.S
DAY 7	26.060	1.702	26.260	2.324	N.S	25.760	1.736	N.S	26.140	1.244	N.S
TER. B.WT.	26.020	1.163	25.920	2.331	N.S	25.440	1.641	N.S	25.500	1.751	N.S
ADR	0.021	0.004	0.017	0.003	N.S	0.017	0.002	N.S	0.022	0.005	N.S
THY	0.017	0.005	0.014	0.002	N.S	0.016	0.005	N.S	0.018	0.002	N.S
EPI	0.102	0.022	0.115	0.034	N.S	0.098	0.013	N.S	0.098	0.036	N.S
SPL	0.175	0.087	0.193	0.080	N.S	0.160	0.031	N.S	0.189	0.056	N.S
HEA	0.142	0.032	0.143	0.034	N.S	0.136	0.026	N.S	0.131	0.016	N.S
BRN	0.165	0.019	0.165	0.023	N.S	0.158	0.009	N.S	0.164	0.016	N.S
KID	0.485	0.013	0.482	0.021	N.S	0.483	0.012	N.S	0.472	0.027	N.S
TES	0.389	0.041	0.369	0.035	N.S	0.363	0.019	N.S	0.379	0.034	N.S
LIV	1.447	0.160	1.398	0.223	N.S	1.413	0.200	N.S	1.418	0.120	N.S
ADR	0.081	0.016	0.065	0.014	N.S	0.066	0.011	N.S	0.086	0.020	N.S
THY	0.065	0.024	0.055	0.011	N.S	0.063	0.025	N.S	0.070	0.011	N.S
EPI	0.394	0.092	0.336	0.170	N.S	0.389	0.063	N.S	0.379	0.129	N.S
SPL	0.679	0.356	0.737	0.256	N.S	0.634	0.155	N.S	0.734	0.186	N.S
HEA	0.549	0.133	0.547	0.087	N.S	0.534	0.092	N.S	0.516	0.057	N.S
BRN	0.635	0.057	0.636	0.038	N.S	0.622	0.042	N.S	0.646	0.063	N.S
KID	1.867	0.062	1.872	0.185	N.S	1.905	0.147	N.S	1.857	0.161	N.S
TES	1.491	0.097	1.424	0.110	N.S	1.429	0.058	N.S	1.489	0.130	N.S
LIV	5.555	0.485	5.373	0.400	N.S	5.548	0.655	N.S	5.564	0.302	N.S

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STUDY N°	SA45	SPECIES	MOUSE
DATA	CLIN. PATH	SEX	MALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	BARTLETT'S TEST			
	CALCULATED VALUE	P = 0.05	P = 0.01	SIGNIFICANCE
GLU	5.696	7.815	11.345	N.S
URE	8.743	7.815	11.345	S *
CRE	7.374	7.815	11.345	N.S
PRO	1.913	7.815	11.345	N.S
ALB	2.265	7.815	11.345	N.S
CHO	3.986	7.815	11.345	N.S
TRI	0.788	7.815	11.345	N.S
PHO	11.725	7.815	11.345	S **
CLO	4.472	7.815	11.345	N.S
BIT	2.199	7.815	11.345	N.S
GOT	3.545	7.815	11.345	N.S
GPT	3.926	7.815	11.345	N.S
ALP	1.966	7.815	11.345	N.S

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STUDY N°	SA45	SPECIES	MOUSE
DATA	CLIN. PATH	SEX	MALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	ANOVA				DUNNETT'S T-TEST							
	CALCULATED F-VALUE	TABLE F-VALUE 0.05	TABLE F-VALUE 0.01	SIGNIFICANCE	T-VALUE 0.05	T-VALUE 0.01	G2	SIGNIFICANCE	G3	SIGNIFICANCE	G4	SIGNIFICANCE
GLU	0.622	3.240	5.290	N.S	2.340	3.170	0.080	N.S	0.711	N.S	0.649	N.S
URE	0.510	3.240	5.290	N.S	2.340	3.170	0.359	N.S	0.359	N.S	0.822	N.S
CRE	1.129	3.240	5.290	N.S	2.340	3.170	0.742	N.S	1.577	N.S	0.000	N.S
PRO	2.489	3.240	5.290	N.S	2.340	3.170	0.370	N.S	2.524	S *	1.127	N.S
ALB	3.232	3.240	5.290	N.S	2.340	3.170	0.189	N.S	2.036	N.S	0.935	N.S
CHO	4.306	3.240	5.290	S *	2.340	3.170	0.601	N.S	1.403	N.S	2.139	N.S
TRI	0.255	3.240	5.290	N.S	2.340	3.170	0.267	N.S	0.153	N.S	0.662	N.S
PHO	4.483	3.240	5.290	S *	2.340	3.170	0.248	N.S	0.189	N.S	2.953	S *
CLO	2.606	3.240	5.290	N.S	2.340	3.170	1.291	N.S	2.020	N.S	0.453	N.S
BIT	1.023	3.240	5.290	N.S	2.340	3.170	0.986	N.S	0.147	N.S	0.759	N.S
GOT	0.814	3.240	5.290	N.S	2.340	3.170	0.519	N.S	0.652	N.S	0.798	N.S
GPT	0.502	3.240	5.290	N.S	2.340	3.170	1.168	N.S	0.373	N.S	0.278	N.S
ALP	2.905	3.240	5.290	N.S	2.340	3.170	1.201	N.S	0.788	N.S	1.532	N.S

TOXICSTAT, VERSION 2005.2
DEPARTMENT OF TOXICOLOGY, OCPL

STUDY N°	SA45	SPECIES	MOUSE
DATA	CLIN. PATH	SEX	MALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

T TEST

PARAMETER	G1		G2			G3			G4		
	MEAN	SD	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE
GLU	128.780	34.866	126.660	26.750	N.S	147.520	67.368	N.S	111.680	21.881	N.S
URE	27.800	8.044	26.400	7.369	N.S	29.200	1.304	N.S	31.000	5.568	N.S
CRE	0.400	0.024	0.432	0.049	N.S	0.468	0.110	N.S	0.400	0.060	N.S
PRO	7.034	1.116	6.842	0.829	N.S	5.726	0.578	S *	6.450	0.648	N.S
ALB	3.768	1.451	3.906	1.110	N.S	2.282	0.641	N.S	4.450	1.255	N.S
CHO	143.800	42.204	154.600	24.358	N.S	118.600	14.381	N.S	182.200	25.352	N.S
TRI	173.200	41.294	181.600	51.786	N.S	168.400	61.211	N.S	194.000	41.851	N.S
PHO	12.250	4.153	12.662	0.608	N.S	11.936	2.873	N.S	7.348	1.298	N.S
CLO	98.020	4.110	101.100	2.064	N.S	102.840	2.341	N.S	96.940	5.505	N.S
BIT	2.286	1.244	2.876	0.560	N.S	2.374	0.998	N.S	1.832	0.850	N.S
GOT	149.660	41.219	162.820	49.563	N.S	133.120	44.371	N.S	169.900	17.476	N.S
GPT	103.960	39.435	83.560	14.933	N.S	97.440	30.156	N.S	99.100	19.047	N.S
ALP	118.200	41.590	145.000	32.381	N.S	135.800	42.056	N.S	84.000	20.893	N.S

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DEPARTMENT OF TOXICOLOGY, OCPL

STUDY N°	SA45	SPECIES	MOUSE
DATA	CLIN. PATH	SEX	FEMALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	BARTLETT'S TEST			
	CALCULATED VALUE	P = 0.05	P = 0.01	SIGNIFICANCE
GLU	2.062	7.815	11.345	N.S
URE	1.144	7.815	11.345	N.S
CRE	0.854	7.815	11.345	N.S
PRO	8.820	7.815	11.345	S *
ALB	10.430	7.815	11.345	S *
CHO	9.084	7.815	11.345	S *
TRI	9.552	7.815	11.345	S *
PHO	2.484	7.815	11.345	N.S
CLO	3.039	7.815	11.345	N.S
BIT	4.083	7.815	11.345	N.S
GOT	3.474	7.815	11.345	N.S
GPT	5.136	7.815	11.345	N.S
ALP	1.419	7.815	11.345	N.S

TOXICSTAT, VERSION 2005.2
DEPARTMENT OF TOXICOLOGY, OCPL

STUDY N°	SA45	SPECIES	MOUSE
DATA	CLIN. PATH	SEX	FEMALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	ANOVA				DUNNETT'S T-TEST							
	CALCULATED F-VALUE	TABLE F-VALUE 0.05	TABLE F-VALUE 0.01	SIGNIFICANCE	T-VALUE 0.05	T-VALUE 0.01	G2	SIGNIFICANCE	G3	SIGNIFICANCE	G4	SIGNIFICANCE
GLU	1.302	3.240	5.290	N.S	2.340	3.170	1.894	N.S	0.474	N.S	0.676	N.S
URE	2.413	3.240	5.290	N.S	2.340	3.170	1.345	N.S	0.000	N.S	1.345	N.S
CRE	0.303	3.240	5.290	N.S	2.340	3.170	0.228	N.S	0.684	N.S	0.228	N.S
PRO	0.568	3.240	5.290	N.S	2.340	3.170	0.598	N.S	0.499	N.S	0.609	N.S
ALB	2.837	3.240	5.290	N.S	2.340	3.170	2.143	N.S	0.457	N.S	2.376	S *
CHO	1.442	3.240	5.290	N.S	2.340	3.170	1.985	N.S	1.390	N.S	1.467	N.S
TRI	0.531	3.240	5.290	N.S	2.340	3.170	0.970	N.S	0.186	N.S	0.211	N.S
PHO	2.022	3.240	5.290	N.S	2.340	3.170	1.348	N.S	2.458	S *	1.334	N.S
CLO	1.147	3.240	5.290	N.S	2.340	3.170	0.324	N.S	1.619	N.S	1.228	N.S
BIT	1.272	3.240	5.290	N.S	2.340	3.170	0.152	N.S	0.088	N.S	1.604	N.S
GOT	0.808	3.240	5.290	N.S	2.340	3.170	1.060	N.S	0.453	N.S	0.111	N.S
GPT	1.213	3.240	5.290	N.S	2.340	3.170	0.571	N.S	0.391	N.S	1.403	N.S
ALP	1.680	3.240	5.290	N.S	2.340	3.170	1.056	N.S	0.917	N.S	0.825	N.S

TOXICSTAT, VERSION 2005.2
DEPARTMENT OF TOXICOLOGY, OCPL

STUDY N°	SA45	SPECIES	MOUSE
DATA	CLIN. PATH	SEX	FEMALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

T TEST

PARAMETER	G1		G2			G3			G4		
	MEAN	SD	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE
GLU	145.140	12.885	127.320	11.253	S *	140.680	11.989	N.S	138.780	21.176	N.S
URE	20.000	2.121	18.200	2.387	N.S	20.000	1.414	N.S	21.800	2.387	N.S
CRE	0.384	0.028	0.388	0.027	N.S	0.396	0.021	N.S	0.380	0.034	N.S
PRO	5.948	1.610	5.598	0.412	N.S	6.240	0.608	N.S	5.592	0.537	N.S
ALB	3.404	0.940	2.392	0.337	N.S	3.188	1.089	N.S	2.282	0.213	N.S
CHO	146.600	42.495	110.600	9.290	N.S	121.400	34.595	N.S	120.000	14.124	N.S
TRI	162.400	21.916	131.200	17.079	S *	156.400	68.755	N.S	169.200	69.694	N.S
PHO	8.876	1.984	10.614	2.899	N.S	12.046	1.544	S *	10.596	1.383	N.S
CLO	98.280	1.455	98.860	3.449	N.S	101.180	3.507	N.S	100.480	2.406	N.S
BIT	1.530	0.584	1.606	0.598	N.S	1.486	0.514	N.S	2.332	1.240	N.S
GOT	146.880	44.743	107.720	33.345	N.S	163.620	56.303	N.S	142.780	85.821	N.S
GPT	60.360	24.822	53.640	16.822	N.S	64.960	21.036	N.S	43.840	6.738	N.S
ALP	166.600	23.480	189.400	34.290	N.S	146.800	44.110	N.S	148.800	31.412	N.S

TOXICSTAT, VERSION 2005.2
DEPARTMENT OF TOXICOLOGY, OCPL

STUDY N°	SA45	SPECIES	MOUSE
DATA	HAEMATOLOGY & FC	SEX	MALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	BARTLETT'S TEST			
	CALCULATED VALUE	P = 0.05	P = 0.01	SIGNIFICANCE
WBC	3.518	7.815	11.345	N.S
RBC	0.675	7.815	11.345	N.S
HGB	2.529	7.815	11.345	N.S
HCT	1.113	7.815	11.345	N.S
MCV	2.342	7.815	11.345	N.S
MCH	7.792	7.815	11.345	N.S
MCHC	21.059	7.815	11.345	S **
PLT	5.033	7.815	11.345	N.S
LYM	1.682	7.815	11.345	N.S
MIX	1.197	7.815	11.345	N.S
NEU	1.965	7.815	11.345	N.S
CLOT	0.166	7.815	11.345	N.S
FC D4	0.978	7.815	11.345	N.S
FC D7	30.800	7.815	11.345	S **

TOXICSTAT, VERSION 2005.2
DEPARTMENT OF TOXICOLOGY, OCPL

STUDY N°	SA45	SPECIES	MOUSE
DATA	HAEMATOLOGY & FC	SEX	MALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	ANOVA				DUNNETT'S T-TEST							
	CALCULATED F-VALUE	TABLE F-VALUE 0.05	TABLE F-VALUE 0.01	SIGNIFICANCE	T-VALUE 0.05	T-VALUE 0.01	G2	SIGNIFICANCE	G3	SIGNIFICANCE	G4	SIGNIFICANCE
WBC	0.464	3.240	5.290	N.S	2.340	3.170	0.154	N.S	0.906	N.S	0.482	N.S
RBC	1.627	3.240	5.290	N.S	2.340	3.170	1.869	N.S	0.633	N.S	1.753	N.S
HGB	0.723	3.240	5.290	N.S	2.340	3.170	0.826	N.S	0.130	N.S	1.086	N.S
HCT	1.250	3.240	5.290	N.S	2.340	3.170	1.680	N.S	0.794	N.S	1.611	N.S
MCV	0.513	3.240	5.290	N.S	2.340	3.170	0.732	N.S	0.266	N.S	0.715	N.S
MCH	1.038	3.240	5.290	N.S	2.340	3.170	1.613	N.S	0.949	N.S	1.423	N.S
MCHC	0.919	3.240	5.290	N.S	2.340	3.170	1.526	N.S	1.194	N.S	1.254	N.S
PLT	1.102	3.240	5.290	N.S	2.340	3.170	0.130	N.S	1.433	N.S	0.206	N.S
LYM	0.185	3.240	5.290	N.S	2.340	3.170	0.306	N.S	0.102	N.S	0.416	N.S
MIX	0.490	3.240	5.290	N.S	2.340	3.170	1.043	N.S	0.777	N.S	0.164	N.S
NEU	0.170	3.240	5.290	N.S	2.340	3.170	0.202	N.S	0.011	N.S	0.482	N.S
CLOT	0.362	3.240	5.290	N.S	2.340	3.170	0.338	N.S	0.338	N.S	1.014	N.S
FC D4	0.383	3.240	5.290	N.S	2.340	3.170	0.627	N.S	0.823	N.S	1.007	N.S
FC D7	0.644	3.240	5.290	N.S	2.340	3.170	0.021	N.S	0.864	N.S	1.025	N.S

TOXICSTAT, VERSION 2005.2
DEPARTMENT OF TOXICOLOGY, OCPL

STUDY N°	SA45	SPECIES	MOUSE
DATA	HAEMATOLOGY & FC	SEX	MALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

T TEST

PARAMETER	G1		G2			G3			G4		
	MEAN	SD	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE
WBC	5.340	0.945	5.180	1.287	N.S	6.280	1.499	N.S	5.840	2.442	N.S
RBC	9.050	0.970	10.018	0.710	N.S	9.378	0.888	N.S	9.958	0.670	N.S
HGB	14.760	0.385	15.140	0.737	N.S	14.700	0.752	N.S	15.260	0.929	N.S
HCT	49.700	4.836	54.100	2.791	N.S	51.780	4.225	N.S	53.920	4.428	N.S
MCV	54.980	1.363	54.100	2.747	N.S	55.300	1.612	N.S	54.120	1.566	N.S
MCH	16.520	2.229	15.160	0.902	N.S	15.720	1.028	N.S	15.320	0.522	N.S
MCHC	30.000	3.924	27.980	0.277	N.S	28.420	1.165	N.S	28.340	0.826	N.S
PLT	1359.600	441.726	1333.200	130.051	N.S	1067.800	374.905	N.S	1401.600	249.077	N.S
LYM	67.000	11.363	69.520	12.983	N.S	67.840	8.871	N.S	63.580	17.320	N.S
MIX	4.620	1.992	3.600	1.170	N.S	3.860	1.316	N.S	4.460	1.581	N.S
NEU	28.380	9.949	26.880	11.954	N.S	28.300	7.703	N.S	31.960	15.820	N.S
CLOT	138.000	12.550	135.000	15.000	N.S	135.000	15.000	N.S	129.000	13.416	N.S
FC D4	1.520	0.614	1.200	0.689	N.S	1.100	0.951	N.S	1.006	0.922	N.S
FC D7	0.140	0.055	0.132	0.064	N.S	0.508	0.428	N.S	0.530	1.101	N.S

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STUDY N°	SA45	SPECIES	MOUSE
DATA	HAEMATOLOGY & FC	SEX	FEMALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	BARTLETT'S TEST			
	CALCULATED VALUE	P = 0.05	P = 0.01	SIGNIFICANCE
WBC	1.332	7.815	11.345	N.S
RBC	3.020	7.815	11.345	N.S
HGB	0.912	7.815	11.345	N.S
HCT	3.015	7.815	11.345	N.S
MCV	1.135	7.815	11.345	N.S
MCH	4.385	7.815	11.345	N.S
MCHC	1.213	7.815	11.345	N.S
PLT	0.563	7.815	11.345	N.S
LYM	2.932	7.815	11.345	N.S
MIX	3.432	7.815	11.345	N.S
NEU	2.684	7.815	11.345	N.S
CLOT	3.016	7.815	11.345	N.S
FC D4	1.188	7.815	11.345	N.S
FC D7	12.627	7.815	11.345	S **

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STUDY N°	SA45	SPECIES	MOUSE
DATA	HAEMATOLOGY & FC	SEX	FEMALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

PARAMETER	ANOVA				DUNNETT'S T-TEST							
	CALCULATED F-VALUE	TABLE F-VALUE 0.05	TABLE F-VALUE 0.01	SIGNIFICANCE	T-VALUE 0.05	T-VALUE 0.01	G2	SIGNIFICANCE	G3	SIGNIFICANCE	G4	SIGNIFICANCE
WBC	0.938	3.290	5.420	N.S	2.360	3.200	0.053	N.S	1.418	N.S	0.622	N.S
RBC	0.608	3.290	5.420	N.S	2.360	3.200	0.524	N.S	0.973	N.S	1.263	N.S
HGB	0.887	3.290	5.420	N.S	2.360	3.200	0.061	N.S	0.911	N.S	1.276	N.S
HCT	0.523	3.290	5.420	N.S	2.360	3.200	0.489	N.S	1.107	N.S	1.006	N.S
MCV	0.109	3.290	5.420	N.S	2.360	3.200	0.229	N.S	0.137	N.S	0.549	N.S
MCH	0.200	3.290	5.420	N.S	2.360	3.200	0.766	N.S	0.479	N.S	0.431	N.S
MCHC	0.984	3.290	5.420	N.S	2.360	3.200	1.254	N.S	0.792	N.S	0.264	N.S
PLT	1.289	3.290	5.420	N.S	2.360	3.200	1.006	N.S	0.934	N.S	0.235	N.S
LYM	1.099	3.290	5.420	N.S	2.360	3.200	0.537	N.S	1.691	N.S	0.723	N.S
MIX	0.628	3.290	5.420	N.S	2.360	3.200	0.075	N.S	1.050	N.S	0.886	N.S
NEU	1.225	3.290	5.420	N.S	2.360	3.200	0.618	N.S	1.791	N.S	0.683	N.S
CLOT	0.595	3.290	5.420	N.S	2.360	3.200	0.319	N.S	0.958	N.S	0.106	N.S
FC D4	0.932	3.290	5.420	N.S	2.360	3.200	0.322	N.S	1.254	N.S	0.193	N.S
FC D7	1.271	3.290	5.420	N.S	2.360	3.200	0.728	N.S	0.728	N.S	1.063	N.S

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STUDY N°	SA45	SPECIES	MOUSE
DATA	HAEMATOLOGY & FC	SEX	FEMALE
T.S.CODE	NQ	N° OF GROUPS	4
STUDY TITLE	REPEATED DOSE (7 DAYS) TOXICITY STUDY	DATE OF ANALYSIS	16-04-2013
STUDY DIRECTOR	MATHULO SHAULI	P VALUE	0.05 , 0.01

T TEST

PARAMETER	G1		G2			G3			G4		
	MEAN	SD	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE	MEAN	SD	SIGNIFICANCE
WBC	4.640	1.527	4.700	1.965	N.S	6.425	2.326	N.S	5.340	1.278	N.S
RBC	9.220	0.647	9.372	0.284	N.S	9.502	0.328	N.S	9.586	0.483	N.S
HGB	14.600	0.453	14.580	0.606	N.S	14.900	0.596	N.S	15.020	0.396	N.S
HCT	50.960	2.092	51.640	1.877	N.S	52.500	3.145	N.S	52.360	1.250	N.S
MCV	55.400	2.661	55.100	1.531	N.S	55.220	2.032	N.S	54.680	1.908	N.S
MCH	15.880	0.811	15.560	0.623	N.S	15.680	0.259	N.S	15.700	0.797	N.S
MCHC	28.640	0.321	28.260	0.467	N.S	28.400	0.570	N.S	28.720	0.522	N.S
PLT	1109.800	131.089	1015.600	122.692	N.S	1197.200	172.967	N.S	1131.800	159.588	N.S
LYM	80.840	5.801	77.320	8.255	N.S	68.450	11.794	N.S	76.100	14.015	N.S
MIX	2.680	1.161	2.760	0.974	N.S	3.925	1.916	N.S	3.620	2.347	N.S
NEU	16.480	4.927	19.920	7.425	N.S	27.625	9.981	N.S	20.280	11.681	N.S
CLOT	138.000	12.550	135.000	10.607	N.S	147.000	22.249	N.S	139.000	10.840	N.S
FC D4	1.740	1.062	1.940	0.907	N.S	0.960	0.684	N.S	1.620	1.203	N.S
FC D7	1.060	0.089	1.320	0.792	N.S	0.800	0.721	N.S	0.680	0.349	N.S

CLINICAL PATHOLOGY RESULTS

A.No	GLU	UREA	CRE	PRO	ALB	CHO	TRI	PHO	CLO	BIT	GOT	GPT	ALP
1	122.9	15	0.36	5.50	2.37	89	124	9.93	95.6	0.67	136.1	73	151
2	148.5	27	0.40	7.25	4.57	134	165	18.0	104.2	3.11	163.3	122.6	133
3	101.0	37	0.42	7.80	3.71	204	237	14.87	99.4	1.75	139.5	65.7	85
4	178.0	30	0.40	6.35	2.42	160	181	10.8	97.5	3.88	98.7	96.3	158
5	93.5	30	0.42	8.27	5.77	132	159	7.63	93.4	2.02	210.7	162.2	64
6	147.2	23	0.43	8.42	4.69	168	200	6.9	96.2	1.02	122.5	97.9	131
7	123.7	18	0.39	5.87	2.31	101	149	9.18	99.1	1.40	149.3	66.8	159
8	146.3	21	0.37	6.4	3.11	184	160	8.5	97.4	2.47	132.2	48.6	173
9	150.2	18	0.36	4.51	2.91	100	158	12.08	99.8	1.10	222.3	57.6	176
10	158.3	20	0.37	4.54	4.00	180	145	7.72	98.9	1.66	108.1	30.9	194
11	119.5	15	0.41	6.47	3.73	137	108	11.89	99.2	3.11	117.5	67.2	118
12	110.9	32	0.44	8.32	5.48	160	209	13.3	99.6	2.49	242.1	96.9	115
13	109.7	30	0.42	6.40	3.60	190	229	12.95	102.8	3.11	167.1	98.8	153
14	119.4	23	0.38	6.44	4.29	159	215	12.15	103.8	2.13	124.5	69.1	144
15	173.8	32	0.51	6.58	2.43	127	147	13.02	100.1	3.54	162.9	85.8	195
16	132.4	21	0.41	5.98	2.25	123	114	9.71	103.5	2.63	166.7	81.6	148
17	134.4	17	0.37	5.35	2.12	108	158	8.01	94.1	1.15	88.2	54.7	194
18	139.2	18	0.41	5.27	2.20	105	133	8.33	98.2	1.45	87.8	40.2	170
19	115.8	15	0.40	6.11	2.43	100	119	12.09	100.4	1.22	98.1	50.7	240
20	114.8	20	0.35	5.28	2.96	117	132	14.93	98.1	1.58	97.8	41	195
21	139.2	28	0.52	5.23	1.30	110	128	13.20	105.7	2.3	177.6	105.3	129
22	45.9	31	0.63	6.14	2.08	108	270	9.68	100.5	2.45	85.4	67.7	70
23	160.7	28	0.40	6.48	2.46	138	170	11.83	104.5	4.0	112.4	64.1	174
24	234.0	30	0.35	5.64	3.01	107	160	8.9	100.5	1.6	107	119.8	135
25	157.8	29	0.44	5.14	2.56	130	114	16.07	103	1.52	183.2	130.3	171
26	143.8	20	0.38	5.76	2.27	108	103	9.91	96.8	0.79	102.3	49.9	202
27	158.0	18	0.39	6.04	2.40	117	117	11.39	99.8	1.33	172	54.3	121
28	126.4	22	0.43	7.30	4.15	182	166	11.87	105.8	2.22	155.9	61.7	122
29	142.2	20	0.40	6.13	4.58	100	124	13.76	103.5	1.56	252.9	101.8	103
30	133.0	20	0.38	5.97	2.54	100	272	13.30	100.0	1.53	135	57.1	186
31	85.7	27	0.36	5.81	4.73	199	144	5.80	87.6	2.03	193.5	129.9	59
32	118.8	32	0.42	6.89	5.2	153	201	7.62	99.4	1.57	175.7	93.2	104

101	28.3
102	30.1
103	22.8
104	29.5
105	24.6
106	22.9
107	28.4
108	28.1
109	23.4
110	27.1
111	27.0
112	23.7
113	25.9
114	27.8
115	26.2
116	25.6
117	26.7
118	26.3
119	25.3
120	25.6

DEPARTMENT OF TOXICOLOGY, OCPL

RANDOMIZATION RESULTS

STUDY N°	SA45	**DATE OF ANALYSIS**	2013/04/01 14:47
BALANCE ID N°	TOX/43	**TOTAL N° OF ANIMALS**	20
T.S.CODE	NQ	**SPECIES**	Mouse
SEX	Female	**N° OF GROUPS**	4
STUDY TITLE	REP. DOSE(7 DAYS) ORAL TOX. ST. IN MICE		
STUDY DIRECTOR	MATHULA		
RANDOMIZED BY	A. J. ZABIULLAH		

Group1			**Group2**			**Group3**			**Group4**		
T.A.	**B.W.**	**P.A.**	**T.A.**	**B.W.**	**P.A.**	**T.A.**	**B.W.**	**P.A.**	**T.A.**	**B.W.**	**P.A.**
125	26.9		135	25.9		132	26		133	26	
130	25.5		131	25.7		126	25.3		123	25.7	
134	24.8		124	24.5		129	25.2		127	24.1	
122	23.4		139	23.6		128	23.5		140	23.8	
121	23.2		137	22.2		138	22.6		136	22.5	
Mean	24.8		Mean	24.4		Mean	24.6		Mean	24.4	
SD	1.534		SD	1.535		SD	1.413		SD	1.441	

121	23.2
122	23.4
123	25.7
124	24.5
125	26.9
126	25.3
127	24.1
128	23.5
129	25.2
130	25.5
131	25.7
132	26.0
133	26.0
134	24.8
135	25.9
136	22.5
137	22.2
138	22.6
139	23.6
140	23.8

DEPARTMENT OF TOXICOLOGY, OCPL

RANDOMIZATION RESULTS

STUDY N°	SA45	**DATE OF ANALYSIS**	2013/04/01 14:44
BALANCE ID N°	TOX/43	**TOTAL N° OF ANIMALS**	20
T.S.CODE	NQ	**SPECIES**	Mouse
SEX	Male	**N° OF GROUPS**	4
STUDY TITLE	REP. DOSE(7DAYS)ORAL TOX. ST. IN MICE		
STUDY DIRECTOR	MATHULA		
RANDOMIZED BY	A. J. ZABIULLAH		

Group1			Group2			Group3			Group4		
T.A.	**B.W.**	**P.A.**	**T.A.**	**B.W.**	**P.A.**	**T.A.**	**B.W.**	**P.A.**	**T.A.**	**B.W.**	**P.A.**
102	30.1		101	28.3		104	29.5		107	28.4	
110	27.1		108	28.1		111	27		114	27.8	
118	26.3		115	26.2		117	26.7		113	25.9	
105	24.6		116	25.6		119	25.3		120	25.6	
112	23.7		103	22.8		109	23.4		106	22.9	
Mean	26.4		Mean	26.2		Mean	26.4		Mean	26.2	
SD	2.486		SD	2.233		SD	2.251		SD	2.163	

CAGEWISE FEED INPUT, LEFTOVER AND FEED CONSUMPTION RECORD

G1 0mg/kg

A. NO	week 1	FDL	AFC		week 2	FDL	AFC		WEEK 3	FDL	AFC		WEEK 4	FDL	AFC
1	285	148	137		285	192	93		285	206.69	78.31		150	111.83	38.17
2	285	57	114		285	30.2	127.4		285	69.13	107.94		150	44.92	52.54
3	285	210	75		285	167.3	117.7		285	182.85	102.15		150	103.35	46.65
4	285	171	114		285	183.8	101.2		285	192.41	92.59		150	89.33	60.67
5	285	162	123		285	192.9	92.1		285	207.58	77.42		150	111.85	38.15
6	285	62	111.5		285				285				150		
7	285	150	135		285	177.1	107.9		285	237.42	47.6		150	132.01	17.99
8	285	230	27.5		285	105.2	89.9		285	124.89	80.05		150	67.55	41.2
9	285	197	88		285	175.1	109.9		285	181.81	103.19		150	113.14	36.8
10	285	152	133		285	174.8	110.2		285	177.08	107.92		150	107.95	42.05

G2 125mg/kg

A. NO	week 1	FDL	AFC		week 2	FDL	AFC		WEEK 3	FDL	AFC		WEEK 4	FDL	AFC
11	285	42	121.5		285	74.9	105.1		285	91.3	96.85		150	56.65	46.73
12	285	146	139		285	185.7	99.3		285	184.25	100.75		150	90.83	59.17
13	285	122	163		285	162.2	122.8		285	202.05	82.95		150	107.73	42.27
14	285	100	185		285	273.8	11.2		285				150		
15	285	24.9	130		285	109.6	87.7		285	90.59	97.2		150	55.28	47.36
16	285	82	203		285	165	120		285	180.59	104.41		150	95.16	54.84
17	285	153	132		285	153.5	131.5		285	189.87	95.03		150	92.22	77.22
18	285	227	58		285	197.4	87.6		285	217.58	67.42		150	103.41	46.59
19	285				285				285				150		
20	285	137.7	147.3		285	167.5	117.5		285	201.52	83.48		150	90.38	59.62

G3 250mg/kg

A. NO	week 1	FDL	AFC		week 2	FDL	AFC		WEEK 3	FDL	AFC		WEEK 4	FDL	AFC
21	285	131	154		285	154.8	130.2		285	196.4	88.5		150	110.44	39.5
22	285	53	232		285	134.3	150.7		285	181.09	103.9		150	98.01	51.99

23	285	117	168		285	234.5	50.5		285	225.11	59.89		150	106.42	43.58
24	285	137	148		285	206.8	78.2		285	223.15	61.85		150	129.43	20.57
25	285	112	173		285	201.8	83.2		285	222.88	62.12		150	117.35	32.65
23	285	50	235		285	146.1	138.9		285	187.83	100.17		150	96.49	53.57
27	285	55	115		285				285				150		
28	285	145	140		285	272.7	12.3		285				150		
29	285	217	68		285	207.3	77.7		285	227.78	57.12		150	116.66	33.34
30	285	200	85		285	167.7	117.3		285	176.71	108.29		150	102.94	47.06

	G4	**500mg/kg**													
A. NO	**week 1**	**FDL**	**AFC**		**week 2**	**FDL**	**AFC**		**WEEK 3**	**FDL**	**AFC**		**WEEK 4**	**FDL**	**AFC**
31	285	173	56		285	152.1	66		285	164.41	60		150	81.11	34.4
32	285	260	12.5		285	51.1	116.95		285	95.98	94.51		150	55.72	47.1
33	285	185	100		285	240.1	44.9		285				150		
34	285	197	88		285	208.6	76.4		285	207.77	77.23		150	120.56	29.44
35	285	217	68		285				285				150		
36	285	219	66		285	237.8	47.2		285	218.33	66.67		150	104.34	45.66
37	285	247	38		285	242.7	42.3		285				150		
38	285	193	92		285	148.3	136.7		285	209.97	75.03		150	121.45	28.58
39	285	214	71		285	216.5	68.5		285	222.49	62.5		150	123.67	26.33
40	285	195	90		285	189.7	95.3		285	218.08	66.92		150	116.77	32.2

	INDIVIDUAL ANIMAL BODY WEIGHT										ABSOLUTE ORGAN WEIGHT											RELATIVE ORGAN WEIGHT
		G1	0mg/kg																	G1	0mg/kg	
A. No	pre-exp	week 1	week 2	week 3	week 4	term. Wt		adrenal	ovaries	thymus	epi/uter	spleen	heart	brain	kidney	testes	liver		adrenal	ovaries	thymus	epi/uter
1	28.3	27.5	26.7	27.5	28.8	27.3				0.08		0.215	0.14	0.352	0.427	0.223	1.634				0.293	
2	25.9	24.5	22	25.2	25.3	22.5		0.015		0.139		0.129	0.116	0.389	0.381	0.261	1.378		0.067		0.618	
3	25.3	27.7	26.8	29.1	29.8	27.4		0.004		0.073		0.183	0.148	0.346	0.521	0.191	1.565		0.015		0.266	
4	23.4	28.3	28.6	30.2	31.3	28.7		0.011		0.045		0.173	0.121	0.423	0.455	0.398	1.741		0.038		0.157	
5	23.3	22.1	22.1	24.7	25.7	23.9		0.012		0.021	0.128	0.073	0.15	0.417	0.396	0.251	1.342		0.05		0.088	0.536
6	25.7	27.6																				
7	24.3	24.9	24.7	23.5	24.1	22.3		0.005		0.074		0.078	0.124	0.272	0.276		1.266		0.022		0.332	
8	23	24.5	23.1	23.8	23.8	22.4		0.008	0.03	0.057		0.15	0.112	0.434	0.241		1.324		0.036	0.134	0.254	
9	22.7	23.8	23.2	25.3	25.6	24		0.006	0.266	0.162	0.198	0.344	0.118	0.443	0.359		1.58		0.025	1.108	0.675	0.825
10	21.5	21.3	21.1	22.9	23.5	21.7		0.003	0.048	0.041		0.19	0.113	0.428	0.311		1.363		0.014	0.221	0.188	
		G2	125mg/kg																	G2	125mg/kg	
11	27.5	28	25.7	27.3	29.5	25.8		0.013		0.025	0.061	0.284	0.129	0.442	0.439	0.208	1.764		0.05		0.097	0.236
12	27.2	29	29.5	29.8	30.6	27.5		0.033		0.078	0.01	0.153	0.203	0.5	0.495	0.226	1.602		0.12		0.284	0.036
13	25.1	28	25.2	27.1	28.3	25.9						0.119	0.138	0.393	0.469	0.286	1.594					
14	23.5	27																				
15	21.4	25.3	26.1	25.3	26.3	26.2		0.061		0.09	0.01	0.402	0.154	0.479	0.438	0.189	1.718		0.233		0.344	0.038
16	27.3	24.8	25	22.9	27.2	25.2		0.02	0.014	0.061	0.045	0.118	0.135	0.5	0.425		1.627		0.079	0.056	0.242	0.179
17	24.2	26.8	26	26.1	27.9	26.5		0.011		0.064		0.116	0.13	0.43	0.408		1.492		0.042		0.242	
18	23.4	18.9	20.1	18.8	20.8	19.4		0.006	0.034	0.065		0.223	0.137	0.377	0.347		1.301		0.031	0.175	0.335	
19	22																					
20	21.6	20.4	19.4	20.9	20.2	19		0.014		0.071	0.159	0.127	0.123	0.489	0.39		1.216		0.074		0.374	0.837
		G3	250mg/kg																	G3	250mg/kg	
21	28.2	29.1	29.7	30.5	31.7	29.1		0.017		0.131		0.185	0.262	0.512	0.501	0.425	1.767		0.058		0.45	
22	25.4	24	24.4	26.9	27.9	25.7		0.031		0.084		0.174	0.144	0.486	0.44	0.198	1.598		0.121		0.327	
23	25.3	23.5	22.5	22.8	23.3	23.1		0.004		0.06	0.148	0.11	0.17	0.457	0.32	0.308	1.16		0.017		0.26	0.641

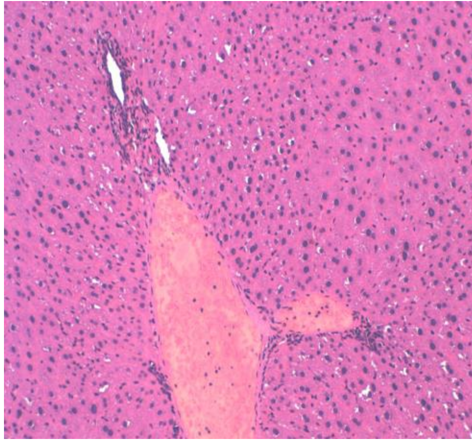
24	23.4	25.4	25.8	28.3	27.7	25.5				0.05	0.122	0.164	0.158	0.441	0.932	0.5	1.67				0.196	0.478
25	22.4	24.4	22	25.9	26.2	22.8		0.054		0.015	0.088	0.109	0.121	0.291	0.418	0.224	1.508		0.237		0.066	0.386
26	25.5	24.4	25.3	28.2	28.3	26		0.028	0.102	0.078	1.764	0.147	0.137	0.447	0.4		1.471		0.108	0.392	0.3	6.785
27	25.2	25.4																				
28	23.3	19.1																				
29	22.6	22.9	20.7	22.6	23.1	21.4		0.116		0.045		0.095	0.199	0.464	0.278		1.374		0.542		0.21	
30	20.5	24.9	22.8	24.3	24.6	23.5			0.065		0.143	0.065	0.123	0.372	0.316		1.316			0.277		0.609
		G4	**500mg/kg**																	**G4**	**500mg/kg**	
31	27.9	25.1	26.4	25.3	26.7	23.4				0.059	0.112	0.168	0.12	0.472	0.396	0.182	1.461				0.252	0.479
32	27	25.9	24.2	24.9	25.1	23		0.024		0.02	0.088	0.146	0.127	0.458	0.334	0.196	1.196		0.104		0.087	0.383
33	24.4	25.5																				
34	23.9	24	24.9	26.8	22.2	21.2		0.059		0.092	0.078	0.132	0.139	0.393	0.348	0.057	1.38		0.278		0.434	0.368
35	21.6																					
36	27.3	25.9	26	25.3	25.7	24.5		0.012	0.022	0.065	0.111	0.201	0.119	0.481	0.368		1.598		0.049	0.09	0.265	0.453
37	24	21	14.8																			
38	24	24.3	22.3	23.6	22.3	21.6		0.013	0.024	0.063	0.172	0.151	0.136	0.525	0.326		1.205		0.06	0.111	0.292	0.796
39	22.1	26	23.3	22.4	26.2	24.9		0.008	0.1	0.063	0.151	0.139	0.094	0.438	0.33		1.493		0.032	0.402	0.253	0.606
40	21.5	21.4	23.4	22.1	23.7	22.2		0.017	0.048	0.086	0.099	0.147	0.122	0.387	0.284		1.134		0.077	0.216	0.387	0.446

					ANIMAL GROUPING: MEAN & SD								
group 1				**group 2**				**group 3**				**group 4**	
TN	WT	new A.N	**dose vol**	TN	WT	new A.N	**dose vol**	TN	WT	new A.N	**dose vol**	TN	WT
118	27.5	1	0.28	107	27.30	11	0.27	110	30.5	21	0.31	114	25.3
115	25.2	2	0.25	105	29.80	12	0.30	108	26.9	22	0.27	101	24.9
112	29.1	3	0.29	104	27.10	13	0.27	117	22.8	23	0.23	103	0
109	30.2	4	0.30	119	0.00	14	0.00	113	28.3	24	0.28	116	26.8
106	24.7	5	0.25	111	25.30	15	0.25	120	25.9	25	0.26	102	0
127	0	6	0.00	121	22.90	16	0.23	139	28.2	26	0.28	126	25.3
132	23.5	7	0.24	125	26.10	17	0.26	137	0	27	0.00	133	0
138	23.8	8	0.24	130	18.80	18	0.19	131	0	28	0.00	123	23.6
122	25.3	9	0.25	140	0.00	19	0.00	135	22.6	29	0.23	136	22.4
129	22.9	10	0.23	134	20.90	20	0.21	128	24.3	30	0.24	124	22.1
		tot vol	**2.322**			**tot vol**	**1.982**			**tot vol**	**2.095**		

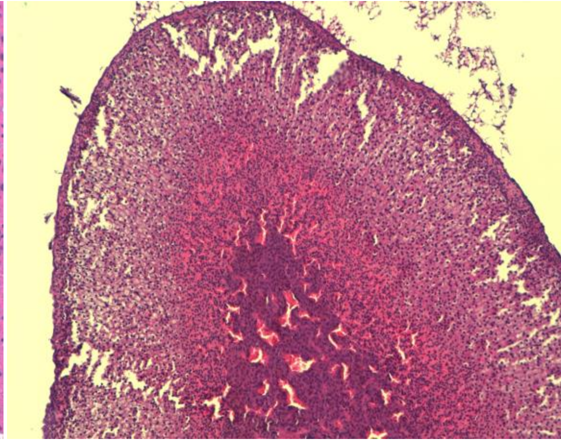
total vol=6 mL for all groups	3.5ml for all groups										
		test sub	dose mg				test sub	20%dmso	80%cmc	t.vol	
G1	0	0mg	0mg/kg				43.8mg	700ul	2.8ml	3500ul	
G2	12.5*3.5	43.8mg	125mg/kg	low			87.5mg	700ul	2.8ml	3500ul	
G3	25*3.5	87.5mg	250mg/kg	mid			175.0mg	700ul	2.8ml	3500ul	
G3	50*3.5	175mg	500mg/kg	high							
									dmso	cmc	tot vol
				due to high viscocity, tot vol was doubled;					700ul	6300ul	7000ul

APPENDIX 2

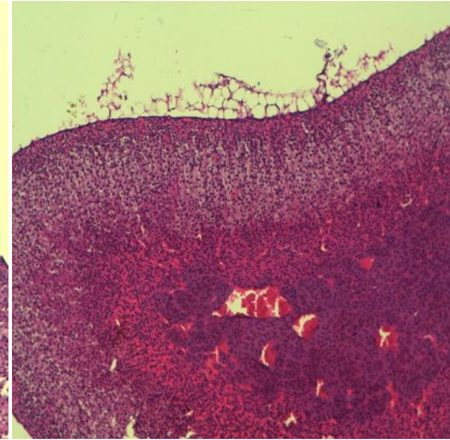
PHOTOMOCROGRAPHS IN MAIN STDY



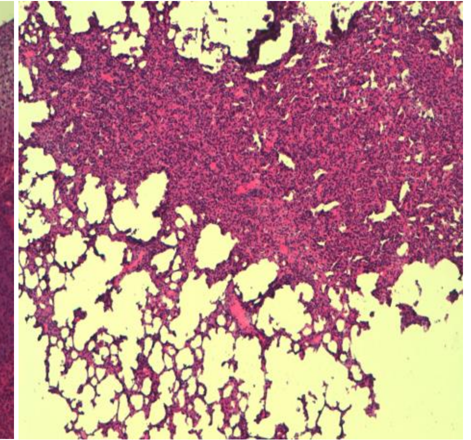
A32 high dose liver x20- male



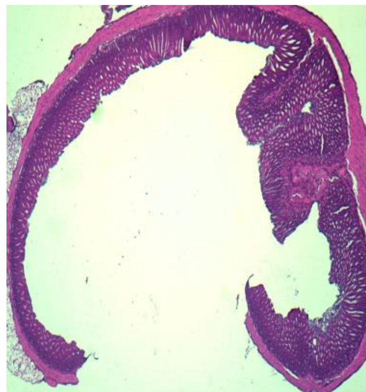
A38 high dose adrenal x10-female



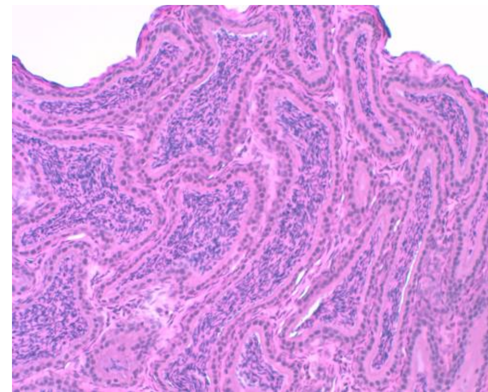
Control A9 adrenal x10-female



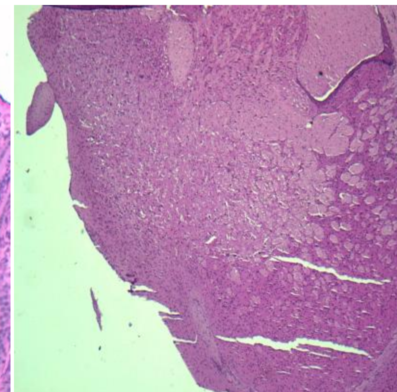
A31 high dose lung x10-male



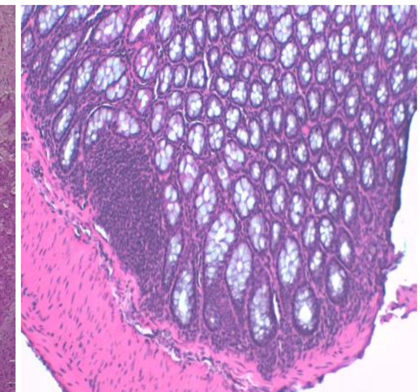
A36 high dose intestines x4 –female.



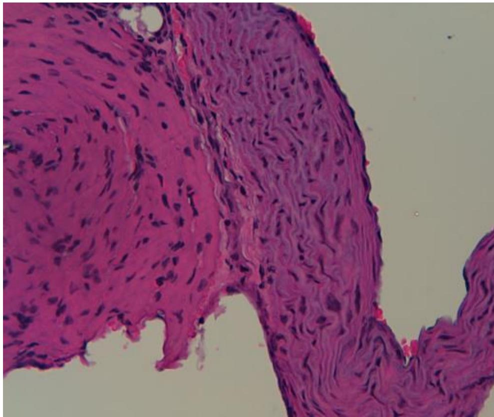
A 32 high dose epididymis x20 – male.



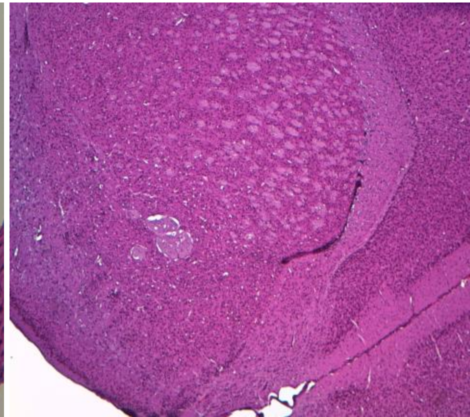
A36 high dose brain x4 –female



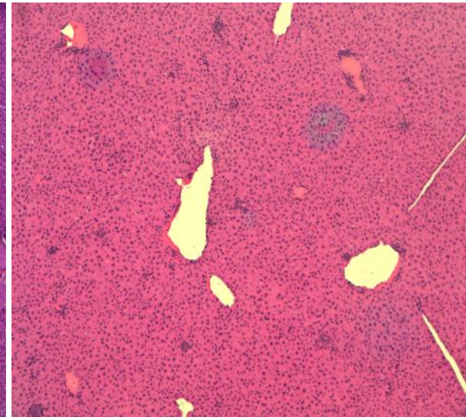
A38 high dose colon x20-female



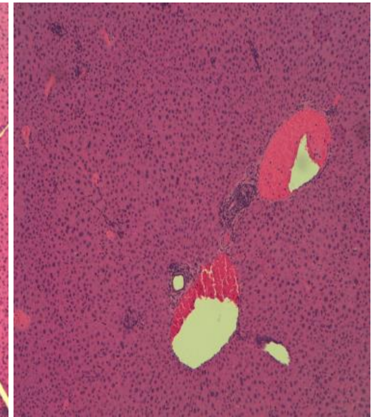
A31 high dose aorta x20- male



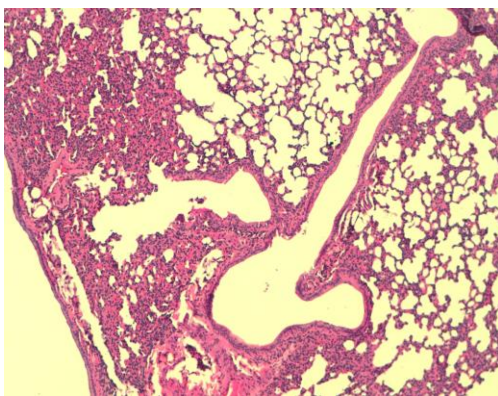
Control 9 brain x4 – female



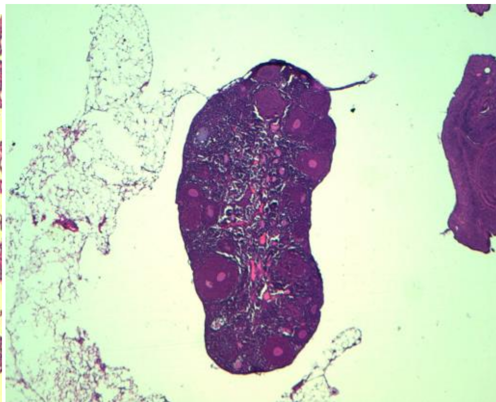
A38 high dose liver x10 –female



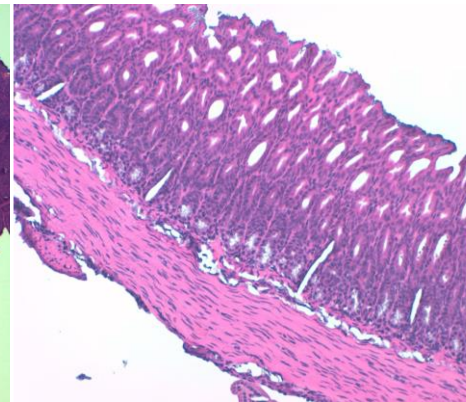
Control 5 liver x10-male



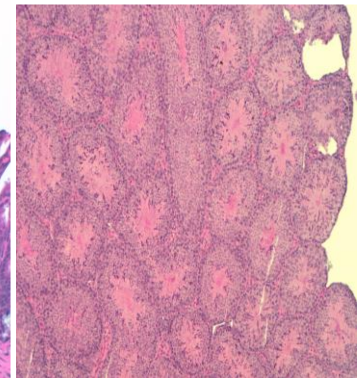
A 39 high dose lung x10- female



Animal 39 high dose ovary x4



Animal 36 high dose smooth muscle x20



Control 4 testes x4-male