ACUTE AND SUB-ACUTE TOXICITY OF DICHLOROMETHANE-METHANOL EXTRACT OF *TECLEA TRICHOCARPA* ROOT-BARK IN RATS

By

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This thesis is my original work and has not been presented for a degree in any other University, and all sources that I have used or quoted have been indicated and acknowledged by means of complete references.

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To my loving son- Denis Muthuma, daughter- Daphine Njoki and wife- Lucy Kirubi, for

their daily commitment and unending inspiration throughout this great exercise. God

bless you.

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LIST OF ABBREVIATIONS

ALT: Alanine aminotransferase

ALP: Alkaline phosphatase

ANOVA: Analysis of Variance

AST: Aspartate aminotransferase

BST: Brine shrimp lethality test

CPK: Creatinine phosphokinase

DMSO: Dimethylsulphoxide

EDTA: Ethylene diamine tetraacetic acid

Hb: Haemoglobin

LD_{50} :   Lethal dose that kills 50 % of the population

Mcg: Microgram

MCHC: Mean corpuscular haemoglobin concentration.

MCV: Mean corpuscular volume

OECD: Organisation for Economic Co-operation and Development

OWI: Organ weight index

PCV: Parked cell volume

RBC: Red blood cell

Rpm: Revolution per minute

STH: Soil transmitted helminths

STN: Screen-to-Nature technique

WBC: White blood cell

WHO: World Health Organization

ABSTRACT

Plant-based medicaments are important therapeutic weapons in the fight against various human and animal diseases. The current study is based on five plants namely *Teclea trichocarpa, Albizia gummifera*, *Crotalaria axillaris*, *Manilkara discolour,* and *Zanthoxylum usambarense* with more focus on *Teclea trichocarpa*. Preliminary work has confirmed that these plants possess anthelmintic activity. *Teclea trichocarpa* is used as a herbal remedy for malaria treatment, as an anthelmintic and a vapour inhalant for treatment of fever. Despite the wide traditional uses of *Teclea trichocarpa,* its adverse effects or toxicity to human and animals have not been reported.

The general purpose of the present study was to rapidly evaluate and compare brine shrimp lethality test of dichloromethane-methanol extracts from different plant parts of *Teclea trichocarpa, Albizia gummifera*, *Crotalaria axillaris*, *Manilkara discolour,* and *Zanthoxylum usambarense.* The results of the brine shrimp lethality test were to be used as the base of selecting the most active and least investigated extract in terms of toxicity. The specific objective was to investigate the acute and sub-acute toxicity of the dichloromethane-methanol extract of the selected extract on Wister rats (*Rattus norvegicus*).

Plant materials were extracted with dichloromethane-methanol (1:1 v/v) to obtain the 14 plants extracts. Viability of brine shrimps eggs was tested and each extracts was evaluated for brine shrimp lethality test at three concentrations (1000, 100 and 10 mcg/ml) against 10 shrimps. The mixtures were maintained at room temperature for 24 hour under the light and surviving larvae

were counted. Brine water was used as negative control. For each concentration the test was carried out in triplicate.

Acute, intraperitoneal and sub-acute toxicity tests were carried out according to the Organization for Economic Co-operation and Development (OECD) guidelines, starting with the limit test concentration of 2000 mg/kg body weight progressively moving to lower doses. In oral acute toxicity, Wister rats were fed with the extract at 2000 mg/kg by gavage. During intraperitoneal acute tests, three sets of rats were each injected with extract at 2000, 300 and 50 mg/kg body weight and for sub-acute, 3 groups of rats were administered with 1000, 300 and 100 mg/kg body weight for 28 days. The parameters investigated in the laboratory animals included; clinical parameters that comprised of skin and fur, eyes, mucous membranes, respiration, the circulation, autonomic and central nervous systems; haematological tests including haemoglobin concentration, mean corpuscular haemoglobin, mean corpuscular volume, total erythrocyte count, haematocrit, and total and differential leucocyte count as well as clinical chemistry parameters such as total protein, albumin, creatinine and the activities of serum alanine aminotranferase (ALT) and creatine kinase. Organs from dead or sacrificed rats were first weighed to determine the OWI and then processed for histopathology as per the standard protocols.

Out of the 14 extracts, three from *Albizia gummifera* pods, *Crotalaria axillaris* twigs and *Teclea trichocarpa* root wood tested using brine shrimps, had LC_{50}>500 and six comprising of *Albizia gummifera* root bark, *Manilkara discolor* root bark and stem bark, *Teclea trichocarpa* twigs, stem bark and root had (LC50>100 < 500 μg /ml). *Teclea trichocarpa* root bark and *Zanthoxylum usambarense* stem bark had LC50 between 30 and 100 μg/ml. The most active

extracts with LC50 < 30 μg /ml were *Albizia gummifera* root and *Zanthoxylum usambarense* root bark. *Teclea trichocarpa* root bark extract was selected for the toxicity test since it was among the most active. Furthermore, no work in terms of its toxicity potential has been found during literature such as compared *Albizia gummifera* root and *Zanthoxylum usambarense* root bark. During the three days acute oral toxicity testing of dichloromethane-methanol extract of *Teclea trichocarpa* root bark, no death occurred at limit dose level of 2000 mg/kg body weight. Similarly, no death occurred during 28 days sub-acute oral toxicity. During acute intraperitoneal acute administration of *Teclea trichocarpa* root bark extract at 2000 mg/kg, all the animals developed un-coordinated, jerky movement and convulsions followed by death within 3 minutes. At 300 mg/kg, convulsions were followed by death within 4 to 48 hours but no death occurred at 50 mg/kg. During oral acute, oral sub-acute and 50 mg/kg intraperitoneal tests, notable clinical signs included transient raised fur and wet feacal droppings. All haematological and clinical biochemistry parameters fluctuated but remained within normal limits corroborating the absence of pathological lesions observed grossly and microscopically.

In oral acute and sub-acute study, there was no evidence of extract-induced signs or death at all the doses of the *Teclea trichocarpa* root bark extract administered. Lack of death at oral treatment of 2000 mg/kg body weight and LD_{50} above 2000 mg/kg body weight but below 5000 mg/kg body weight suggests that the *Teclea trichocarpa* root bark extract is practically non-toxic. The absence of toxic effects during acute and sub-acute studies support the use of this plant as a traditional herbal remedy for the treatment of helminthiasis but intraperitoneal administration of the extract requires further study.

CHAPTER ONE : INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

1.2 Herbal remedies

Medicinal plants are an integral component of ethnomedicine for both human and animal uses all over the world. They have been in use for centuries to treat illness and improve health, and still account for approximately 80 % of medical treatments in the developing world (Glesler, 1992; WHO, 2008). By use of a few cases, one can easily appreciate the long history of ethnomedicine which has continued to have an impact on today's field of medicine. For example, the Chinese Angelica or Dang Gui (Apiaceae) is currently used as a blood tonic to improve blood circulation. Visnasa or Khella (Apiaceae) is mentioned in the Egyptian 'Ebers Papyrus' in about 1500 B.C. as a traditional treatment for kidney stones. Today, it is also employed to treat kidney stones. The Milk Thistle (*Silybum marianum,* Asteraceae) was consumed in Europe to increase breast-milk production in human.

The Cinchona bark (*Cinchona officinalis,* Rubiaceae) was used as far back as 1633 to cure all kinds of fever. It is presently a well known anti-malarial drug. Ginseng (*Panax ginseng*, Araliaceae) is the most popular herb known for over 7000 years and in written records, it is mentioned in Chinese Herbal 'Shen Nung Pen Tsao Ching' (1st Century B.C.). It was known for improving stamina and resistance to stress and restorative for old age. In present times, it is administered as a tonic. In China, it is used as tonic herb for athletes and sufferers of physical stress (Patil *et al.,* 2011).The use of herbal remedies has gained a lot of attention due to its perceived benefits such as safety, affordability and client satisfaction which can generally be classified as therapeutic and economic benefits (Iwu *et al*., 1999). Herbs are generally defined as

any form of plant or plant product, including leaves, stems, flowers, roots and seeds (Stephen, 2008). Herbal products may contain a single herb or a combination of several different herbs believed to have complementary effects.

According to WHO definition, there are three forms of herbal medicines namely; raw plant materials, processed plant materials and medicinal products that contain active ingredients such as aerial or underground plants material or a combination thereof, whether in crude state or as plant preparations (WHO, 2013). Some formulations such as Chinese medicinal formulations may also contain animal products and minerals (Pamela and Paul, 2001). The reliance on medicinal plant products is not confined to Africa or the developing world, but it also exists in the developed nations (Smith-Hall *et al*. 2012). However their widespread use in the developing world may be attributed to financial constraints which make the modern life-saving drugs unaffordable and inaccessible to the poor (Nyanzema, 1986). The use of herbal medicine has thus gained popularity in primary health care of the poor in developing countries and even in countries where conventional medicine is predominant in national health care system (WHO, 1997). Majority of the population in developing countries therefore rely on medicinal plants to meet health care needs, and this has been going on even in situations where conventional medicine is available due to historical and cultural reasons (WHO, 1999).

In Kenya, nearly 80 % of people live within 5 kilometres of a health facility but medical services are not always available as health facilities often lack basic drugs, basic services and amenities and the cost of modern medicine is high (NCAPD, 2008). Further, there is shortage of health professionals and the ratio of doctors to the population remains low at 15 per 100,000 (NCAPD, 2005). These factors result in promoting the usage of herbal remedies in Kenya.

Some pharmaceutical companies are investing in research on how to use these traditional remedies as 'lead' compounds for new drugs. For example, it has been noted that of newly approved drugs reported between 1983 and 1994, drugs of herbal origin predominated (78 %) in the antibacterial area, while 61 % of the 31 anticancer drugs approved in the same period were either natural products, nature-derived products or compounds modeled on natural product parents or "leads". Further, 50 % of the best-selling pharmaceuticals in 1991 were either natural products or their derivatives (Gupta and Amartya, 2012). Advances in pharmaceutical technology have led to investigation and ultimate isolation of pure active compounds from crude drugs which are now produced at commercial levels. The most common examples of modern medicines derived from natural lead compounds include anticancer agents like vincristine and vinblastine from *Catharanthus roseus,* antimalarial quinine from *Cinchona spp* and the anticholinergic agent atropine from *Atropa belladona* and *Atropa acuminata* as well as opioid analgesic morphine (Gupta and Amartya, 2012). Herbal remedies reputed to possess anthelmintic activity may also be investigated with a view to developing potent and efficacious anthelmintic agents.

1.3 Helminthiasis

1.3.1 Classification of helminths

Helminths are found in two phyla of the subkingdom Metazoa (multicellular animals). The metazoa are classified into two phyla: Platyhelminthes and Nemathelminthes. Platyhelminthesis divided into two classes: Cestodea (tapeworms) and Trematodea (flukes) while Nemathelminthes has only one class Nematodea (roundworms). Helminthiasis is the infestation with one or more intestinal worms which include roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris*

trichura) hookworms (*Necator americanus* or *Acylostoma duodenale*). The worms reside in the gastrointestinal tract but may also burrow into other organs like liver as in *Fasciola hepatica*, lung by *Paragonimus westerman*, and muscle in case of cysticercosis by *Taenia solium*, skin in case of strongyloidiasis by *Strongyloides stercoralis*, lymph by *Wuchereria bancrofti,* eye by *Oncercus volvulus,* brain by *Paragonimus spp* and other tissues.

1.3.2 Aetiology and prevalence of helmithiasis

The majority of infection may be due to inadequate sanitation, ingestion of eggs or larvae from contaminated food or through penetration of larvae through the skin (WHO, 2013). Therefore, the most commonly occurring helminthes in man are endemic with high prevalence in much of the tropical and sub-tropical world. These are the regions where conditions of poor hygiene and sanitation co-exist with the environmental factors favoring survival of extra-human parasitic stage. It therefore means that improvement of socio-economic conditions such as sanitation, personal hygiene and health education are important in helminth control.

The three major soil-transmitted helminths (STH), roundworms, hookworms and whipworms are amongst the most widespread parasites worldwide. An estimated 4.5 billion individuals are at risk of STH infection and more than one billion individuals are thought to be infected, of whom 450 million people suffer morbidity from helminths infection, with the majority being children. An additional 44 million infected pregnant women suffer significant morbidity and mortality due to hookworm-associated anemia. Approximately 135,000 deaths occur per year, mainly due to infections with hookworms and/or *A. lumbricoides* (WHO, 2013). Studies have also suggested that the morbidity in children has been underestimated and that moderate intensities of infection

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may have important developmental consequences, particularly for children of school age (Bundy, 1994).

A study carried amongst school going children in Nigeria showed that 54.7 % of the stool samples examined were infected by soil transmitted helminthes (STH) (Egunyenga and Ataikiru, 2005). In western Kenya, it was realized that the mean school prevalence of *Schistosoma mansoni* infection was 16.3 % with study further revealing that the separate distributions of schistosome and geohelminth infections have important implications for combined mass-treatment programs (Handzel *et al.,* 2003).

The effects of helminth infections depend on the infective load and the virulence of the parasite as well as the host immunological status. Local effects include mechanical tissue damage by hookworms, inflammatory response by whipworms and luminal obstruction by roundworms especially in children. Others include malabsorption, stimulation of reflex peristalsis, space occupying lesions and malignancy. In paediatrics, roundworms have been proved to be the major causes of intestinal colic and roundworm boluses are the most common cause of small bowel obstruction (Walker *et al*., 2000). Systemic effects include iron deficiency anaemia by hookworks; eosinophilia and malnutrition by hookworms, roundworms and tapeworms, which lead to mental and physical growth impairment. In addition to physical manifestations, host response may lead to immunopathologic lesions such as schistosome eggs granulomas (Mandell *et al*., 1995; WHO, 2013). Malnutrition may also compromise immunity and facilitate HIV/AIDS and other infections. In HIV/AIDS patients, hyperinfection by parasites like strongyloides and *Toxocara spp* is frequently fatal as compared to immunocompetent patients (Miller, 1970; Ajao and Ajao, 1979; Kinoti, 1982; Chunge *et al*., 1985; Bukenya, 1987 and

Morrone *et al*., 2004). Of concern is that helminthiasis in children has been proved to cause poor growth, reduced physical activity, impaired cognitive function and learning ability (Nokes *et al*., 1992, Egwunyenga and Ataikiru, 2005). Proper management of helmintiasis is therefore crucial.

1.3.3 Anthelmintics

Treatment remains the main option in reduction of morbidity and mortality caused by worms (Pamba, 1980). Drugs for treatment of intestinal worms are few as compared to other human ailments. The major classes of these drugs available in Kenyan market include benzimidazoles such as mebendazole, albendazole and thiabendazole, imidazothiazoles which are represented by levamisole hydrochloride and heterocyclics like piperazines. Other classes available locally include prazinoisoquinolin like praziquantel and salicylanilides like niclosamide (Figure 1.1). Each class of helminths is sensitive to a particular class of anthelmintic. Nematodes are more sensitive to benzimidazoles and imidazothiazoles, trematodes and cestodes to prazinoisoquinolins. Salicylanilides are generally broad-spectrum anthelmintics (Martindale, 2000). These anthelmintic agents destroy or expel parasitic intestinal worms from the body, by either stunning or by killing by vermicides. However, most of these drugs are expensive and the emergences of drug resistant and extra-drug resistant strains of parasites now pose a major challenge in their anthelmintic effectiveness.
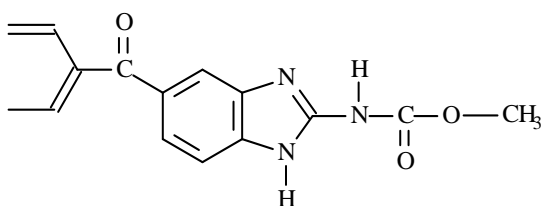
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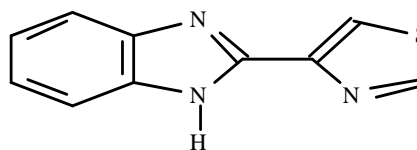
c) . T h i a b e n d a z o l e
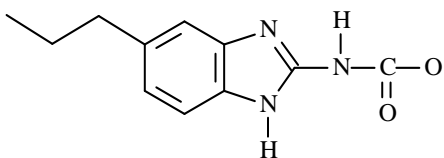
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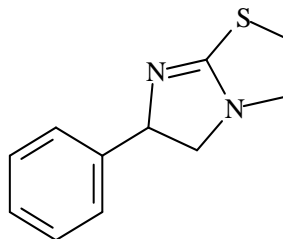
d). Levamisole
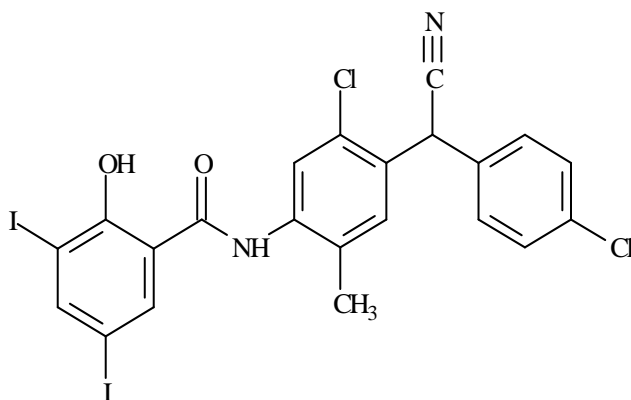
e). Niclosamide
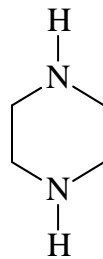
f). Piperazine
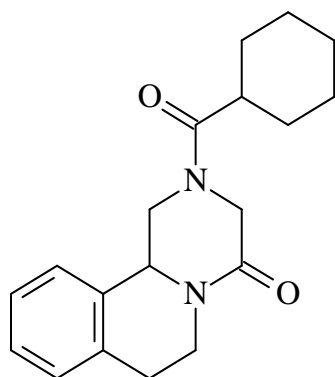
h). Praziquantel

Figure 1.1: Structures of some of the common conventional anthelmintics

A World Health Organization-World Bank meeting on ''monitoring of drug efficacy in large scale treatment programs for human helminthiasis'', held in Washington DC at the end of 2007, highlighted the need to closely monitor anthelmintic drug efficacy and to develop standard operating procedures for this purpose. It is therefore important to seek alternative remedies through exploration.

1.3.4 Anthelmintic drugs of herbal origin

A number of anthelmintic drugs of herbal origin have been used for centuries and are recognized in various pharmacopoeias. These include alantolactone, areca, chenopodium oil, cucurbita and desaspidin (Martindale, 2000). The pumpkin, *Cucurbita maxima* (Cucurbitaceae), a common vegetable in Kenya is reported to possess anthelmintic activity. Its seeds are reputed in Ayurvedic medicine as anthelmintic especially against tapeworms. They act by reducing the motility of the helminthes leading to temporary paralysis (Anita and Ravindra, 2007).

 In Kenya, some more plants have been reported to have been used for treatment of intestinal worms in man and domestic animals with some of them investigated for phytochemical and biological activity. Steroids, triterpenoids, anthraquinones, such as emodin, 2-hydroxychrysophanol, nepodine and 5-methoxy-7-hydroxypthalide are some of secondary metabolites isolated from one of the most frequently used plant, *Myrsine africana* (Manguro *et al*., 1997). The phytochemical essential oil constituent eugenol has been found in plants such as *Ocimum sanctum* (Laminaceae), also known as Sacred Basil. This essential oil has been suggested as the anthelmintic principle (Bundy, 1994). The anthelmintic potential of aqueous extract of *Carica papaya* (Caricaceae) has also been evaluated using *Ascaris lumbricoides* (Nematode). The phyto-principle benzyl isothiocyanate was isolated from the extract and is said

to be responsible for the anthelmintic activity (Anita and Ravindra, 2007). The essensial oils from *Piper longum* (Piperaceae) was found to cause paralytic action on the nerve mascular preparation of *Ascaris lumbricoides* and that its activity was greater than the piperazine citrate used as a standard in the study (Anita and Ravindra, 2007).

1.4 Toxicity studies

Toxicity is the degree to which a substance can harm humans or animals. It can be measured by its effects on the target organism, organ, tissue or cells. The toxic effects of a substance on animal physiology can range from minor changes such as reduced weight gain, small physiological alteration or change in the levels of circulating hormones, to severe effects in organ functional loss leading to death. Intermediate levels of toxicity may cause pain and suffering (Home office, 2004). During toxicity studies, five major aspects are put into consideration. These include organs affected by the chemical, relevance of quantification effect, concentration of chemical to be tested, *in vitro* markers of toxicity that are relevant to the chemical and how to use the data from *in vitro* test for risk assessment. Toxicological studies in the pharmaceutical field have been growing exponentially. These developments have been prompted by discovery of teratogenic effects of drugs such as thalidomide, exposure of chemicals to the environment and employees and by conduct and assessment of toxicity studies as part of good manufacturing practice (Traina, 2006).

In the study of herbal remedies, it has been found that toxicity may result from inadvertent substitution of one plant species another. For example, rapidly progressive renal failure resulting in end-stage renal disease has been reported in women who have taken weight-reducing pills containing the Chinese herbs *Stephania tetrandra* and *Magnolia officinalis*. This so called

Chinese–herb nephropathy is characterized by a pattern of interstitial fibrosis. The cause of the disease was later noted to be due to inadvertent inclusion of *Stephania fangchi* containing the nephrotoxic and carcinogenic aristolochic acids instead of *S. tetrandra* that contains weight reducing tetrandrine (Nortier, 2000; Rotblatt and Zimet, 2002).

Another reported case on herbal toxicity involved patients using a dietary supplement containing herbal constituents yohimbine in addition to norephendrine, sodium usiniate and 3,5-diiodothyronine. All patients developed hepatotoxicity within 3 months, recovering spontaneously on withdrawal of the supplement (Flavreau *et al*., 2002). Another study carried out in Zimbabwe confirmed an increase in incidences of poisoning by herbal remedy since 1971 (Nyanzema, 1986). These facts support the need to have toxicity profiling of all herbal remedies.

Qualitative toxicity assessment of a chemical substance in laboratory animals or *in vitro* toxicity testing gives information on its potential to cause toxic effects in humans or animals (Descote, 1996). To succeed in this exercise, an appropriate choice of control group must be selected, sufficient number of laboratory animals used and good selection of rigorous experimental protocols. Furthermore, the severity of the effect described on major organs and the relevance of the mechanisms involved including the variations in different species assist in extrapolation of toxicological findings from laboratory animals to man (Descote, 1996). During the study, the target organ of toxicity in laboratory animals are identified, the mechanism of induced changes are noted and compared to the properties of the target site in man.

Determination of the toxic potential of new compounds constitutes a major part in drug development and it involves both *in vivo* and *in vitro* toxicological tests. These tests are very critical in the assessment of the safety of all pharmaceutical products before they are released for

general use. Animal models are used in *in vivo* studies as indicators of human toxicity (Magna and Alan, 2007).

Toxicity testing on herbal extracts is carried out on the same principles as the conventional medicine. *In vitro* toxicity testing employs the use of models such as the brine shrimp lethality test (BST) whereas in *in vivo* methods, animals such as mice or rats are used. The advantage of the brine shrimp in toxicity testing is that the shrimp has a lot of homogeneity in eggs and in newly born nauplii which are highly sensitive to chemicals. The eggs are easily available and hatch with ease within 16-24 hours to the nauplii. The nauplii are utilized within 24-48 hours post hatching. At the 24th hours post exposure of nauplii to the test chemical, the concentration causing 50 % lethality (LC_{50}) is determined.

The BST method is used to screen for bioactivity, anticancer, cytotoxicity, toxicity, pesticidal activity or gastroprotective action among other pharmacological effects of plant extracts. Brine shrimp lethality is a rapid method that is reliable, inexpensive and convenient as an in-house general bio-assay tool (Meyer *et al.,* 1982). This method is basically used to predict toxicity and the results obtained are compared with oral acute toxicity in rodents (Amenya, 2011). *In vivo* toxicity testing mainly employs the use of rats although other rodents may be used. Dogs and monkeys are restricted to advanced stages of testing (Amenya, 2011).

Organization for Economic Cooperation and Development (OECD) guidelines are used during acute and sub-acute oral toxicity testing (Diener *et al*., 1995). It is important to optimize the information obtained by using the smallest number of animals to comply with animal welfare regulations. Further, it is important to avoid excessive pain or tissue damage in the animals, pharmaceuticals with irritant or corrosive characteristics should not be administered in

concentrations that produce severe toxicity after administration. During toxicity studies, all the animals must be checked for morbidity, mortality and specific signs of toxicological relevance. For example, neurofunctional and neurobehavioral, opthalmological observation, body-weight and food/water intake. The key haematological parameters investigated are mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), haemoglobin levels, haematocrit levels, packed cell volume (PCV) total and differential leukocytes, erythrocytes and platelet counts.

Clinical biochemistry is crucial to investigate major toxic effects on organs especially the kidney and the liver. Some of the parameters include total protein, albumin, major electrolytes, total cholesterol, alanine aminotransferase, arspatate aminotransferase, creatinine and alkaline phosphatase that aid in hepatocellular evaluation. All long term studies such as chronic toxicity must include urinalysis (urine output, color, protein and osmalarity). Pathological studies and gross necropsy are done by examining the body, orifices, abdominal cavity, body weight and organ weight changes among others. In addition, histopathological studies are done on adrenals, lung, liver, kidney, testis, ovaries among others (OECD 407, 2008). These organs are considered to be the most important during toxicity studies in rodents and non-rodents (Michael *et al.* 2007). Taking the weights of organs is necessary because organ to body weight ratios or organ weight index (OWI) are commonly calculated and are considered more useful when body weights are affected (Michael *et al*. 2007). By carrying out toxicity tests, the effects of increase in dose on the mortality and other effects of the lethal dose that kills are determined. Estimating different levels of toxicity by use of LD_{50} for instance can help in estimating the probabilities of an outcome for a given individual in a population. The determination of acute, sub-acute, sub-chronic and chronic toxic effects of the test compounds is therefore crucial (Traina, 2006).

1.4.1. Acute toxicity

Acute toxicity is caused by an agent when it is administered in one or more doses over a period not exceeding 24 hour and involves harmful effects to the organism through a single or short-term exposure**.** Acute toxicity studies have also been used during the selection of starting doses for phase-I human and animal studies, and provide information relevant to acute overdosing in humans and animals. The testing is based on the route of substance administration to the animal and therefore it is classified from Class-1 to Class-5 for oral, dermal, gas inhalation, vapor/dust/mist inhalation and injection. Dosing can be repeated during the administration of test material by a variety of routes of exposure, including gavaging which involves stomach intubation or forced feeding, injection, skin, painting and inhalation. The acute toxic class method, a step-wise procedure, involves the use of three animals of a single sex per step. Depending on the mortality and/or moribund status of the animals, on average 2 to 4 steps may be necessary to allow judgment on the acute toxicity of the substance. The OECD Guideline 423 (2001) provides a reproducible method that uses few animals as per appendices 2, 3 and 4.

1.4.2. Sub-acute toxicity

The sub-acute toxicity test in the study was based on OECD guideline 407 (2008), repeated dose 28-day oral toxicity study in rodents. In this form of toxicity, adverse effects occur as a result of repeated daily dosing of a chemical or exposure to the chemical, for part of an organism's life-span usually not exceeding 10 % of the animals' lifespan. With experimental animals, the period of exposure may range from a few days to 6 months. Exposure for 28 days provides a first-hand indicator of potential sub acute toxicity. The test is intended to investigate effects on a very broad variety of potential targets of toxicity. It provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time, including

effects on nervous, immune and endocrine systems. The duration of exposure is normally 28 days in rodents where results are used for hazard identification and risk assessment (OECD, 2008). All the knowledge gathered from the studies is used in selecting doses for repeat-dose studies as a source of preliminary identification of target organs of toxicity, and may also reveal delayed toxicity. Sub-acute toxicity studies in animals are essential for any pharmaceutical products especially those intended for human use.

1.4.3. Sub-chronic toxicity

This is the ability of a toxic substance to cause effects for more than one year but less than the lifetime of the exposed organism. This form of toxicity is studied for at least 90 days in animal models notably rodents. The test is carried out after getting initial information on toxicity from acute or 28 day sub-acute toxicity studies. It provides information on possible hazards likely to arise from repeated exposures over a prolonged period of time covering post-weaning maturation and growth well into adulthood. The study provides information on the major toxic effects, indicates target organs and the possibility of accumulation. It can also provide an estimate of a non-observed-adverse effects level (NOAEL) of exposure which can be used in selecting dose levels for chronic studies and to establish safety criteria for human studies (OECD 408, 1998).

1.4.4. Chronic Toxicity

This is the ability of a substance or mixture of substances to cause harmful effects over an extended period, usually upon repeated or continuous exposure, sometimes lasting for the entire life of the exposed organism. The three main routes for chronic study include oral, dermal and inhalation depending on the characteristic of the test substance and the predominant route of exposure in human. The objectives of chronic toxicity studies include; identification of the

hazard properties of a chemical, identification of target organs, characteristic of dose-response relationship and identification of NOAEL or point of departure of Benchmark Dose (BMD). It also helps in identification of chronic toxic effects in human exposure levels and provision of data to test hypotheses regarding mode of action (OECD 452, 2008).

1.5. Literature review

1.5.1. Plants under study

1.5.1.1. Albizia gummifera

Albizia gummifera (Fabaceae), (locally known as Mùkùrùe amongst the Agikuyu, 'Seyet' by Nandi, 'Omgonjoro by Kisii and 'Mukhonzuli' in Kakamega among others), is a large deciduous plant that grows up to about 30 meter tall with large-spreading flat topped crown and a smooth grey bole that grows wildly in most African countries including Kenya. Its leaves are bipinnate with leaflets with a diagonal midrib. The flowers are white and at the head. Their pods are flat in shape. The species is widely distributed and its inner bark produces lather like soap. Leaves are used for covering mature banana to hasten ripening (Gachathi, 1989). A bark decoction is used for malarial properties which have been validated in *in-vitro* tests (Kokwaro, 1976). The Embu and the Mbeere communities of Eastern province in Kenya have traditionally been using *A. gummifera* barks in treatment of malaria (Kareru *et al*, 2007). An extract of crushed up pods is taken for stomach pain and the powdered roots extract put in water bath used to cure skin diseases (Kokwaro, 1976). Other species in this genus with medicinal values include *Albizia anthelmintica* which is used as anthelmintic, *Albizia petersiana* bark for treating rheumatic pain and *Albizia zimmermannii* roots boiled to treat sore eyes (Kokwaro, 1976).

Lipophilic extracts of *A. gummifera* have revealed very promising antitrypanosomal activity. Four new macrocyclic spermine alkaloids have been isolated and were active against two Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* (Rukunga *et al*. 2000).

In East Africa, extracts from the crushed pods are taken for stomach pains and the bark decoction for malaria. A study carried out in Kenya concluded that the aqueous and chloroformic extracts of *A. gummifera* are slightly and moderately toxic, respectively and that the target organs for the chloroformic extract were mainly the lungs, brain and liver (Mwihia, 2013). This could be related to a study carried out earlier that found the presence of cytotoxic triterpenoid saponins called gummiferaosides A, B and C (Shugeng *et al*, 2007). Earlier recorded phytochemical study (Asfaw *et al*. 1999) confirmed that *A. gummifera* contains triterpenoid saponins, saponin, lactones and triterponin sapogenin in their sterm bark. These chemicals are believed to be the ones associated with antitrypanosomal activity (Freiburgaus *et al*. 1996). The triterpenoid saponins and macrocyclic alkaloids have also been isolated from other species of *Albizia* such as *A. amara* (Mar *et al*. 1991).

1.5.1.2. Crotalaria axillaris

Crotalaria axillaris (Engl.*)* Aiton, is a shrub that grows to a height ranging from 1 to 4 m high. It is widely distributed in Kenya and Ethiopia especially in farmlands and grazing fields. *Clotalaria* species are widely found in the tropical countries including Africa. *Crotalaria axillaris* is known as 'Mùchingiri' amongst the Agikuyu of Kenya. *Crotalaria axillaris* leaf infusion is applied to the eyes to treat opthalmia while a poultice made from crushed up seeds is applied to the back for kidney troubles (Kokwaro, 1976). A different species of this genus, *Crotalaria juncea* which is

mostly found in Asian countries has been found to be pharmacologically active in treatment of ulcers and inflammation. The anti-inflammatory effects compares to that of indomethacin (Ashok *et al.,* 2006). Phytochemical toxicity studies on the plant are of importance. First, in Ethiopia some *Crotalaria* species are used either alone or in combination with other plants in the traditional medical practices, and due to recurrent drought, grazing animals are often tend to consume these plants which are known to thrive in dry and arid climates leading to ailments and even death.

Clotalaria species have been found to contain pyrrolizidine alkaloids which are considered to be important secondary metabolites largely on account of their biological activities, which include acute-hepatotoxic, carcinogenic, teratogenic, anticancer and neuroactive properties. The cytotoxicity of the pyrrolizidine alkaloids is due to their pyrrolic metabolites formed by microsomal bioactivation. Herbal preparations from these species may thus cause acute or chronic toxicity although the extent of damage caused by consuming such preparations has not been assessed. It has been reported that a single episode of pyrrolizidine alkaloids toxicity and possibly a long term low level exposure may lead to cirrhosis of the liver (Kaleab *et al*., 2004).

1.5.1.3. Manilkara discolor

Manilkara discolor belongs to the genus of trees in the family Sapotaceae that are collectively known as Manilkara trees and occurs throughout the tropics. *Manilkara discolor* is also known as Forest Milkberry and 'Nchogis' among the Dorobos, 'Mgama' by the Shambaa of Tanzania whereas the the Agikuyu call the tree 'Mùgambwa'. It is a big tree measuring up to 28 metres tall with rough dark grey bark and milky latex. The leaves are dark-green on top and silvery grey on bottom and are crowded at the end of a branch. Flowers are yellow, appearing as clusters of 4

to 6 (Gachathi, 1989). In other parts of Africa, it is mainly distributed in Natal in South Africa, in Malawi, Zimbabwe and Tanzania. It grows well in lowland mixed evergreen forest and mountain rainforest usually with good drainage. In Tanganyika, it is found in areas such as West Usambara mountains, Kwai, Iringa and Sao Hills. In Kenya, the plant is mainly found in Nairobi Arboretum, Machakos, Kiambu and Ngong Forest. *Manilkara discolor*'s medicinal uses include bark infusion which is drunk for stomachache and as astringent. The roots are also used medicinally for backache. The plant has been studied for its antiplasmodial activity against *Plasmodium falciparum* (Kigondu, 2011). From literature searches, another species, *Manilkara zapota,* was found to have antioxidant activity due to its high content of phenolic compounds (Shanmugapriya *et al*., 2011).

1.5.1.4. *Zanthoxylum usambarense*

Zanthoxylum usambarense (Engl.) is a short tree with scattered prickles and imparipinnate aromatic leaves with transluscent gland dots. The genus Zanthoxylum belongs to the family Rutaceae, which is represented by 28 species in 10 genera in Kenya. They are common trees of the dry lands with this Zanthoxylum genus referred to as 'prickly ashes' because of the shape of the leaves. Their flowers are small and white set in small clusters. *Zanthoxylum usambarense* (also known as *Fagara usambarensis*) have world-wide importance as natural medicines, and a few of these are used in Kenya. This particular species is known as 'Mùgùcùa' among the Agikuyu tribe of Kenya (Gachathi, 1989) 'Loisuki' by the Dorobo and 'Mhamba-muungu' by the Shambaa of Tanzania. The bark and leaves of *Z. usambarense* and *Z. chalybeum* have been used in the treatment of malaria, fever, severe colds and to alleviate stomachache and toothache. A decoction made from its bark is drunk for relief from rheumatism (Gachathi, 1989) while an infusion made from its fruits is mixed together with milk and the mixture drunk for relief from fevers, sore throats, tonsillitis and chest pain. Both its bark and root extracts exhibit fungicidal and insecticidal properties. The *Zanthoxylum usambarense* species also been used in the highlands of central Kenya as a folk remedy by the Kikuyu and Kamba tribes.as well as the Maasai, Digo, and Shimbaa tribes in the hills of the East Coast District of Kenya. Studies carried

out in the University of Nairobi (He *et al.* 2002) confirmed that the crude extract of *Zanthoxylum usambarense* have larvicidal activity against the second and forth instar larvae of *Aedes aegypti*, the vector for malaria, with ED_{50} values of 1.27 and 7.17 µg/ml, respectively.

Another study that was conducted on *Warburgia ugandensis* and *Zanthoxylum usambarense* confirmed that the two plants possess bioactive compounds against malaria parasites and could be exploited for further development into malaria therapy (Were *et al*. 2010). Much of the pharmacological activity of these species may be attributed to their alkaloidal constituents as many pharmacologically active quaternary alkaloids have previously been isolated from various *Zanthoxylum* species (Atsushi *et al. 1996*). Phytochemical studies carried out in Kenya led to isolation of eleven compounds. The isolated pure compounds as elucidated using NMR, EIMS, and UV spectroscopy included benzophenanthridine alkaloids; dihydrochelerythrine, chelerythrine, angoline and 6-oxynitidine; anthranilic acid derived alkaloid (canthin-6-one) triterpine stigmasterol, bis-epoxy lignans, *O*-prenylpruviatilol and yangamin (Ayoo, 2001). Another earlier study had isolated usambanoline alkaloid (Atsushi *et al.* 1996). Studies carried out on the essential oils of *Zanthoxylum alatum* and *Zanthoxylum limonella* found better antihelmitic activity than piperazine phosphate against roundworms, tapeworms and hookworms (Akhtar *et al*. 2000).

1.5.1.5 *Teclea trichocarpa*

Teclea trichocarpa[Vepris trichocarpa (Engl.) Mziray] common name is the furry-fruited Teclea. Its local name is Mùnderendu wa ikurarĩ among the Kikuyu of Kenya, 'Mulela by the Akamba, 'Olerai by the Maasai and 'Mndizi by the Shambaa of Tanzania. Its synonym is *Vepris trichocarpa.* It is a shrub mostly found in coastal, upland forests and grasslands especially near rivers. The trees are usually 8 m tall with trifoliated leaves which have flattened petiole and dotted with numerous lenticels (Figure 1.2). Their fruits are hairy and wrinkled when dry. It is common in the dry forest. It is commonly used for tool handles and walking sticks (Gachathi,

1989). *Teclea trichocarpa* is used by traditional healers of the Akamba tribe of East Africa for malaria treatment, as an anthelmintic and as a vapour inhalant for treatment of fever. Another species, *Teclea nobilis* has been used by the Maasai of Kenya as a traditional herbal remedy against malaria (Bussmann *et al*. 2006)**.** Other species of *Teclea* are used medicinally in various parts of Africa. For example, *T. nobilis* bark and leaves are used as analgesics in Ethiopia and as anthelmintic by the Kipsigis of Kenya (Kokwaro, 1976), while *Teclea ouabanguiensis* is used as a remedy for coughs and asthma in Cameroon. Further, previous biological studies of *Teclea ouabanguiensis* revealed potent insect antifeedant activity against the African armyworm, *Spodoptera exempta*, as well as antifungal, antibacterial activity and *in vitro* antiplasmodial activities.
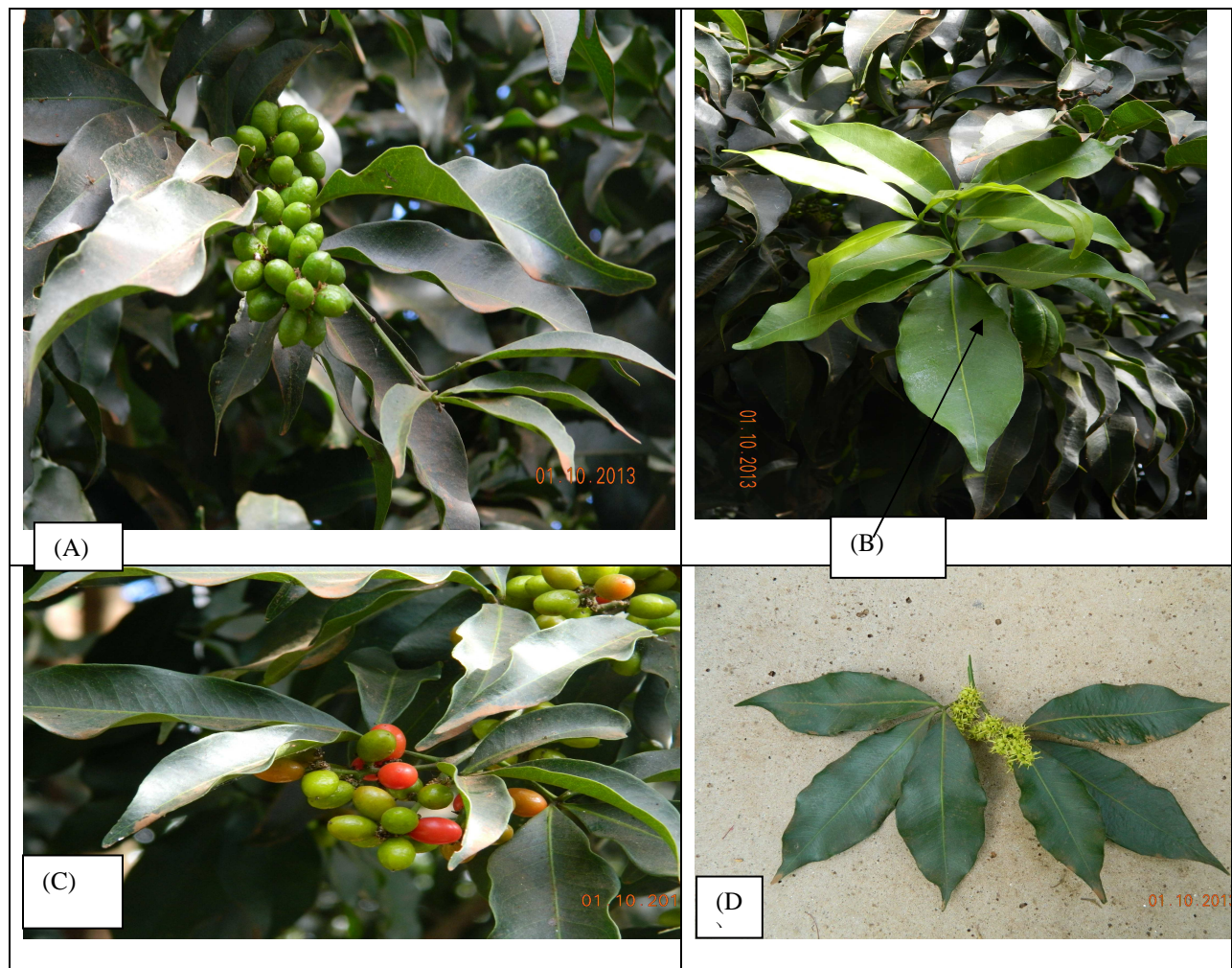
Figure 1. 2: Specimens of *Teclea trichocarpa* plant parts. **(A) Unripe fruits**, **(B) Trifoliate leaves (C) Ripening fruits (D) Srouting flowers (Photo by Denis Kirubi)**

Phytochemical studies have been done on *Teclea trichocarpa.* The studies indicated presence of several alkaloids including melicopicine, normelicopine, arborinine, skimmianine, diatamnine, tecleanthine and 6-methyltecleanithine, some of which are illustrated in Figure 1.3. The structure of normelicopine was determined by means of X-ray crystography (Mureithi et al., 2002).
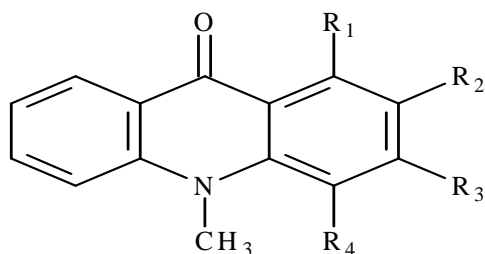
M elicopicine $(R_1, R_2, R_3, R_4 = OCH_3)$

N ormelicopicine $(R_1 = OH; R_2, R_3, R_4 = OCH_3$

A rborinine $(R_1 = OH; R_2, R_3 = OCH_3; R_4 = H)$
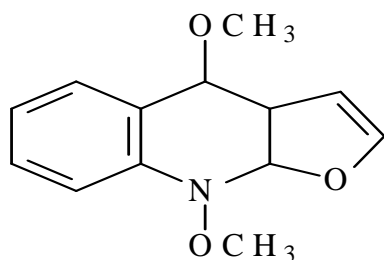
S kim m ianine

Figure 1.3: Examples of some of the phytochemicals isolated from *Teclea trichocarpa*.

In another study, *n*-hexane, dichloromethane and methanol crude extracts of *Teclea trichocarpa* were investigated for anti-trypanosomal, anti-leishmanial and cytotoxicity activities. The total methanol extract of the leaves of this plant and the isolated compounds were screened *in vitro* for

cytotoxicity and against parasitic protozoa, *Plasmodium falciparum*, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi* and *Leishmania donovani*. Among the compounds a-amyrin had the best anti-plasmodial activity, normelicopicine and skimmianine had the best anti-trypanosomal activity against *T. b. rhodesiense* and *T. cruzi*. Normelicopicine also exhibited best anti-leishmanial activity (Mwangi *et al.,* 2010).

1.6. Problem statement and justification

The most important aspects during the usage of drugs from all sources are their quality, efficacy and safety. Assumptions have been made that the use of herbal drugs in treatment of parasitic diseases in humans is going on without any noticeable toxic effects (Sushma *et al*., 2012). The assumption is not based on scientific evidence but on the fact that herbs are often believed to be safe because they are 'natural' (Glesler, 1992). Thus they are often used indiscriminately and in unstandardized manner. However, many dangerous or even lethal side effects have been reported including direct toxic effects, allergic reactions, effects from contaminants, and interactions with drugs and other herbs (Ernst, 1998). It is noteworthy that toxicity related to drugs is common in both conventional and herbal therapeutic agents. For example, a study carried out on 548 compounds marketed between 1975 to1999 showed that 10.2 % were withdrawn or acquired a black box warning due to toxicity (Boone *et al.,* 2005). This emphasizes the need to carry out toxicity testing on all drugs intended for human and animal consumption.

Less than 10 % of herbal products in the world are not standardized to known active components, and therefore no strict quality control measures are followed. The active constituents of most herbs or the toxicants are rarely known. Herbs contain complicated mixtures of organic chemicals, the level of which may vary substantially depending upon many factors

related to the growth, production, and processing of the herbal product (Stephen and Richard, 2004). Most of the toxic constituents in herbs are secondary metabolites produced by plants as their natural defense to adverse conditions but may end up poisoning the human (Obidike and Salawy, 2013). Deaths have been reported on individuals who have consumed drugs that have been empirically identified *via* trial and error methods (Pamela *et al*., 2001). It is very important to put more effort in the study of herbal medicine and especially in the areas of standardization. It is documented that herbs as medicine have not gained enough momentum in the scientific community due to lack of specific standards being prescribed for herbal medicines (Sarika *et al.,* 2006). The challenges faced by herbal formulations arise because of their lack of complete evaluation since evaluation is necessary to ensure quality and purity of the herbal product (Gupta and Amartya*,* 2012). Besides studies on quality and efficacy of herbal medicines, it is necessary to ensure the safety of a product and this entails toxicity testing.

The empirical process of identifying medicinal agents by trial and error is not efficient and countless individual have died following treatments with plant products that were poisonous and/or ineffective. Thus, the scientific medicine of 20th century should depend on rational codified principles, providing an understanding of why some treatments are effective and others are not. It is very important to ensure that each drug derived from plant products be evaluated for safety and efficacy by methods identical to those used for novel synthetic entities (Rotblatt and Zimet, 2002).

Toxicity testing of herbal drugs has also been found to have a lot of benefits. Notably, it is easy to identify the toxic effects and thus determine the limit of exposure levels especially to sensitive

population. Once these toxicants are known they may be discarded or modified *via* dosage adjustment, chemical group or structural adjustments (Obidike and Salawy, 2013).

From the literature review, there is no prior documented scientific research especially on sub-acute toxicity testing on the root bark extracts of *Teclea trichocarpa*. *Teclea trichocarpa* has been used in herbal medicine and preliminary studies have shown that it has antihelmintic activity. The possibility of any potential lethal side effects justifies the necessity for this work. There are reports on the cytotoxicity of the leaf extract on protozoa (Mwangi *et al*., 2010). Although photochemical investigation has been carried out, there are no reports on the safety of its root bark extract and hence the need to carry out this study.

1.7. Objectives

1.7.1. Broad Objective

To investigate the toxicity of dichloromethane-methanol (1:1) extracts from different plant parts of *Teclea trichocarpa, Albizia gummifera*, *Crotalaria axillaris*, *Manilkara discolor,* and *Zanthoxylum usambarense.*

1.7.2. Specific Objectives

1. To investigate the brine shrimp lethality test of the plant parts from *Teclea trichocarpa, Albizia gummifera*, *Crotalaria axillaris*, *Manilkara discolor,* and *Zanthoxylum usambarense*.

2. To investigate the acute and sub-acute toxicity of dichloromethane-methanol (1:1) extract of *Teclea trichocarpa* root bark on rats with reference to clinical, haematological, pathological/histopathological and clinical biochemistry investigation parameters.

CHAPTER TWO: PRELIMINARY TOXICITY SCREENING OF FIVE PLANTS

2.1.Introduction

Toxicity testing on herbal extracts is carried out on the same principles as the conventional medicine. *In vitro* toxicity testing employs the use of models such as the brine shrimp lethality test (BST) whereas in *in vivo* methods, animals such as mice or rats are used. The advantage of the brine shrimp in toxicity testing is that the shrimp has a lot of homogeneity in eggs and in newly born nauplii which are highly sensitive to chemicals. The eggs are easily available and hatch with ease within 16-24 hours to the nauplii. The nauplii are utilized within 24-48 hours post hatching. At the 24th hours post exposure of nauplii to the test chemical, the concentration causing 50 % lethality (LC_{50}) is determined.

The BST method is used to screen for bioactivity, anticancer, cytotoxicity, toxicity, pesticidal activity or gastroprotective action among other pharmacological effects of plant extracts. Brine shrimp lethality is a rapid method that is reliable, inexpensive and convenient as an in-house general bio-assay tool (Meyer *et al.,* 1982). This method is basically used to predict toxicity and the results obtained are compared with oral acute toxicity in rodents (Amenya, 2011). *In vivo* toxicity testing mainly employs the use of rats although other rodents may be used. Dogs and monkeys are restricted to advanced stages of testing (Amenya, 2011).

Preliminary work was carried out at the University of Nairobi in 2010 using 'Screen-to Nature' (STN^{TM}) technology to screen plants for anthelmintic activity. Screen-to-Nature technology is an innovation of Global Institute for Bio-exploration (GIBEX), that uses effective and portable drug

discovery tools and technique to screen bioactive properties in plants directly in the field by using local resources (GIBEX, 2008).

The study plants were selected based on previous traditional knowledge on Kenyan medicinal plants gathered at the Mitishamba Drug Research Centre of the School of Pharmacy, University of Nairobi for more than twenty years. The information was from herbalists and literature on their use for common ailments like malaria, diarrhea, skin infections, coughs, abdominal ailments and intestinal worms. A study carried out on *Albizia gummifera*, *Crotalaria axillaris, Manilkara discolour, Teclea trichocarpa* and *Zanthoxylum usambarense* found them to have high activity against the roundworm *Panagrellus redivivus*. This is a free living nematode that serves as a model organism to determine the lethality of plant extracts to roundworms (GIBEX, 2008).

2.2. Objectives

2.2.1. Main Objective

To carry out pleliminary toxicity sreening of five plants

2.2.2. Specific objectives

1. To carry out plant identification, correction and preparation

2. To determine LC_{50} of the plants extracts using brine shrimp lethality test

2.3. Materials and reagents

2.3.1. Plant materials

The 5 plants used during this study were obtained from Ngong Hills Forest which is known for its richness in biodiversity. Specimens of the plant parts and the voucher numbers were preserved at the School of Biological Science herbarium in Chiromo, University of Nairobi.

2.3.2. Brine shrimps eggs

The shrimp eggs for use in lethality test were purchased locally from an aquarium shop (Aquapet Ltd, Westgate Mall, Nairobi, Kenya).

2.3.3. Apparatus and equipments

2.3.4. Equipment for plant material preparation

Tools used at the site of plant material collection included "pangas", knives and a hand-saw. A ginding mill was used during powdering of the dried plant materials and various glassware and laboratory equipment were used during extraction. Gauze, Whatmann's filter papers and a rotor evaporator (Heidoph VV2000, Schwabach, Germany) were used during the extraction process.

2.3.5. Apparatus for BSL test

The BST was carried out in a small tank or hatching chamber with a dividing tank that was locally fabricated. The other essential was overhead lamp.

2.3.6. Reagents for BST

The reagents used included sea salt from Aquapet Ltd Westgate mall, Nairobi.

2.3.7. Solvent for BST

The solvents used at this stage included dichloromethane, methanol and dimethylsulphoxide (DMSO) all of which were of analytical grade in quality. These reagents were obtained from Lobachemie, Mumbai, India.

2.4. Procedures

2.4.1. Plant collection, identification and extraction

The site for plant collection is stated in section 2.2.1. Different plant parts were collected as indicated in appendix 1. The roots were washed in running water to remove soil matter and the root bark peeled-off when the sample was still fresh. The plant parts were then air dried with regular turning, ground to powder and the powder weighed. The powders were then packed in well labeled air-tight polythene bags to avoid moisture and growth of fungi. Cold extractions were done on several batches, each using dichloromethane-methanol (1:1). About 1500 ml of solvent mixture was added to 1000 g of powdered plant materials and thoroughly mixed. Periodical stirring of the mixture was performed for the next 48 h before filtering. A golden-yellow coloured liquid extract was obtained. Further 500ml of the solvent mixture was added to the plant material and the procedure repeated for another 48 hour. This was repeated three times until the disappearance of the colour of the extract indicated that exhaustive extraction. The extracts were then each filtered through gauze, centrifuged at 5000 r.p.m. and then filtered through a double layer filter paper. Each extract was dried *in vacuo* using a rotor evaporator and then in the oven at a temperature of 40 ^{o}C. The percentage yield were calculated and recorded. The extracts were kept at 4°C until use.

2.4.2. Brine shrimp lethality assay

2.4.2.1. Shrimp's eggs/cysts viability test

The viability test on the shrimp eggs/cysts was conducted to confirm their hatchability. A high volume beaker was filled up to approximately two thirds full with sea water. About a teaspoon of brine shrimp eggs/cysts was added and the beaker with eggs put into a cultivator for 48 hours. The ability of the eggs to hatch was assessed by visually checking for the swimming *Artemia* naupliis.

2.4.2.2. Hatching the shrimps

The Artemia salina eggs were incubated in natural sea water (33 g / L) at room temperature under constant aeration for 48 hours, the phototropic nauplii were then ready for use in assays.

2.4.2.3. Brine shrimp lethality assay

Different concentrations of the dichloromethane-methanol extracts from the 5 different plants were prepared in dimethyl sulphoxide (DMSO) to obtain each of the three concentrations at 10, 200 and 1000 μg/ml. Ten of the napuliis were collected by a pipette from the lighter side of the hatching chamber and were put in vials containing 4.5 ml of sea water each with different concentration of extracts for the tests. Ten of the naupliis were put in 4.5 ml sea water with 0.2 % of DMSO for the control set up. After 24 hours, surviving shrimps in each vial were viewed with a magnifying glass, counted and the survival data recorded.

2.5. Data Analysis

Data collected was entered into Microsoft Excel spreadsheets (Microsoft Inc, Washington, USA) before exporting to the relevant statistical packages. The data obtained from brine shrimp lethality test was exported to Graphpad Prism 4 (April 3, 2003) computer program for analysis to determine the IC_{50} values at $p < 0.05$, 95 % confidence interval.

2.6. Results

2.6.1. Extract description

The dried *Teclea trichocarpa* extract was obtained as a tar-like semi-solid substance with dark to golden brown colour. Further, the *Teclea trichocarpa* turned filter paper transluscent confirming that the extract contained oils.

2.6.2. Yields of extracts

The yields of dried solid extract from the dichloromethane-methanol extracts of the 14 plant parts ranged from as low as 0.2 % to 9.9 %. *Clotalaria axillaris* twigs had the lowest yield (0.2 %), *Teclea trichocarpa* root bark had 7.9 % and *Albizia gummifera* pods had the highest yield of 9.9 % (Appendix 1).

2.6.3. Results from brine shrimp lethality tests

The dichloromethane-methanol (1:1) extracts of *Albizia gummifera*, *Crotalaria auxillaris*, *Manilkara discolour*, *Teclea trichocarpa* and *Zanthoxylum usambarense* brine shrimp lethality test results are as shown in Table 2.1. The results obtained from the study indicated that *Albizia gummifera* pods and *Clotaralia axillaris* twigs extracts had $LC_{50} > 1000$ μg/ml while that of

Teclea trichocarpa root wood was >500 μg /ml. *Albizia gummifera* root bark, *Manilkara discolor* root bark and stem barks and *Teclea trichocarpa* stem bark, root and twigs had LC$_{50}$ >100 < 500 μg /ml. The LC$_{50}$ for *Teclea trichocarpa* root bark and *Zanthoxyllum usambarense* stem bark extracts were between 30 and 100 μg /ml. Extracts from *Albizia gummifera* root & stem bark and *Zanthoxyllum usambarense* root barks were the least, below 30 μg/ml.

Table 2.1: The LC$_{50}$ values in μg/ml of various extracts under study on brine shrimps.

Plant parts	LC$_{50}$ Values (μg/ml)	Level of toxicity *
Albizia gummifera pods	>1000	Practically non-toxic
Albizia gummifera root	12.97	Moderately toxic
Albizia gummifera root bark	385.7	Very low toxicity
Albizia gummifera stem bark	28.67	Moderately toxic
Crotalaria axillaris twigs	>1000	Practically non-toxic
Manilkara discolor root bark	183.2	Very low toxicity
Manilkara discolor stem bark	190.8	Very low toxicity
Teclea trichocarpa twigs	196.5	Very low toxicity
Teclea trichocarpa root wood	768.7	Non-toxic
Teclea trichocarpa stem bark	446.4	Very low toxicity
Teclea trichocarpa root	454.4	Very low toxicity
Teclea trichocarpa root bark	41.64	Mildly toxic
Zanthoxylum usambarense root bark	12.99	Moderately toxic
Zanthoxylum usambarense stem bark	31.14	Mildly toxic

*Reference: (Meyer et al., 1982 and Moshi et al., 2010)

2.7. Major findings

The results obtained from the study indicated that *Albizia gummifera* pods and *Clotaralia axillaris* twigs extracts were practically non-toxic or non active (with LC_{50} >1000 μg/ml). *Teclea trichocarpa* root wood (LC_{50} >500 μg /ml) was non-toxic while *Albizia gummifera* root bark, *Manilkara discolor* root bark and stem barks and *Teclea trichocarpa* stem bark, root and twigs (LC_{50} >100 < 500 μg /ml) had very low toxicity. *Teclea trichocarpa* root bark and *Zanthoxyllum usambarense* stem bark extracts (LC_{50} results between 30 and 100 μg/ml) were mildly toxic to the shrimps. Extracts from *Albizia gummifera* root & stem bark and *Zanthoxyllum usambarense* root barks were moderately toxic with LC_{50}< 30 μg/ml.

2.8. Conclusion

From the results obtained from the brine shrimp lethality test, 5 extracts namely *Teclea trichocarpa* root bark, *Zanthoxyllum usambarense* stem bark, *Albizia gummifera* root and stem bark and *Zanthoxyllum usambarense* root barks were comparatively the most cytotoxic with LC_{50} < 100 μg/ml. These findings corroborates with earlier studies that that guide on the best herbal extracts to be selected for further work as leads in development of novel drugs.

Further, from the literature survey, much work has been done on *Zanthoxyllum usambarense* and *Albizia gummifera.* It is on this basis that the acute and sub acute toxicity tests were focused basically on *Teclea trichocarpa* root bark whose toxicity profile had not been documented despite its appreciable anthelmintic activity.

CHAPTER THREE: ACUTE AND SUB-ACUTE TOXICITY STUDIES ON

TECLEA TRICHOCARPA ROOT BARK EXTRACT ON RATS

3.1. Introduction

Teclea trichocarpa has been used as herbal medicine in the treatment of helminthiasis and preliminary studies have shown that it has antihelmintic activity. As noted earlier in this report at 1.5.1.5, *Teclea trichocarpa* is used by traditional healers of the Akamba tribe of East Africa for malaria treatment, as an anthelmintic and as a vapour inhalant for treatment of fever.   However, there are no reports on the safety of its root bark extract and hence the need to carry out this toxicity studies.

The potential of chemical substances to cause toxic effects can be assessed qualitatively in laboratory animals. This is an *in vivo* toxicity test that gives information on the possibility of a conventional drug or herbal extract to cause toxic effects in humans or animals (Descote, 1996). To succeed in this exercise, an appropriate choice of control group must be selected, sufficient number of laboratory animals used and good selection of rigorous experimental protocols. Furthermore, the severity of the effect described on major organs and the relevance of the mechanisms involved including the variations in different species assist in extrapolation of toxicological findings from laboratory animals to man (Descote, 1996). During the study, the target organ of toxicity in laboratory animals are identified, the mechanism of induced changes are noted and compared to the properties of the target site in man.

The findings on toxic potential of new compounds constitute a major part in drug development and it involve *in vivo* toxicological test, which is very critical in the assessment of the safety of

all pharmaceutical products before they are released for general use. Animal models are used in *in vivo* studies as indicators of human toxicity (Magna and Alan, 2007).

Organization for Economic Cooperation and Development (OECD) guidelines are used during acute and sub-acute oral toxicity testing (Diener *et al*., 1995). It is important to optimize the information obtained by using the smallest number of animals to comply with animal welfare regulations. Further, it is important to avoid excessive pain or tissue damage in the animals, pharmaceuticals with irritant or corrosive characteristics should not be administered in concentrations that produce severe toxicity after administration. During toxicity studies, all the animals must be checked for morbidity, mortality and specific signs of toxicological relevance. For example, neurofunctional and neurobehavioral, opthalmological observation, body-weight and food/water intake. The key haematological parameters investigated are mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), haemoglobin levels, haematocrit levels, packed cell volume (PCV) total and differential leukocytes, erythrocytes and platelet counts.

Clinical biochemistry is crucial to investigate major toxic effects on organs especially the kidney and the liver. Some of the parameters include total protein, albumin, major electrolytes, total cholesterol, alanine aminotransferase, arspatate aminotransferase, creatinine and alkaline phosphatase that aid in hepatocellular evaluation. All long term studies such as chronic toxicity must include urinalysis (urine output, color, protein and osmalarity). Pathological studies and gross necropsy are done by examining the body, orifices, abdominal cavity, body weight and organ weight changes among others. In addition, histopathological studies are done on adrenals, lung, liver, kidney, testis, ovaries among others (OECD 407, 2008). These organs are considered

to be the most important during toxicity studies in rodents and non-rodents (Michael *et al.* 2007). Taking the weights of organs is necessary because organ to body weight ratios or organ weight index (OWI) are commonly calculated and are considered more useful when body weights are affected (Michael *et al.* 2007). By carrying out toxicity tests, the effects of increase in dose on the mortality and other effects of the lethal dose that kills are determined. Estimating different levels of toxicity by use of LD_{50} for instance can help in estimating the probabilities of an outcome for a given individual in a population. The determination of acute, sub-acute, sub-chronic and chronic toxic effects of the test compounds is therefore crucial (Traina, 2006).

3.2. Objectives

3.2.1. Main objective

To investigate acute and sub-acute toxicity studies on 1:1 dichloromethane-methanol extract of *Teclea trachocarpa* root bark in rats.

3.2.2. Specific objectives

1. To investigate acute toxicity study of *Teclea trachocarpa* root bark extract in rats

2. To investigate intraperitoneal acute toxicity of *Teclea trachocarpa* root bark extract in rats

3. To investigate sub-acute toxicity of *Teclea trachocarpa* root bark extract in rats

3.3. Methods

3.3.1. Materials and reagents for toxicity tests

3.3.2. Laboratory animals

The experimental animals consisting of Wister rats aged 6-8 weeks, were obtained from the animal house at School of Pharmacy, University of Nairobi.

3.3.3. Reagents

The clinical chemistry kits for total protein, creatine kinase and creatinine were from Diagnostic Systems International, Holzheim, Germany and for ALT, AST and Albumin from Thermo Electron, Scoresby Vic, Australia and Fisher Diagnostics, Massanchusetts, USA). Eosin and haematoxylin dyes were sourced from Kobian Kenya Limited.

3.4. Solvents

The solvent used in this work included diethylether from Lobachemie, Mumbai India and phosphate buffered normal saline.

3.5. Equipment for toxicity testing

The equipments used during toxicity testing included a Visual[R] spectrophotometer (Biomerieux, Paris, France) for clinical chemistry analysis, a microscope (Leica, Wetzlar, Germany) for histopathological work, MS4 Vet[R] haematology blood counter (Melet Schloesing Laboratories, Cergy-Pontoise Cedex, France) and a locally fabricated anaesthetizing chamber.

3.6. Experimental animals

The study was carried out as per the OECD 407, 2008 guidelines. Wister rats were bred at the School of Pharmacy, University of Nairobi. They were randomly allocated and housed in clear polycarbonate cages with stainless mesh lids, each containing 5 rats, male and females separately. Wood shaving bedding was changed twice weekly to maintain hygiene. The animal room was well ventilated, with functional windows that were either opened or shut depending on the weather conditions to ensure that temperature in the experimental animal room was maintained at 22°C (± 3°C). Lighting was artificial, the sequence being 12 hours light and 12 hours darkness. The rats were fed with mice pencils (Unga Farmcare (EA) Ltd, Nairobi, Kenya) and water *ad libitum*. All the experiments were conducted as per the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Animal Health (NACLAR, 2004).

3.7. Procedures for toxicity tests

3.7.1. Acute toxicity after oral administration

The experimental animals, Wister rats, aged 6-8 weeks, were randomly allocated and housed in cages, each containing 3 rats; males and females separately with bedding changed twice a week to maintain hygiene. Feed and water were given *ad libitum*. The animals were randomly selected, marked with picric acid to permit individual identification, and observed for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions. Animals were fasted overnight prior to dosing but water was not withheld. Following the period of fasting, the animals were weighed and the test substance administered on the basis of individual animal weight. The extract was suspended in 2.5 % Tween 80 in normal saline with volume of the

aqueous suspension not allowed to exceed 1 mL/100 g of body weight. The test dose was administered in a single dose by gavage using suitable intubation canula. Food was withheld for a further 3-4 hours after dosing.

For each dose used, the volume administered was calculated using the equation; $V = (D \times P) / C$, Where; D = Dose used (g/kg body weight), P = Body weight of rat (g), C = Concentration of extract in g/ml and V = Volume (ml) (Tendong *et al.* 2007).

The Globally Harmonized Classification System (GHS) in Acute toxicity category (ATC) method of the OECD was used to determine the LD_{50} range (OECD Guidelines, 2001) as per appendix 2. Since there was no prior information on toxicity of *Teclea trichocarpa* extract, for animal welfare reasons, the starting dose was selected to be 300 mg/kg body weight (Appendix 3). Since no death occurred at this dosage level, then the next higher dose, 2000 mg/kg was used. In 2000 mg/kg category, all the three rats were treated with 2000 mg of extract per kg body weight of the rat. The volume of the extract given was calculated according to the weight of each rat, ensuring that the volume fed to the rat did not exceed 2 mL.

Mortality and other clinical signs were recorded. The test was designed in such a way that if one or no animal died after 24 hour, the procedure was repeated by using three rats at the same dose level. Absence of death or death of only one animal during the repeat dose implied that the LD_{50} range is more than 2000 mg/kg but lies between 2000 mg/kg and 5000 mg/kg. Moribund rats, those obviously in pain or showing signs of severe and enduring distress were humanely killed and considered in the interpretation of the test results in the same way as animals that died on test.

The rats were observed individually after dosing at least once within the first 30 minutes, then periodically during the first 24 hour. The parameters of interest were changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems for signs of toxicity that may include; tremors, convulsions, salivation, diarrhea, lethargy, somnolence, or coma or death. The mortality rates for each dose group were recorded for the first 24 hours. Standard pathology procedures were followed to examine the dead or sacrificed animals and the pathological changes recorded. Microscopic examinations were done for those organs showing gross pathology and in organs from all animals at the highest dose group and in the control groups. Pertinent lesions were recorded at all dosage levels. Lesions were then followed through in animals at other lower dose group as was necessary.

3.7.2. Acute toxicity after intraperitoneal administration

The procedure used conformed to the OECD guideline that is used in acute toxicity testing (OECD 423 guidelines, 2001) and as per Appendices 2 to 4. The extract was dissolved in phosphate buffered saline with 3 % DMSO. The extract was first filtered through filter paper, and then through 0.2 µm Millipore filters to ensure sterility of the solution for intraperitoneal administration. The concentration of the solution was adjusted to ensure that the volume delivered per animal based on individual body weight was between 1 ml and 2 ml, the volume recommended for rats. The solution was then injected via intraperitoneal route starting with the 2000 mg/kg body weight dosage level.

The GHS/ATC method was then used to estimate the LD_{50} range. For example, for a starting dose of 2000 mg/kg body weight (Appendix 2), three animals were injected with 2000 mg/kg each. If 0-1 death occurred within 24 hours, the experiment was repeated with 3 more animals. If

during the repeat exercise 0-1 death occurred, it was concluded that the LD_{50} range for the extract falls between 2000 and 5000 mg/kg body weight. On the other hand, if 2-3 deaths occurred during the first or repeat exercise it was assumed that the LD_{50} range was below 2000 mg/kg and therefore the experiment was shifted to test procedure with a starting dose of 300 mg/kg body weight (Appendix 3).

3.7.3. Sub-acute toxicity

Feed and water were provided *ad libitum* and the Wistar rats were allowed 7 days for acclimatization. The rats aged 6-8 weeks, were randomly allocated and housed in cages, each containing 5 rats; males and females separately with wood shaving bedding changed twice a week to maintain hygiene. The animals were assigned at random to three treatment groups of 5 animals per sex and a control group. A total of 40 animals were used. Each treatment group received a different concentration of the plant extract by gavage as described in the acute toxicity study. The dosage levels were logarithmically spaced as follows; 100 mg/kg, 300 mg/kg and 1000 mg/kg body weight daily. Controls were administered with untreated vehicle comprising 2.5 % Tween 80 in normal saline. Animals were dosed daily for 28 days with the test material on the basis of weekly mean group weight in accordance with OECD guideline 407 (2008). All animals were weighed weekly.

3.7.3.1. Clinical observations

Animals were observed individually for clinical signs twice daily after dosing. Clinical observations were recorded daily. The parameters of interest were changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems for signs of toxicity. Signs of toxicity included tremors, convulsions, salivation, diarrhea, lethargy,

somnolence, or coma or death. The mortality rates for each dose group were recorded for the first 24 hours. Animals found moribund or showing clinical signs of pain or distress were euthanized using diethylether.

3.7.3.2. Haematological tests

For haematological studies, 2-3 ml of blood was collected using a capillary tube from the orbital sinus of the lightly ether-anaesthetized Wister rat into a test tube containing ethylenediamine tetraacetic acid (EDTA). Haematological parameters of interest included; haemoglobin concentration (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), total erythrocyte count (RBC), haematocrit (PCV). Also collected was total white blood cell (WBC) and differential leucocyte count. Blood was collected before treatment and then thereafter fortnightly for 28 days.

3.7.3.3. Clinical chemistry tests

About 2-3 ml of blood samples per animal were collected in heparinized tubes. Plasma was obtained by centrifuging heparinized blood at 12,000 rpm for 5 min. Plasma was separated and stored at -20 ^{o}C until use. Clinical chemistry parameters included concentration of total proteins, albumin, creatinine and the activities of aspartate aminotransferase (AST), alanine aminotranferase (ALT), and creatine kinase (CK). These parameters were measured using the liquid-chemistry photometric methods. The plasma protein concentration was determined colorimetrically at 540 nm using the biuret method while total plasma albumin was determined by the bromocresol green method at 630 nm. The enzyme activity was determined as per the guidelines from the International Federation of Clinical Chemistry and Laboratory Medicine

(IFCC) (Walter, 2002). The plasma ALT and AST activity was determined by a photometric method with absorbance read at 340 nm while the activity of CK was determined at 492 nm.

3.7.3.4. Necropsy and histopathologic evaluation

A complete necropsy was performed on all treated and control animals that either died or were sacrificed *in extremis*. The weight of the liver, kidney, spleen, adrenals, thymus, testicles and ovaries was taken using electronic weighing balance.

Samples of major organs including liver, kidney, stomach, intestines, lungs, heart, brain and spinal cord were preserved in 10 % buffered formalin for histopathological evaluation. These organs were processed for histopathology through standard protocols. They were trimmed, embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin and observed under the microscope. The severity of gross and histopathologic morphological changes were graded as minimal (1), mild (2), moderate (3) and marked (4) according to a standard criteria (Sharkleford, *et al*. 2002). Frequency of each lesion category was determined in two sections per organ at randomly selected sites at x10, x100 and x400 objective magnification under the microscope, and scored according to the category of severity. Pertinent lesions and their frequency of occurrence were followed along all dosage levels and benchmarked against their occurrence in the highest dose group**.**

3.8. Data Analysis

Data collected was entered into Microsoft Excel spreadsheets (Microsoft Inc, Washington, USA) before exporting to the relevant statistical packages. The data obtained from brine shrimp lethality test was exported to Graphpad Prism 4 (April 3, 2003) computer program for analysis to

determine the IC_{50} values at $p< 0.05$, 95 % confidence interval. Continuous data such as organ weight indices, weight changes, biochemistry and haematological mean values were analyzed by descriptive statistics and two way analysis of variance to compare values in the control and the treated rats carried out. The categorical data such as pathological lesion frequency and severity and differential leucocytes counts were analyzed by comparison of dose level proportions and by use of a logistic regression model. The SPSS Program number 17, USA, was used for data analysis.

3.9. Results

3.9.1. Acute oral toxicity of *Teclea trichocarpa* root bark extract

3.9.1.1. Clinical effects.

There was no death during acute oral toxicity testing of *Teclea trichocarpa* root bark extract at 2000 mg/kg. All the animals showed clinical signs such as piroerectile, rubbing of nose and mouth and avoided feeds for the first 10 min post dosing. All the animals rubbed their mouth and nose with their front pawns and against the walls of the cage soon after dosing. All these symptoms disappeared completely after 30 min post dosing. The extract did not cause diarrhoea but the droppings in all test animals were wet and not well formed like pellets (Figure 3.1).
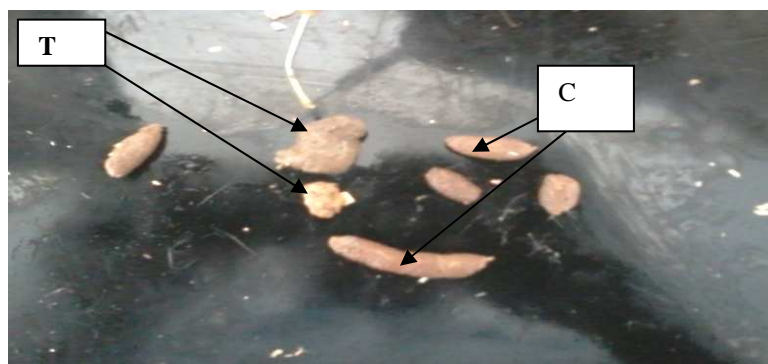
Figure 3.1: A specimen showing faecal droppings from the test laboratory rats (T) and control rats (C) gavaged orally with 2000 mg/kg of root bark extract.

3.9.1.2. Gross pathology

Body weight changes during the 5 treatment days for the three animals dosed at 2000 mg/kg were not significantly different from the control group. All extracts treated animals showed a stable increase in body weights after 5 days (Table 3.1). There was no evidence of exudates in the peritoneal cavity during autopsy.

Table 3.1: Effects of *Teclea trichocarpa* root bark extract on weight of rats (n = 3)

Rat's group	Weight at day 0 (g)	Weight at day 5 after treatment (g)	Weight gain (g)
1	118	130	+12
2	116	130	+14
3	152	162	+10
Control	178	180	+12

3.9.1.3. Histopathological changes

An oral dose of 2000 mg/kg did not cause any pathological changes in the main organs of toxicity. The sections of the heart, spleen, intestines, adrenal, lungs, stomach, liver and kidney appeared normal under the microscope. The microscopic picture of the liver (Figure 3.2) and the intestine (Figure 3.2) showed well-formed and distributed hepatocytes and villi, respectively. The cells around the central vein, that is continuous with the hepatic veins, and which suffers the heaviest burden of gut derived toxins, had no signs of toxicity.
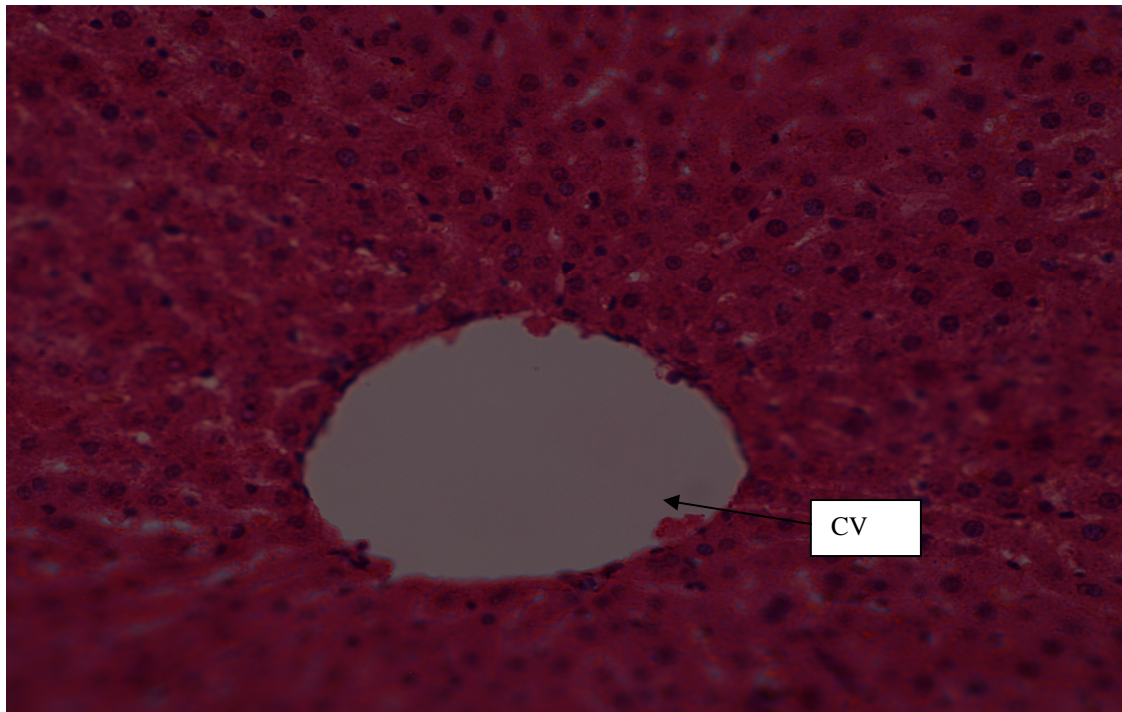
Figure 3.2: **A photomicrograph of a liver 24 hour after oral dose of 2000 mg/kg body weight of *Teclea trichocarpa extract*. Note the normal architecture and intact cells even those contiguous to the central hepatic vein (CV) (×400, Haematoxylin &Eosin).**
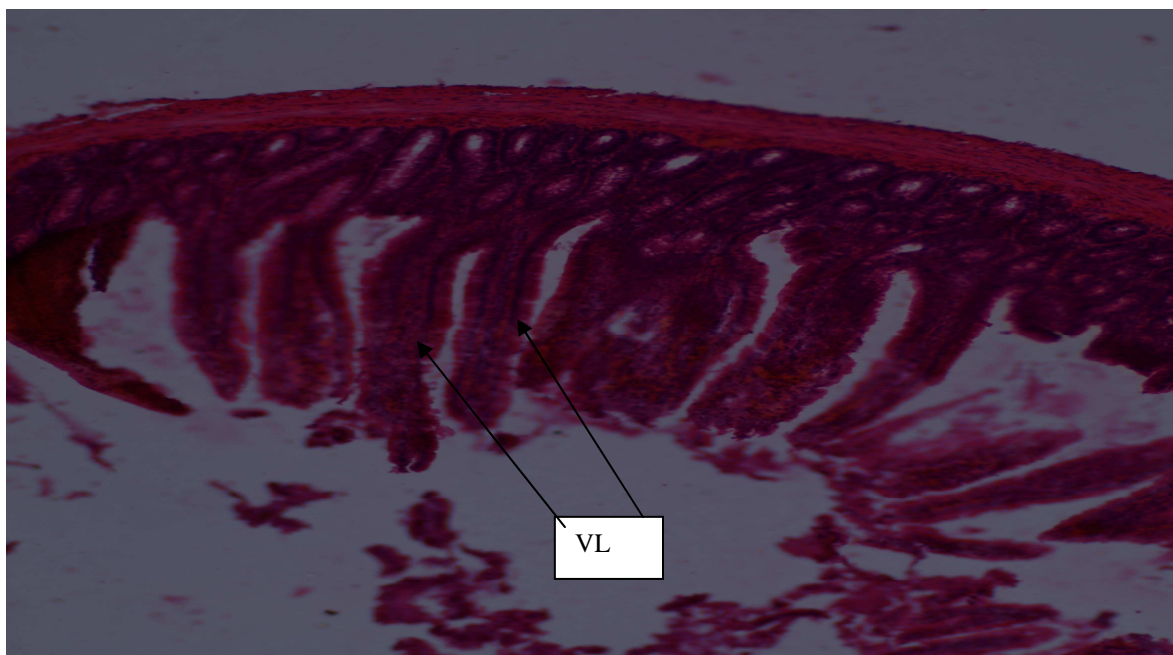
Figure 3.3: A photomicrograph of rat's intestine 24 hour after oral dose of 2000 mg/kg of *Teclea trichocarpa* extract. Note the normal architecture, well-formed and intact villi (VL) (×400, Haematoxylin & Eosin).

3.9.2. Intraperitoneal acute toxicity.

3.9.2.1. Clinical effects.

All the rats dosed at 2000 mg/kg body weight intraperitoneally died within two to three minutes. Immediately after dosing, the animals became restless, developed uncoordinated, jerky movements, then convulsed with their tails stretched and raised upward. The death mimicked that of strychnine toxicity. In the 300 mg/kg per body weight category, one rat died after 45 minutes, the second one died after 4 hours and the third after 48 hours. The symptoms were similar to 2000 mg/kg category but milder. The next group of 3 rats was dosed at 50 mg/kg body weight survived without any observable symptoms for the next 24 hours. Dosing was continued for the next 5 days till the cumulative dose was equal to the next toxic dose of 300 mg/kg body

weight but the rats survived. The only observable transient signs that occurred within 30 minutes post extract administration included raised fur and mouth rubbing.

3.9.2.2. Histopathological changes

The histopathological pictures of the main organs did not portray any abnormal features. The liver and the kidney cells and the general architecture of the organs were normal. The extract acted fast in those animals that were dosed at 2000 mg/kg body weight by affecting the central nervous system and thus the convulsions but there were no histological changes of toxicity in the organs, that is, the liver and kidney examined (Figure 3.4 and 3.5 respectively).
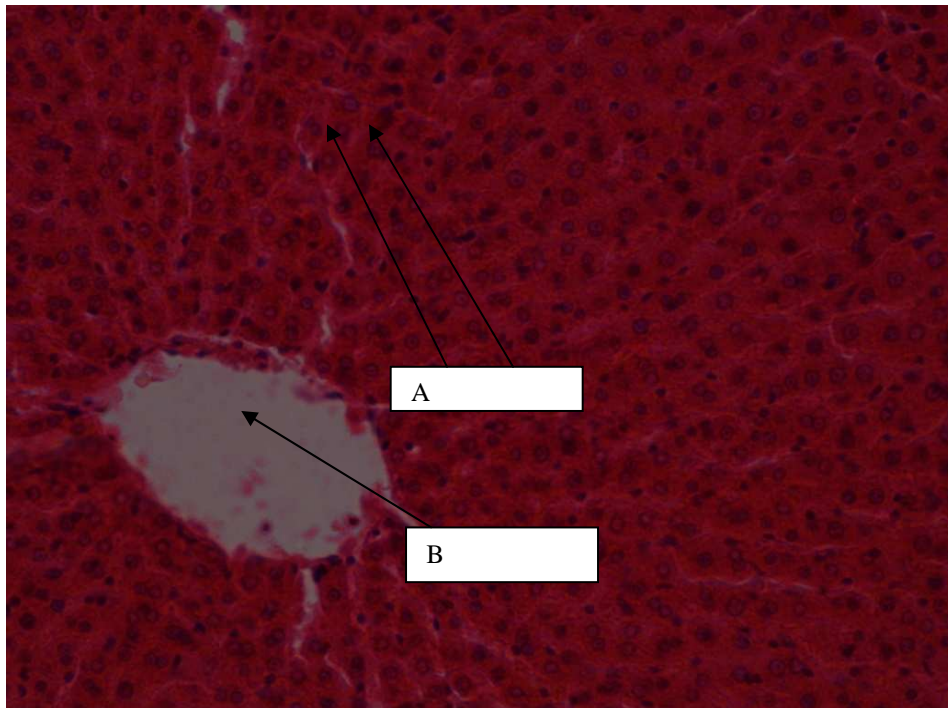
Figure 3.4: A photomicrograph of the liver of a rat injected intraperitoneally with 2000 mg/kg body weight extract. Note the normal histoarchitecture and columnar layers of hepatic cells (A) can be seen radiating from the central portal vein (B) (×100, H & E).
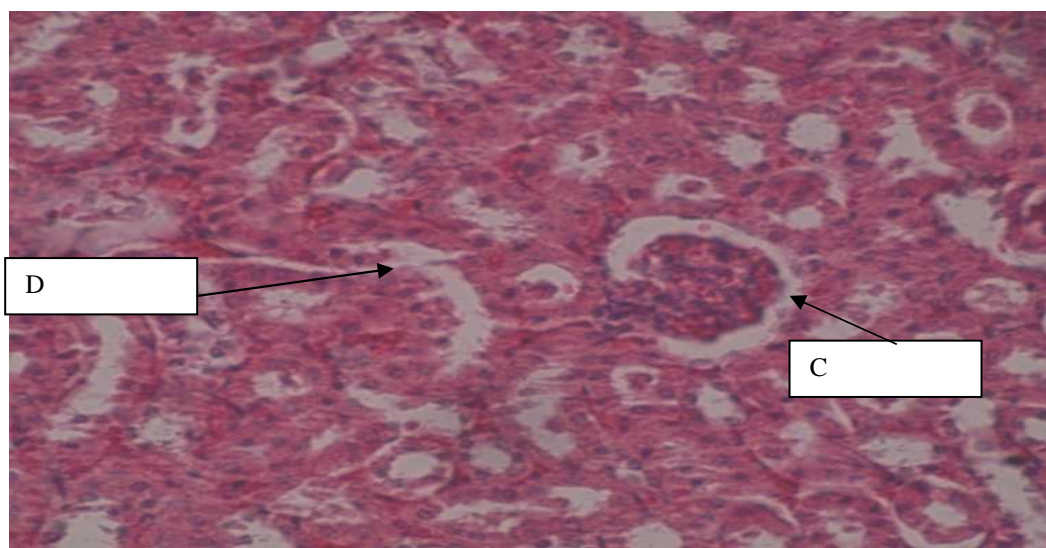
Figure 3.5: A photomicrograph of the kidney of a rat injected intraperitoneally with 2000 mg/kg body weight of extract. Note the normal histoarchitecture; the Bowman's capsule (C) and the renal tubules (D) are intact (×400, H&E).

3.9.3. Sub-acute toxicity

3.9.3.1. Clinical signs

All the animals in 100 mg/kg, 300 mg/kg and 1000 mg/kg dose categories did not exhibit any abnormality throughout the 28 days oral administration of *Teclea trichocarpa* root bark extract. The only transient clinical signs that were most pronounced at 1000 mg/kg and lasted for about 30 minutes included raised fur, fast rates of respiration and rubbing at the oral cavity indicating irritation. The animals looked dull and inactive immediately after dosing but this signs disappeared after a few minutes. The motor functions were normal with no signs of gait abnormality. Before the animals were sacrificed they were placed on examination table for proper observation. The mucous membranes were normal in all animals and there were no noticeable changes to the color of the eyes. All the animals except the control groups defaecated

semi-formed wet droppings/pellet that could not fit the description of outright diarrhoea. All the treatment groups gained weight progressively as compared to the control. There was thus a significant weight difference from week 0 to week 1, week 1 to week 2, week 2 to week 3 and week 3 to week 4. Although there was a moderate increase in weight in all groups, the weight changes in the treatment groups 100 to 1000 mg/kg did not differ significantly. Weight changes between the test groups and control were significantly different, the control group gaining significantly more weight than any of the test groups at each time point. The animals in 100 mg/kg category showed a mean drop in weight during the first week of treatment and this is corrroborated by a negative percentage body weight change. However, this phenomenon could not be explained to be dose-related since the weight at high dosage levels was not affected at this time point (Table 3.2 and Figure 3.6). The control showed a higher increase in weight compared to all the test groups indicating that the extract had some effects either on the feeding patterns or on absorption of nutrients from the gut.

The organ weight indices for various organs remained almost at a constant across the four treatment categories, that is, 1000, 300, 100 mg/kg body weight and in the control group implying that the oral doses tested had little or no impact on the weighed organs. For example, the OWI for the liver ranged between 2.98 to 3.67 across all the treatment groups, a difference of 1.23 units. The OWI for the adrenal glands lay between 0.20 to 0.30 across all the treatment groups, a difference of 0.10 units reflecting a no significant increase ($p = 0.0701$) in all the organs weighed (Table 3.3 and Figure 3.7).

Table 3.2: Effects of the *Teclea trichocarpa* root bark extract on mean body weight (g) of rats.

Dosage mg/kg b.wt.	Mean weights at				
	Week 0	Week 1	Week 2	Week 3	Week 4
1000	184 ±10.7	186.6 ± 10.9	196.3 ± 10.2	202.5 ± 11.2	209.9 ± 10.7
300	177.1 ± 6.9	189 ± 7.6	187.6 ± 9.4	199.7 ± 9	212 ± 7.7
100	190.4 ± 9	186 ± 11.4	208.1 ± 9.9	211.9 ± 10	221.3 ± 8.8
Control	174.3 ± 7	190.5 ± 10.2	203.3 ± 7.9	225.5 ± 4.2	235.7 ± 3.3

The values are expressed as mean ± standard error of the mean (SEM), one way analysis of variance (ANOVA), n = 10. Significant difference from pre-treatment means and control mean ($p<0.05$).
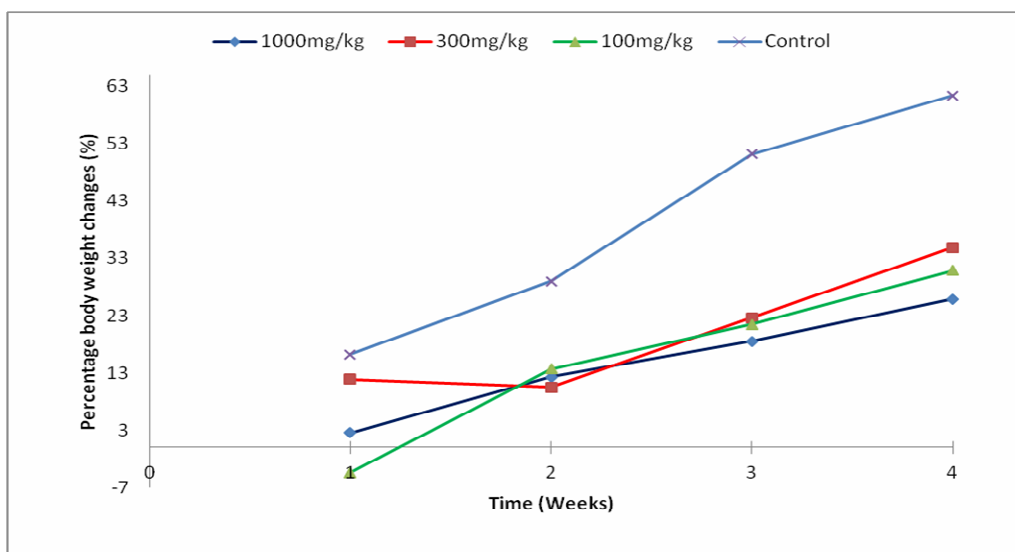
Figure 3.6: Percentage Body weight changes in rats treated with *Teclea trichocarpa* root bark extract in respect to weight at week 0. (Weight gain noted as from week 1 onward)

Table 3.3: Effects of *Teclea trichocarpa* root bark extract on actual weights and organ weight indices of rats.

Dosage	1000 mg/kg		300 mg/kg		100 mg/kg		Control	
Organ	Actual mean weight (mg)	OWI	Actual mean weight (mg)	OWI	Actual mean weight (mg)	OWI	Actual mean weight (mg)	OWI
Liver								
	7600 ± 710	3.62	7130 ±540	3.36	7800 ±670	3.67	7040 ±280	2.98
Kidney								
	1290 ±09	0.61	1. 22 ±90	0.57	1.24 ±80	0.58	1.40 ±540	0.59
Adrenals								
	50 ± 10	0.02	60 ±10	0.03	60 ±0	0.03	60 ±10	0.03
Heart								
	630 ±20	0.30	570 ±40	0.28	590 ±20	0.26	490 ±20	0.21
Spleen								
	740 ±240	0.35	780 ±90	0.37	800 ±40	0.36	770 ±30	0.33
Thymus								
	240 ±30	0.11	240 ±30	0.11	290 ±30	0.13	180 ±10	0.08
Testis								
	1680 ±280	0.80	2000 ±260	0.90	1690 ±760	0.78	2090 ±120	0.89
Ovaries								
	100 ±20	0.04	110 ±10	0.05	120 ±10	0.05	70 ±10	0.03

OWI = Organ Weight Index. These values are expressed as mean ± standard error of the mean (SEM).
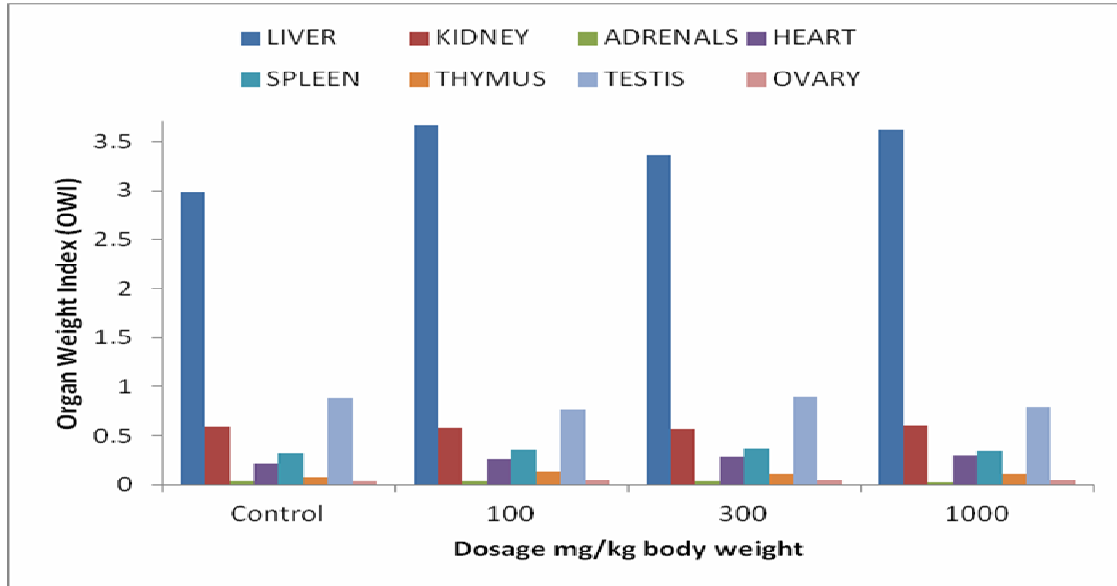
Figure 3.7: The effects of the *Teclea trichocarpa* root bark extract on rat's organ weight index (OWI)

3.9.3.2. Gross pathological changes

The *Teclea trichocarpa* root bark extract did not induce any notable pathological changes in rats at the highest dosage level tested. The gross pictures of the internal organs such as the liver, lungs, kidney, adrenal glands and the gastrointestinal track were normal in both colour and architectural appearances. One animal in the 100 mg/kg per body weight category however had an abscess in the one half of the spleen. The animal did not look sick and this was considered as incidental findings.

3.9.3.3. Histopathological changes

The gross histopathology of all organs was noted to be normal.

3.9.3.4. Haematological effects

The haematological variations during the 4 weeks of sub-acute testing in rats are summarized in the table on Table 3.4. The extract caused a modest increase in RBC count at dosages 100, 300 and 1000 mg/kg compared to the control. However, this increase was neither dose nor time related. There was a slight significant difference in RBC values ($p<0.0001$.) at dose 1000 mg/kg and the control in the fourth week at which point RBC dropped significantly below the control values dropped. All the treatment groups and the control had a gradual rise in haemoglobin, then a slight fall during week 2 to 4 but the levels remained higher than those taken before treatment. The variations between treatment groups were not significant in all time points. Red blood cell levels (Figure 3.8) and haemoglobin levels (Figure 3.9) showed an almost common trend. PCV levels did not show any significance difference between all the treatment groups and the controls. The values for immature RBC in doses 100, 300 and 1000 mg/kg were below a digit whereas that for control was zero (Table 3.4). There was dose unrelated fluctuations in the levels of MCH in all treatment groups with that of control experiencing a moderate fall from week 1 to week 4. In 1000 mg/kg category, the MCH level fell during week 1 to 2 then rose steadily during week 3 to 4.

Table 3.4: The overall cumulative effect of the *Teclea trichocarpa* root bark on haematological values in rats.

Dosage level	WBC (/µL)	RBC (×10^6 /µL)	PCV %	Haemoglobin (g/dL)	MCV (fL)	MCHC (g/dl)	Thrombocytes (×10^3/µl)	Total Neutrophils (%)	Mature Neutrophils (%)	MCH (pictogram)	Lymphocyte (%)	Immature Neutrophil (%)	Eosinophil (%)	Monocyte (%)	Basophil (%)	Nucleated RBC
1000 mg/kg	24057 ±3170	6.63 ±0.4	40.16 ±2.2	15.84 ±0.4	60.9 ±1.1	39.95 ±2.6	433.3 ±46.1	22.7 ±4.7	22.6 ±4.7	23.3 ±4.2	77 ±2.3	0.1	0	0	0	0.8
300 mg/kg	24122 ±3553	6.9 ±0.2	42.2 ±1	15.6 ±0.2	61.4 ±0.8	37.1 ±0.9	448.5 ±55.5	28.8 ±5.5	28.8 ±4.3	28.4 ±4.7	70 ±2.1	0	0	0	0	0.1
100 mg/kg	12112 ±5410	6.6 ±0.3	39.6 ±2.1	15.5 ±0.7	60.3 ±1	39.5 ±1.3	415.6 ±55.4	25.2 ±4.3	25 ±4.3	23.8 ±3.8	74.4 ±2.4	0.2	0	0	0	0.3
Control	28092 ±4016	6.7 ±0.2	41.4 ±1	16 ±0.3	61.6 ±1.2	38.6 ±0.9	430 ±30.9	25 ±4.2	25 ±4.2	23.7 ±3.3	73.3 ±2.0	0	0	0	0	0

Significant difference from the control mean ($p < 0.05$), one way ANOVA followed by Student t test
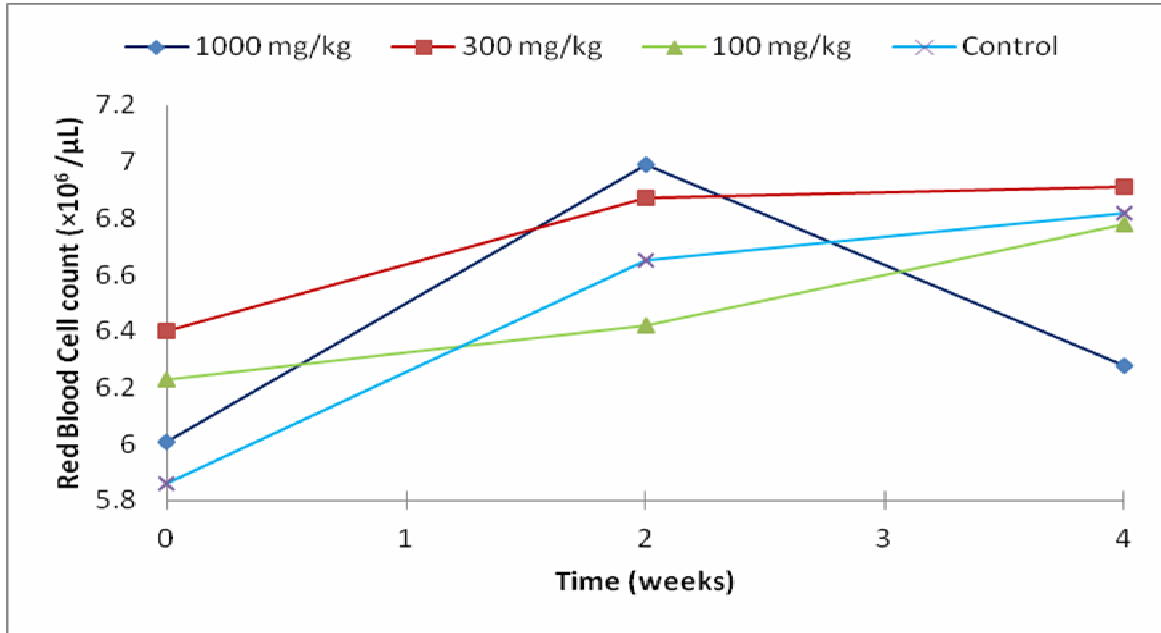
Figure 3.8: Mean RBC count level in rats treated with different concentrations of *Teclea trichocarpa* root bark extract.

Figure 3.9: Mean haemoglobin concentration levels in rats treated with different concentrations of *Teclea trichocarpa* root bark extract.
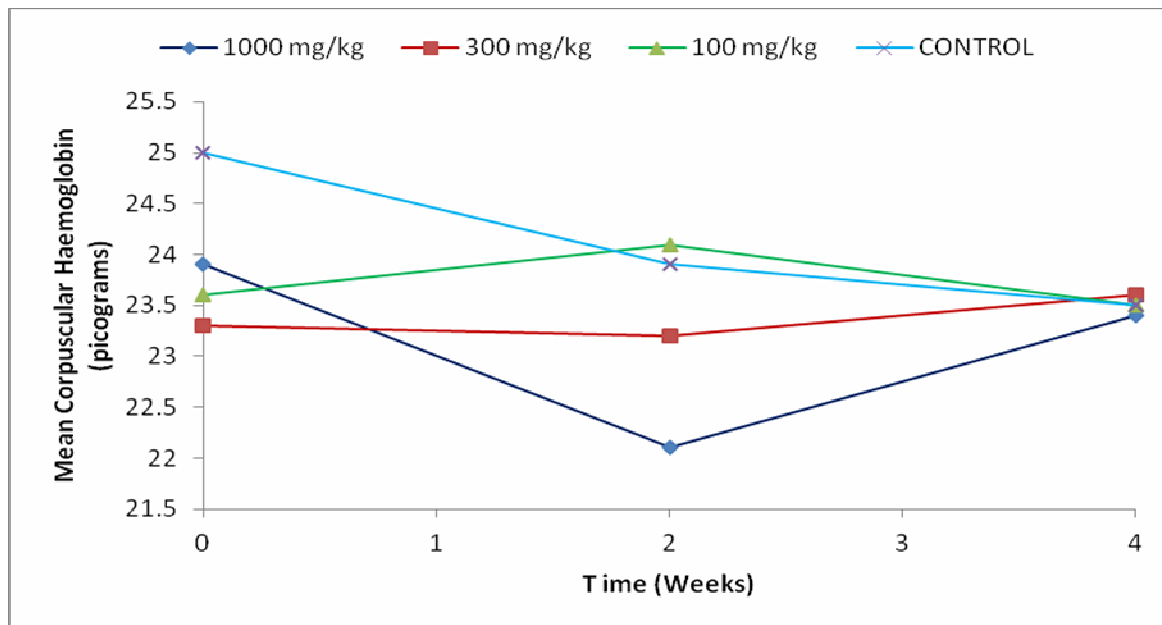
Figure 3.10: Mean corpuscular haemoglobin (MCH) level in rats treated with different concentrations of *Teclea trichocarpa* root bark extract.

The MCHC values fluctuated slightly around 40 g/dL in all dosage levels and the control and then showed a non-dose related variation (rise) at 1000 mg/kg and a fall at dose 300 mg/kg but remained steady at doses 100 mg/kg and control values between week 2 and 4 (Figure 3.11).
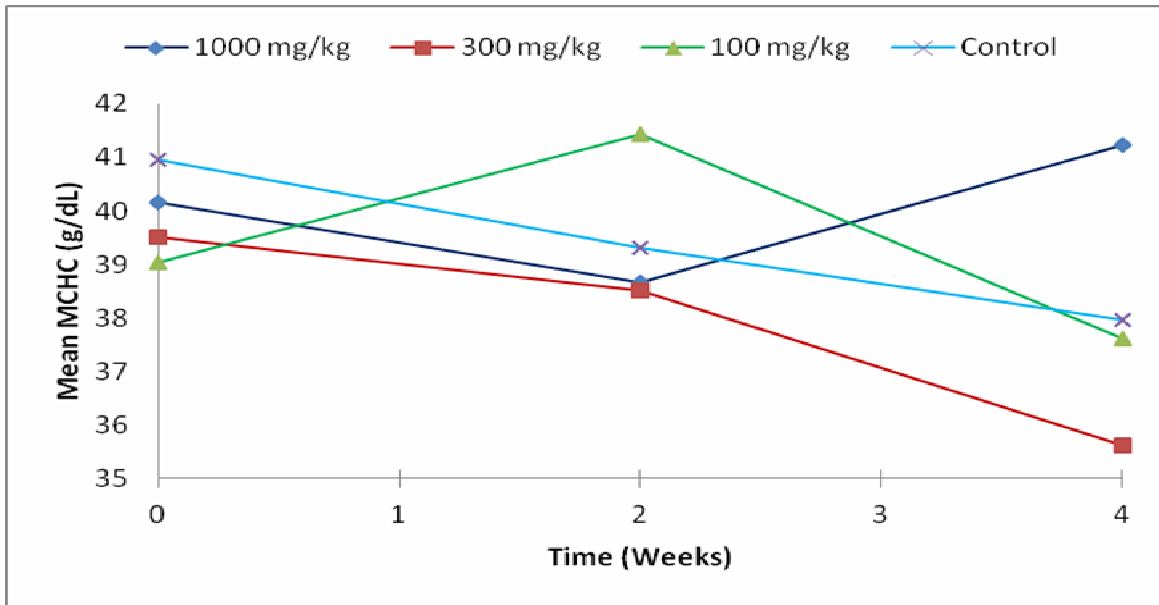
Figure 3.11: Mean corpuscular haemoglobin concentration levels in rats treated with different concentrations of *Teclea trichocarpa* root bark extact

Thrombocyte levels experienced a gradual increase in all the treatment groups together with their controls but these trends were not significantly different from the control and between treatment and at different times (Figure 3.12). There was a significant difference ($p<0.001$) in the levels of thrombocyte at 1000 mg/kg treatment group and in the levels of WBC in the 300 and 100 mg/kg categories.
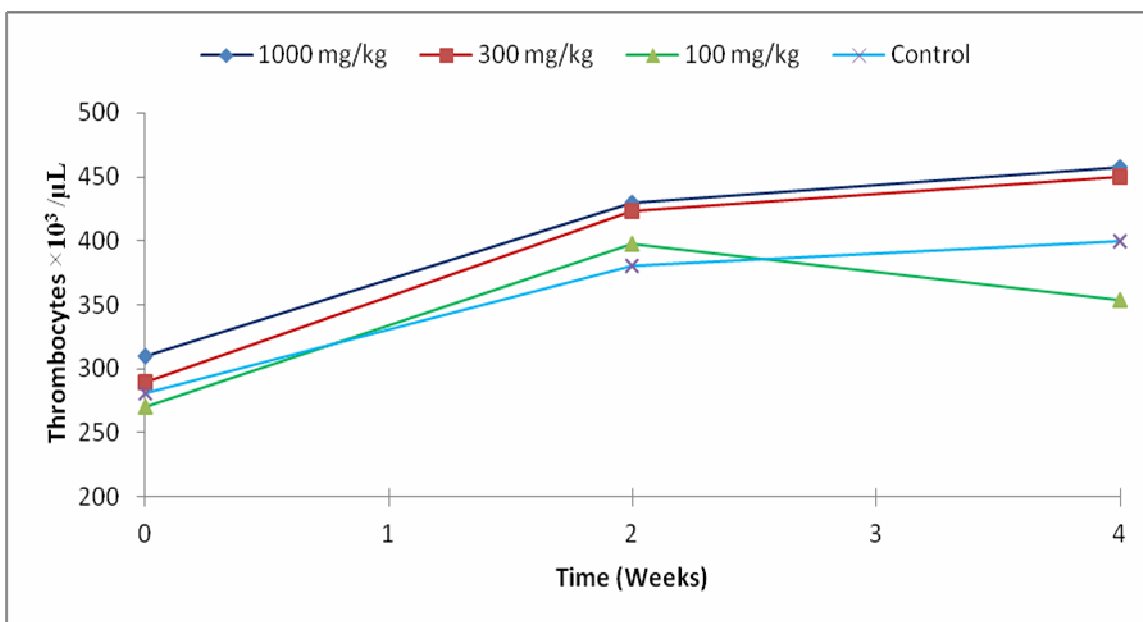
Figure 3.12: Mean thrombocytes count levels in rats treated with different concentrations of *Teclea trichocarpa* root bark extract.

The WBC values at dosage levels of 300 and 1000 mg/kg remained steady around 25,000 cell/μL between week 2 and 4 (Figure 3.13). The increase in WBC values at 100 mg/kg compared closely with that of the control group between week 2 and 4 with the control group showing a slight increase above those values at dosage 100 mg/kg. WBC levels experienced a drop in week 0 to week 2 and then a small rise until week 4 (Figure 3.13). The high WBC levels for dosage 100 and 300 mg/kg at the beginning of the experiment could have been due to laboratory errors or individual animal factors since they could not be related to extract administration.
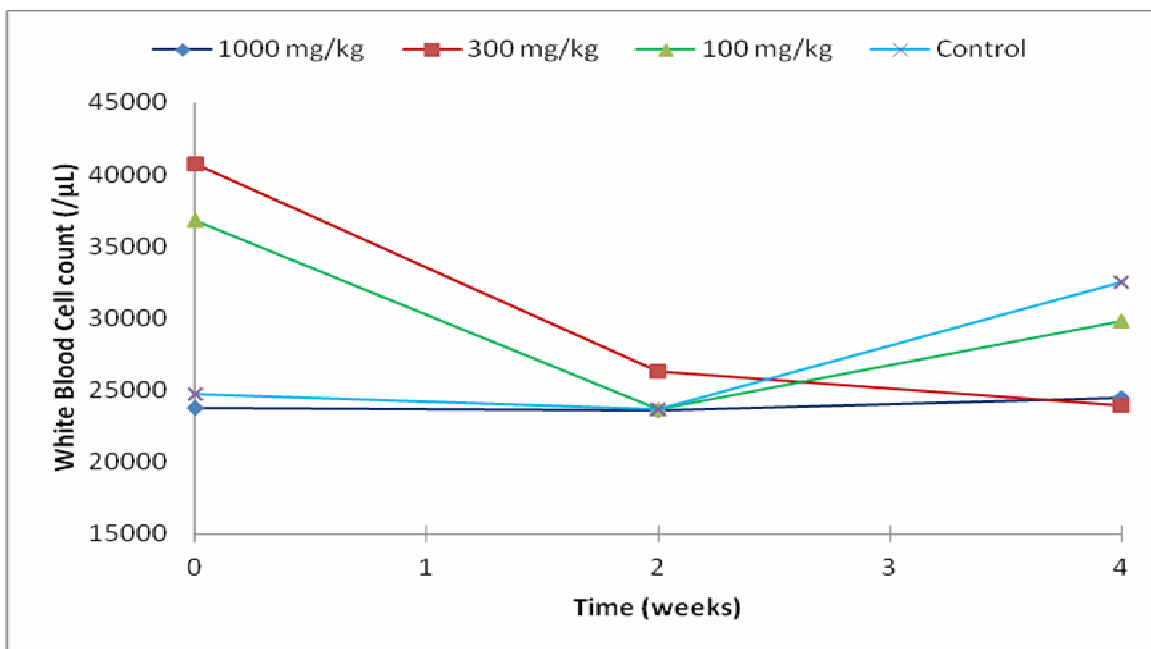
Figure 3.13: Mean white cell (WBC) count levels in rats dosed with different concentrations of *Teclea trichocarpa* root bark extract.

In general, the lymphocyte levels remained almost a constant in all treament groups, fluctuating between 65 and 75%. The was no noticable change in lymphocyte level in control group, but there was a slight increase in 300 and 1000 mg/kg categories. The levels in 100 mg/kg group fell slightly from week 1 to 2 then rose from week 3 to 4 (Figure 3.14).
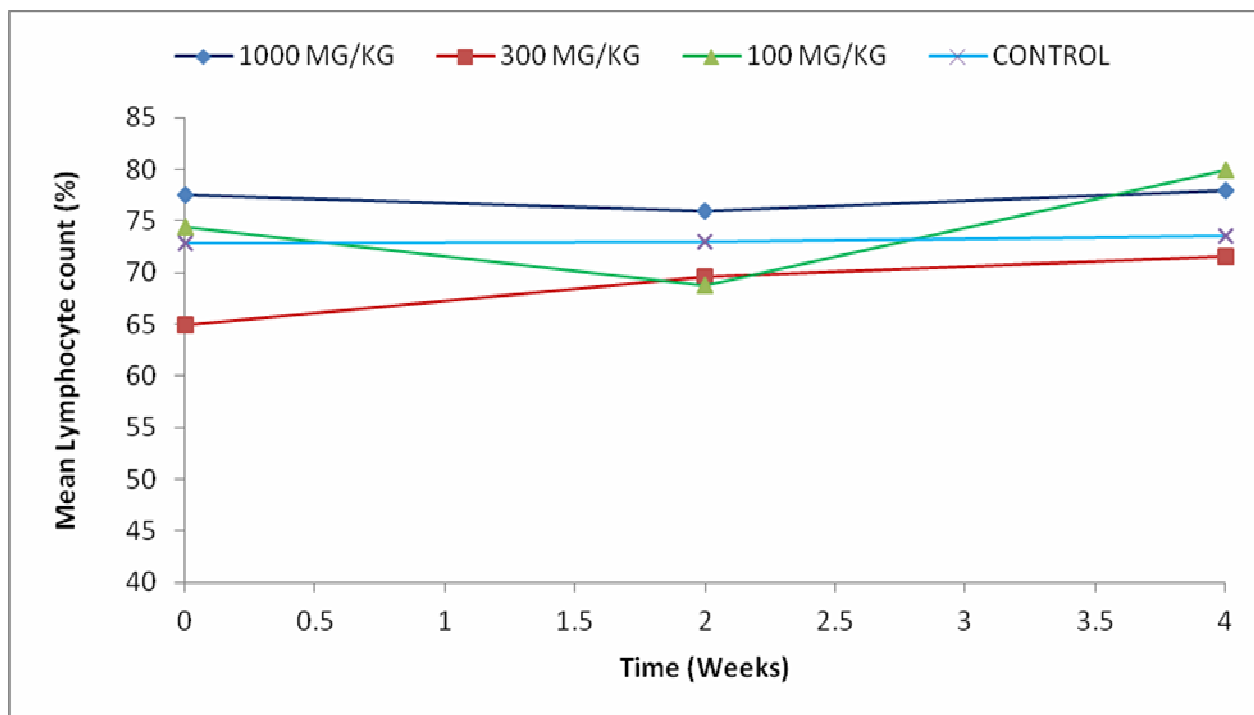
Figure 3.14: Mean lymphocyte level in rats treated with different concentrations of *Teclea trichocarpa* root bark extract.

There was a non-dose related slight MCV increase in all treatment groups between week 2 to 4 with control almost remaining as a constant. However, the level in 100 mg/kg category experienced a drop from week 1 to 2 followed by a rise during week 3 and 4 (Figure 3.15).
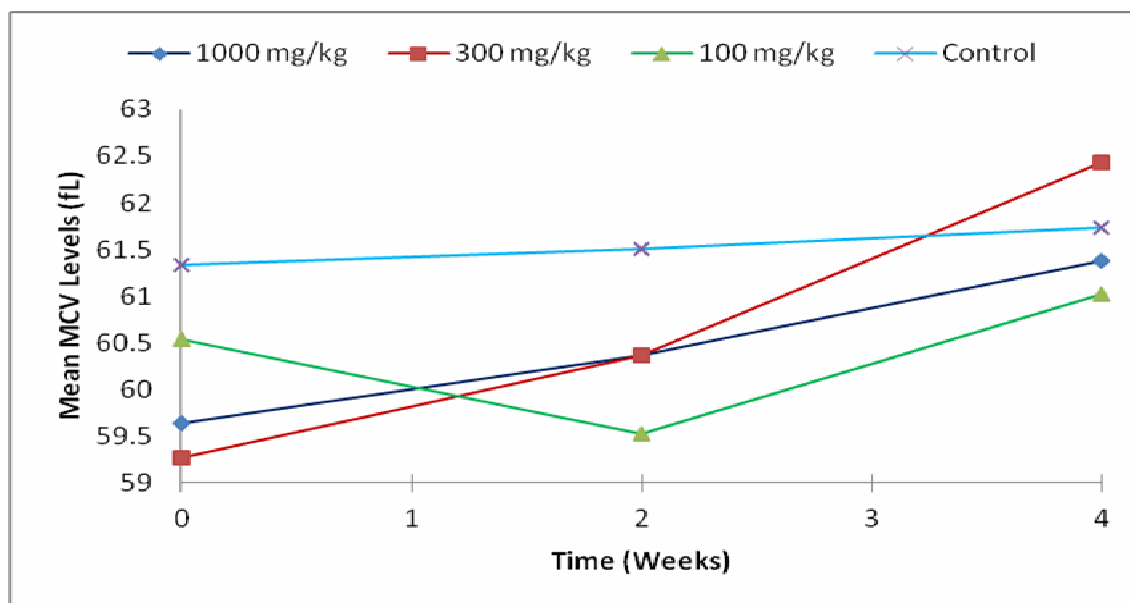
Figure 3.15: Mean corpuscular volume (MCV) level in rats dosed with different concentrations of *Teclea trichocarpa* root bark extract.

Total neutrophils and mature neutrophils levels did no exhibit any appreciable differences amongst all the groups. Their values fluctuated between 22.5 and 34% in all treatment groups. The percentage of both total and mature neutrophils in the control remained a constant (Figure 3.16 and 3.17 respectively). The values were a constant during week 1 to 2 in 100 mg/kg category but there was a slight reduction during week 3 to 4. In 300 mg/kg group the values fell steadily throughout the treatment period. In 100 mg/kg category, there was a small increase followed by a decrease during week 1 to 2 and week 3 to 4 respectively. The percentage of immature neutrophil levels was negligible, zero at both 300 mg/kg and control categories and 0.2 and 0.1% in 100 and 1000 mg/kg respectively.
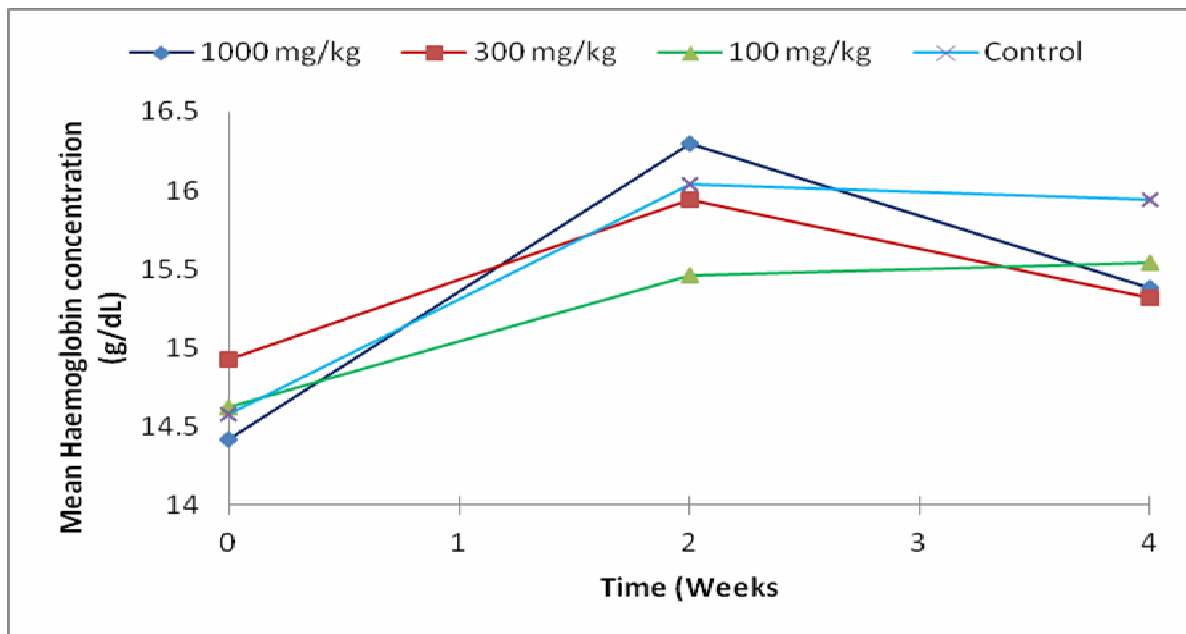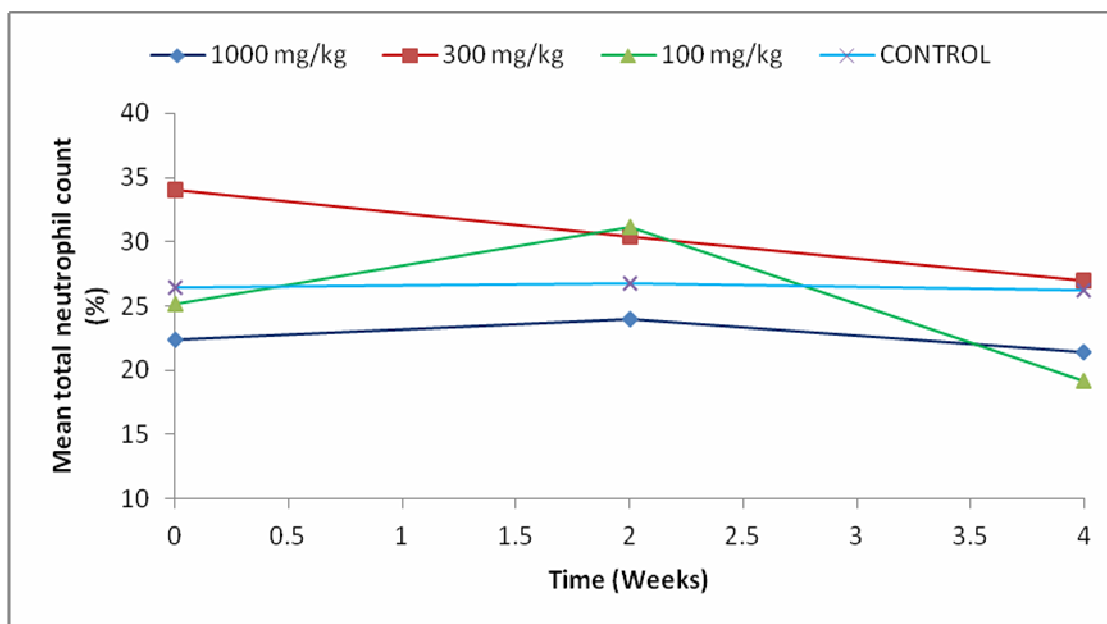
Figure 3.16: Mean total neutrophil level in rats treated with different concentrations of *Teclea trichocarpa* root bark extract.
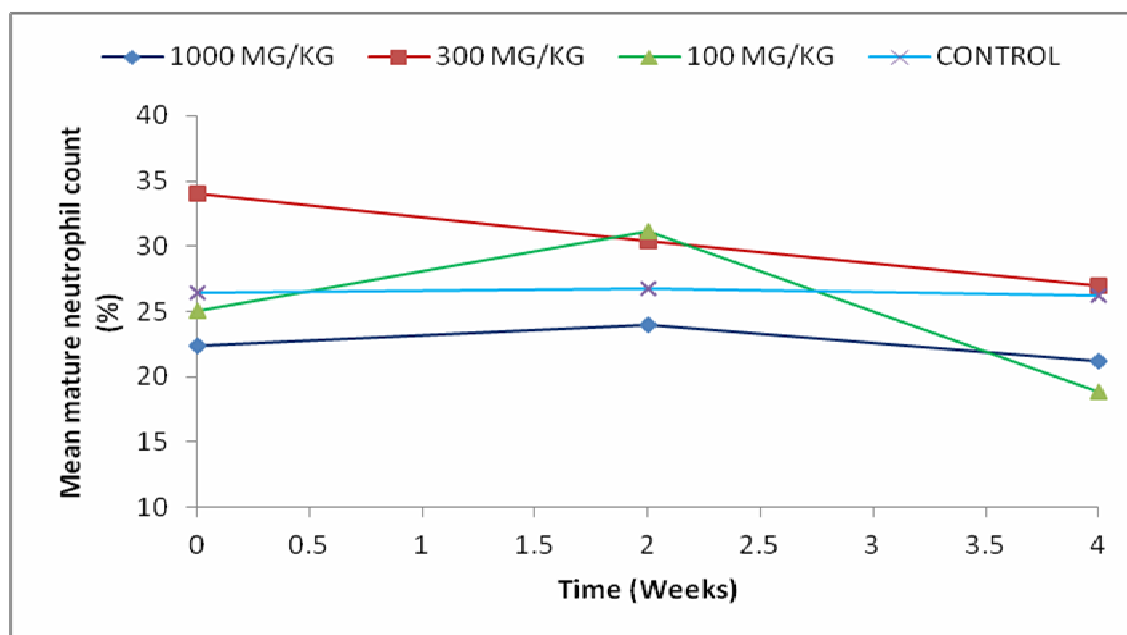
Figure 3.17: Mean mature neutrophil level in rats treated with different concentrations of *Teclea trichocarpa* root bark extract.

3.9.4. Clinical chemistry parameters

The effects of the *Teclea trichocarpa* root bark extract on clinical chemistry parameters are summarized in Table 3.5a, 3.5b and 3.6 below.

Table 3.5a: Effects of dichloromethane-methanol (1:1) extract of *Teclea trichocarpa* root bark on the clinical chemistry in rats.

Dosage(mg/kg body wt)	Total protein level (g/dL)			Mean Albumin levels (mg/dl)			Mean AST levels IU/L		
	Wk 0	Wk 2	Wk 4	Wk 0	Wk 2	Wk 4	Wk 0	Wk2	Wk 4
1000	6.8	7.2	7.2	3.1	2.9	3.9	23.6	14.2	7.5
	± 0.8	± 1	± 1	±0.2	± 0.2	± 0.2	± 12.1	± 3.1	± 5.5
300	7.3	7.1	7.2	3.5	3.2	4.1	16.2	9.8	4
	± 1.2	± 0.6	± 1.1	± 0.2	± 0.1	± 0.2	± 7.3	± 3.3	± 1.2
100	7.5	7.4	7.2	3.4	2.7	4	9.2	5.6	2
	± 1.5	± 0.9	± 1.1	± 0.2	± 0.2	± 0.1	± 3.6	± 1.9	± 1
Control	6.9	7.2	7.4	3.4	2.7	3.8	13.8	6	12.6
	± 0.5	± 0.4	± 0.7	± 0.1	± 0.3	± 0.2	± 3.5	± 2.7	± 5.6

Table 3.5b: Effects of dichloromethane-methanol (1:1) extract of *Teclea trichocarpa* root bark on the clinical chemistry in rats.

Dosage(mg/kg body wt)	Mean ALT levels (IU/L)			Mean CPK levels (IU/L)			Creatinine (g/dl)		
	Wk 0	Wk 2	Wk 4	Wk 0	Wk 2	Wk 4	Wk 0	Wk 2	Wk 4
1000	11.8	4.2	4.8	89.7	23.6	77.4	0.5	0.4	0.5
	± 6.9	± 0.6	± 1.0	± 17.3	± 3.6	± 27.6	± 0.07	± 0.03	± 0.08
300	12	9.6	8.5	75.7	22.6	55.4	0.4	0.4	0.4
	± 4.1	± 2.9	± 3.1	± 13.5	± 7.6	± 8.2	± 0.03	± 0.04	± 0.03
100	8.4	6.2	6.5	88.5	19.5	72.3	0.4	0	0.2
	± 2.7	± 1.0	± 12.6	± 12.5	± 4.3	± 7.1	± 0.04	± 0.04	± 0.04
Control	18	6.7	12.6	93	23.4	71.6	0.4	0.4	0.5
	± 3.4	± 2.7	± 3.8	± 10	± 3.9	± 14.7	± 0.03	± 0.03	± 0.13

Table 3.6: Overall cumulative effect of dichloromethane-methanol (1:1) extract of *Teclea trichocarpa* root bark on clinical chemistry parameters in rats.

Dosage of extract mg/kg	Clinical chemistry parameter					
	Total protein g/dL	Albumin g/dL	AST (iu/L)	ALT ()iu/L	CPK (iu/L)	Creatinine g/dL
1000	7.2	3.4	10.1	4.4	50.5	0.5
300	7.2	3.7	6.9	9.1	78	0.4
100	7.5	3.7	6.9	9.1	78	0.4
Control	7.3	3.3	9.3	9.7	47.5	0.5

During the treatment of laboratory rats with the extract, there was a small dose un-related change in protein levels during week 0 to week 2. The protein levels for dosage 1000 mg/kg and the control increased by less than half a unit whereas those for dosage 100 and 300 mg/kg reduced the same margin. From week 2 to week 4, the levels of protein in all treatment groups increase by a minute value. Overall, the changes in protein levels in all treatment groups and the control did not change significantly from week to week and amongst the groups (Figure 3.18).
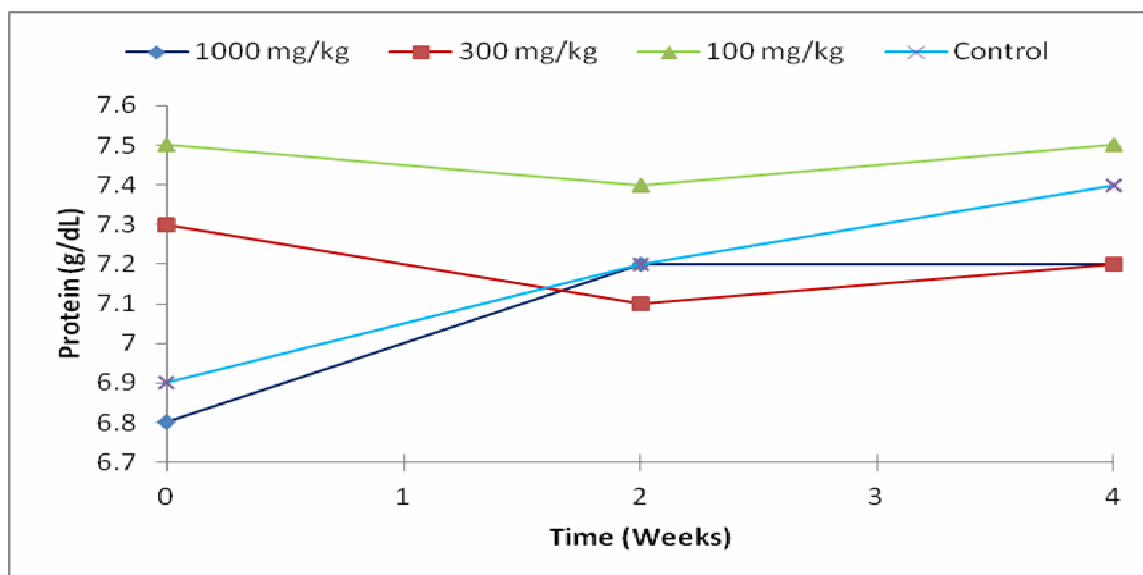
Figure 3.18: Protein level in rats dosed with different concentrations of *Teclea trichocarpa* root bark extract.

All the dosage levels of the extract and the control showed similar trends in albumin concentration that was closely similar to those in control group with values in all groups and the control fluctuating around 3.5 g/dL from weeks 2 to 4 (Figure 3.19). There was therefore no significant difference in albumin levels between all the treatment groups and the control. This corroborates effects of the extract to the trend of the total protein levels during the 4 weeks of treatment.
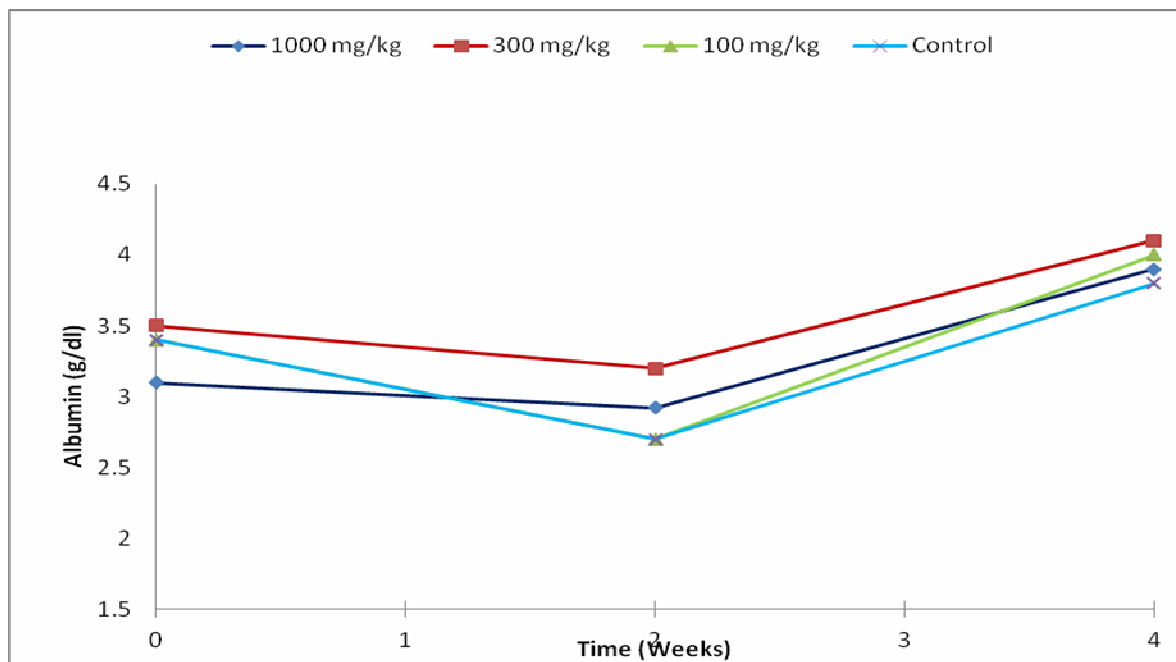
Figure 3.19: Albumin level in rats dosed with different concentrations of *Teclea trichocarpa* root bark extract.

Aspartate aminotranferase (AST) levels dropped steadily from week 1 to week 4 but levels in control group rose again moderately between weeks 2 and 4. The drop in AST was dose related, highest in 1000 mg/kg and lowest in 100 mg/kg body weight. . There was therefore a significant difference (p<0.0021) in all treatment groups as compared to the control. The decreasing trend in AST levels predicted that any continued dosing of the animals beyond week 4 could cause further decrease in the levels of AST (Figure 3.20).
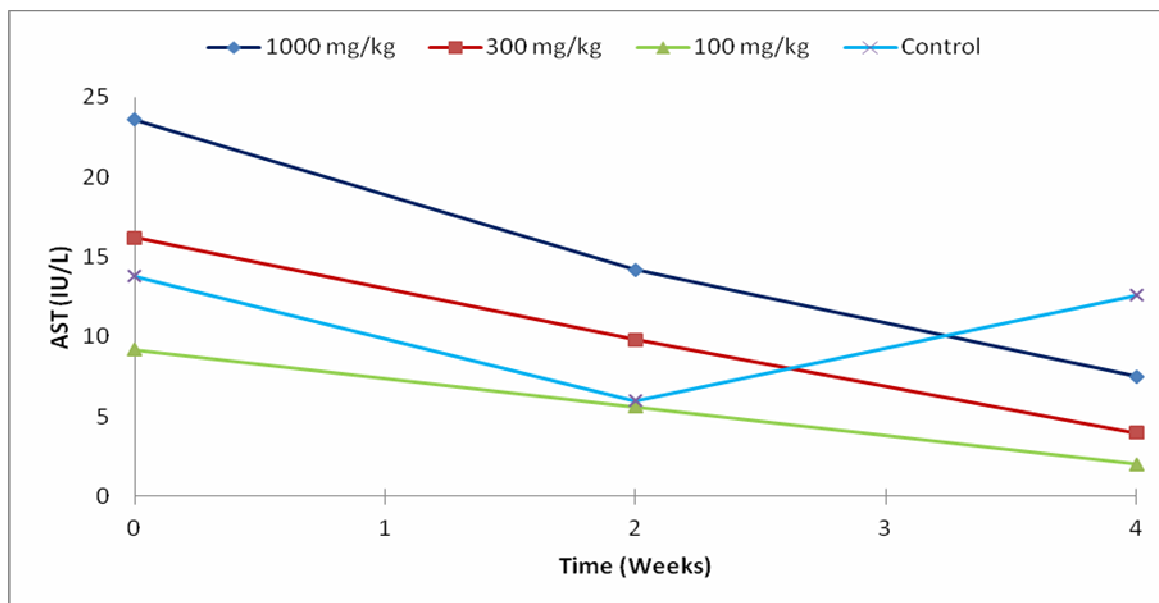
Figure 3.20: Aspartate aminotransferase level in rats dosed with different concentrations of *Teclea trichocarpa* root bark extract.

Alanine aminotransferase (ALT) levels also showed a decreasing trend as compared to AST levels. The level of ALT in the control group decreased in week 1 and 2 then rose gradually between weeks 2 to 4 similar to AST. ALT levels had less decreasing rate as compared to AST levels, and unlike in AST, ALT the almost leveled from week 2 to 4 (Figure 3.21).
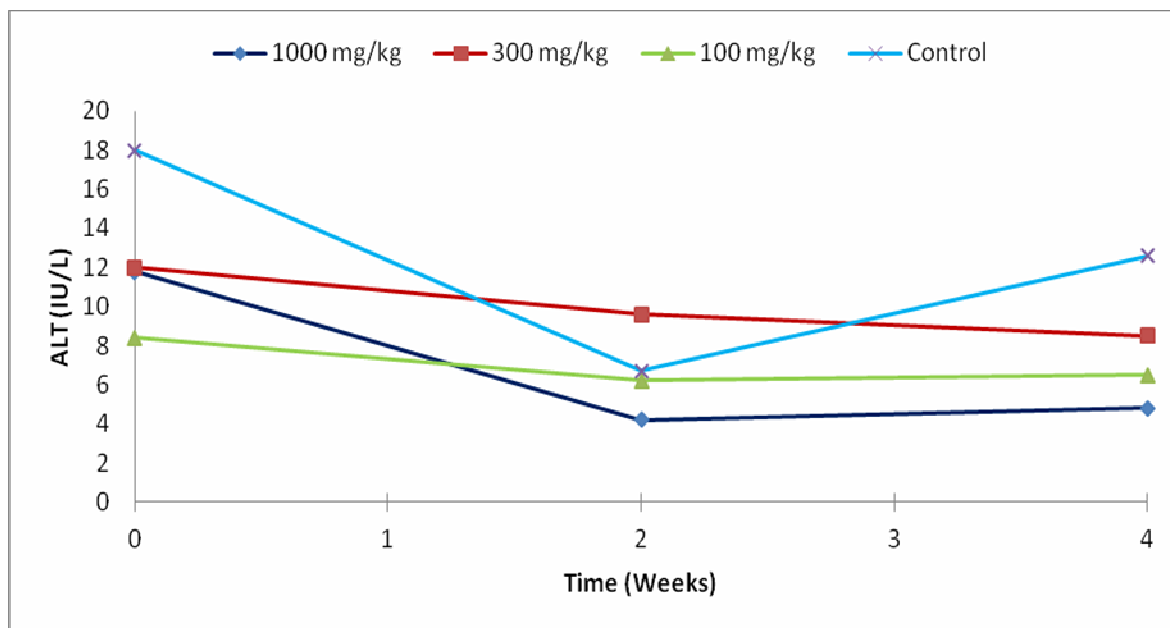
Figure 3.21: Alanine aminotranferase level in rats dosed with different concentrations of *Teclea trichocarpa* root bark extract.

The levels of creatinine phosphokinase (CPK) experienced a rapid drop from the pre-treatment level to the levels in week 2, and then a rise in week 2 to 4. The trend in CPK for all the 3 test groups was very close at all dose levels and the control (Figure 3.22). The drop and rise in the levels of CPK was quite significant though within the normal range which is wide for this parameter.
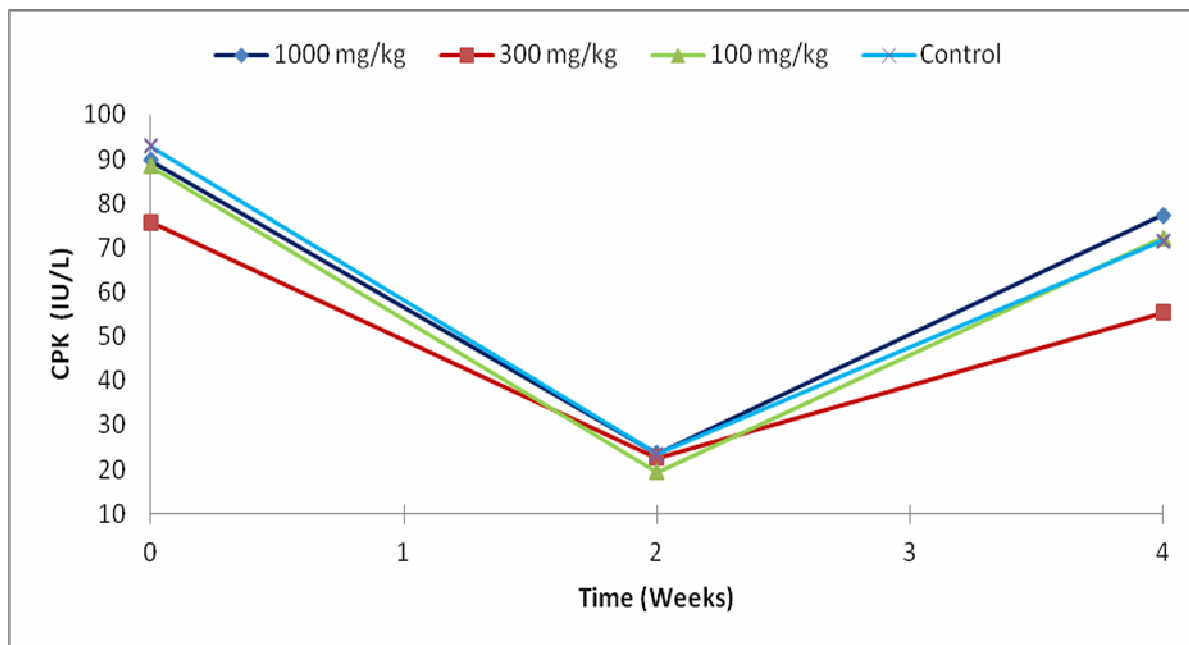
Figure 3.22: Creatine phosphokinase level in rats dosed with different concentrations of Teclea trichocarpa root bark extract

The values of creatinine for the rats in 100 and 300 mg/kg and categories and in the control were the same during the first two weeks and remained at a constant but during week 2 to 4, those for control started rising steadily matching the values for the 400 mg/kg during the same period. The levels for 100 mg/kg dropped steadily whereas those for 300 mg/kg continued to be in constant state during the same period. There was an initial drop in the levels of creatinine during the first two weeks followed by a steady rise up to week 4. Creatinine levels remained almost constant throughout the treatment period (Figure 3.23) within 0.2 to 0.5 g/dl levels.
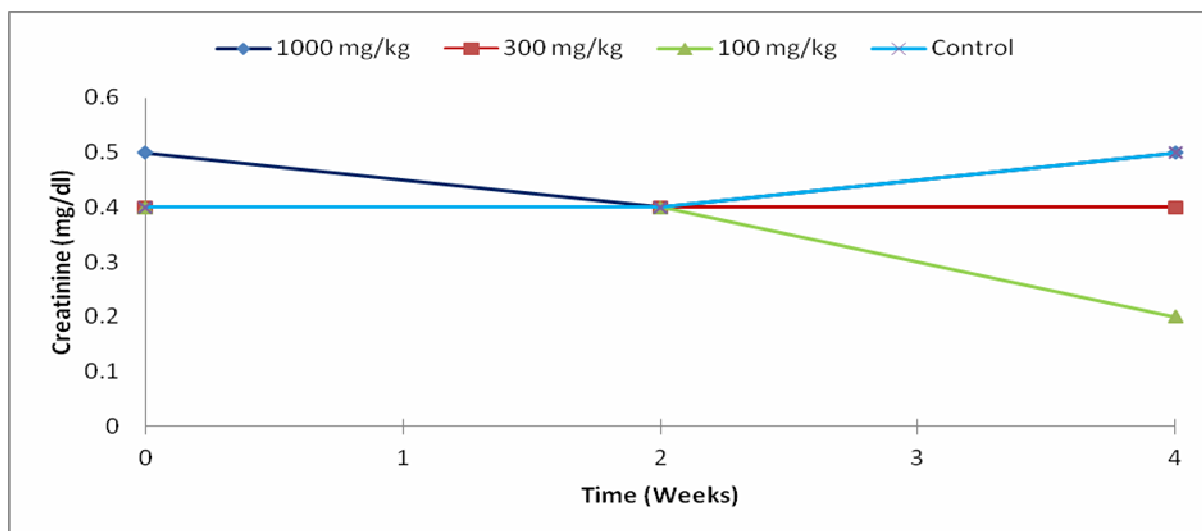
Figure 3.23: Creatinine level in rats dosed with different concentrations of *Teclea trichocarpa* root bark extract.

3.9.3.5. Major findings from toxocoty studies

The oral dose of 2000 mg/kg body weight did not cause death to laboratory rats and there was an increase in body weight during treatment at the high oral dose. The data collected on organ weight was used to calculate the organ weight index, (OWI) for each organ harvested. According to Society of Toxicologic Pathology (STN), the preferred organs include the liver, heart, kidney, brain, adrenal glands and the testis. The organ weight indices for various organs remained almost a constant across the various dosage levels and in the control groups. The pathological picture observed during toxicity studies is the best indicator of the harm done to a particular organ by the chemical. The liver is usually the first casualty during oral dosing because all chemicals pass through it once absorbed from the gut and undergo first pass metabolism (Frank and Robert, 2005). The liver enzymes especially the ALT and AST values were actually declining and no increases in these parameters were noted. The haematological parameters tested did not show any significant variations associated with toxicity to haemopoietic organs. The modest variations

noted in any parameter were well within the normal biological range since none of the parameter increased or decreased significantly in relation with the control values at all dosage levels. There was a small non-dose related increase in the mean haemoglobin levels and thrombocytes count with time in all treatment levels and the control. In a situation whereby the the haemopoietic organs are affected by the toxic agents, the blood film shows an elevated levels of immature neutrophils and nucleated RBC.

3.9.3.6. Conclusion and recommendation

The results showed that no death in laboratory rats occurred at an oral dose of 2000 mg/kg body weight suggesting that the dichloromethane-methanol extract of *Teclea trichocarpa* root bark is practically non-toxic after oral dose exposure presuming that its components were absorbed. The extract can then be concluded to be safe for oral use as a traditional herbal remedy for the treatment of helminthiasis. This safety margin confirms the reason why most communities in Kenya use it as a traditional herbal remedy without any harmful effects.

The increase in body weight observed during treatment at the high oral dose confirms the non-toxicity of the extract. Organ weight is one of the most sensitive drug toxicity indicators, and it changes often precede morphological changes. The organ weight indices for various organs remained almost a constant across the various dosage levels and in the control groups. This implies that the presence of the extract in the animal and the various oral concentrations used during the test had little or no impact on the particular organs. For example, the OWI for the liver (4.34, 3.75, 3.81 and 3.35) and the adrenal glands (0.04, 0.03, 0.03 and 0.03) at the dosage levels 1000, 300, 100 mg/kg and the control group respectively remained almost at constant. This fact suggests that there could not have been any damage to the organ. Any alterations in liver weight may suggest treatment-related changes including hepatocellular hypertrophy either due to

enzyme induction or peroxisome proliferation (Greaves, 2000). Liver weights may be elevated in studies of less than 7 days duration for potent hepatic enzyme-inducing compounds. Elevated heart weight may be the only evidence of myocardial hypertrophy that is often macroscopically and microscopically difficult to recognize. Changes in kidney weight may reflect renal toxicity, tubular hypertrophy or chronic progressive nephropathy (Greaves, 2000). It has been noted that 7 % of nephrotoxic conditions are related to medication toxicities because kidneys are routinely exposed to high concentrations of medications or metabolites because their intrinsic function is to metabolize, concentrate and excrete compounds (Guo and Nzerue, 2002). The OWI values of the kidney in this study (0.57 to 0.61) are too close to warrant any fear of nephrotoxicity. The histopathology of the kidney during acute testing and the gross pathology at all dosage levels during sub-acute toxicity testing showed no signs of toxicity. Variations in adrenal gland weight which may indicate hypertrophy, hyperplasia, or atrophy of the organ associated with stress, endocrinopathies, or test article effects (Greaves, 2000), did not occur in this study.

Histopathological studies showed that the extract had no harmful effects on the wall of the gastrointestinal tract. Herbal remedies taken orally have been associated with gastrointestinal disturbances. The 1:1 mixture of methanol and dichloromethane extract of *Teclea trichocarpa* did not induce any notable side effects during both acute and sub-acute toxicity testing. Pathological picture observed during toxicity studies is the best indicator of the harm done to a particular organ by the chemical. The liver is usually the first casualty during oral dosing because all chemicals pass through it once absorbed from the gut and undergo first pass metabolism (Frank and Robert, 2005). The importance of investigating the effects of new drugs in the liver cannot be over-emphasized because majority of drugs that cause hepatic injury during pre-clinical studies do not progress to clinical trials or are developed with substantial patient

monitoring (Boone *et al*., 2005). Alanine aminotransferase (ALT) and Aspartate aminotranferase (AST) are the markers of liver function and are only released from cytosol and sub-cellular organelles during cell injury. Alanine aminotransferase is more hepatocellular specific whereas creatinine is an indicator of muscle wasting. Alanine aminotransferase is a critical parameter for identification of potential drug-induced injury in both pre-clinical studies and human patients (Boone *et al*., 2005 and Salawu *et al.,* 2010). It is apparent from the results that the ALT and AST values were actually declining and no increases in these parameters were noted. The decrease in enzymatic parameter was unexpected. It is traced to the fact that during the assays, freeze thawing of the sample was occasioned by workload exigencies. Notably, the non-enzymatic parameters, proteins, albumin and creatinine remained unchanged.

All organs that were sectioned and prepared showed no lesions when observed under the microscope. The 28 days oral toxicity test of the extract therefore had no effects on the cellular integrity of these organs. The fact that the non-enzyme parameters analyzed namely creatinine, protein and albumin was within normal limits corroborates the absence of pathological lesions observed grossly and microscopically. The haematological parameters tested did not show any significant variations associated with toxicity to haemopoietic organs. The modest variations noted in any parameter were well within the normal biological range since none of the parameter increased or decreased significantly in relation with the control values at all dosage levels. There was a small non-dose related increase in the mean haemoglobin levels and thrombocytes count with time in all treatment levels and the control. In a situation whereby the the haemopoietic organs are affected by the toxic agents, the blood film shows an elevated levels of immature neutrophils and nucleated RBC. Absence of such a phenomenal corroborates the fact that the extract was not toxic the rats.

CHAPTER FOUR: GENERAL DISCUSSION AND CONCLUSIONS

Lack of Pharmacological and toxicological knowledge on herbal medicine has direct limitation on the usage of these products. Man in his effort to find an alternative remedy has turned to indiscriminate use of herbs without paying attention to their possible toxicity levels. This is usually encouraged by the belief that 'natural' is synonymous to 'harmlessness' (Bandaranayake, 2006). The World Health Assembly in resolution WHA 31.33 of 1978, WHA 40.33 of 1987 and WHA 42.43 of 1989 has emphasized the need to ensure the quality of medicinal plant product and in their resolutions they describe a series of tests for assessing the quality of such products (Bandaranayake, 2006).

Prudent use of rodents in toxicity studies is recommended in various guidelines (OECD, 1998, 2001 & 2008). A study carried out on the concordance of the effects of pharmaceuticals in human and experimental animals showed the true positive human toxicity concordance rate of 71 % for rodent and non-rodent species. With non-rodents alone, concordance rate was predictive for 63 % of human toxicities and rodents alone for 43 %. The highest incidence of overall concordance was seen in hematological, gastrointestinal, and cardiovascular human toxicities, and the least was seen in cutaneous human toxicity (Olson *et al*., 2000). The parameters from these body systems were targeted during this study.

Brine shrimp lethality test is highly recommended because it is a convenient preliminary toxicity test since brine shrimp is highly sensitive to several chemical substances. Hence this *in vitro* assay method is considered a useful tool for preliminary toxicity assessment of plant extracts (Lachumy *et al*., 2010). Compounds that are bioactive are often toxic to *Artemia salina*, the

shrimp larvae, and therefore brine shrimp lethality test is used to monitor lethality of different chemicals to shrimp larvae. It has also been noted that *Artemia salina* toxicity test results have a correlation with rodent and human acute toxicity data (Arslanyolu and Erdemgil, 2006). The dichloromethane-methanol (1:1) extracts of 5 plants (constituting of 14 plant parts) that are used as traditional medicines in Kenya were evaluated for brine shrimp lethality. Literature indicates that extracts showing LC$_{50}$ < 1000 µg/ml during BSL test are considered biologically active (Meyer et al., 1982). Therefore, 12 out of the 14 plant part extracts that were used during this study cytotoxic. The results obtained from the study indicated that *Albizia gummifera* pods and *Clotaralia axillaris* twig extracts were practically non-toxic (with LC$_{50}$ >1000 µg/ml). *Teclea trichocarpa* root wood (LC$_{50}$ >500 µg /ml) was non-toxic while *Albizia gummifera* root bark, *Manilkara discolor* root bark and stem bark and *Teclea trichocarpa* stem bark, root and twigs (LC$_{50}$ >100 < 500 µg /ml) had very low toxicity. *Teclea trichocarpa* root bark and *Zanthoxylum usambarense* stem bark extracts (LC$_{50}$ results between 30 and 100 µg/ml) were mildly toxic to the shrimps. Extracts from *Albizia gummifera* root & stem bark and *Zanthoxylum usambarense* root bark were moderately toxic with LC$_{50}$ < 30 µg/ml.

The incorporation of BSL test during this study simplified the work in that it was possible to zero on the most active extracts and the carry out an intensive work on it and thus aided in saving resources.

The fact that an oral dose of 2000 mg/kg body weight did not cause death to laboratory rat suggest that the dichloromethane-methanol extract of *Teclea trichocarpa* root bark is practically non-toxic after oral dose exposure presuming that its components were absorbed. The extract can then be concluded to be safe for oral use as a traditional herbal remedy for the treatment of

helminthiasis. This safety margin confirms the reason why most communities in Kenya use it as a traditional herbal remedy without any harmful effects.

The increase in body weight observed during treatment at the high oral dose confirms the non-toxicity of the extract. Organ weight is one of the most sensitive drug toxicity indicators, and it changes often precede morphological changes. The data collected on organ weight was used to calculate the OWI for each organ harvested. According to Society of Toxicologic Pathology (STN), the preferred organs include the liver, heart, kidney, brain, adrenal glands and the testis. The organ weight indices for various organs remained almost a constant across the various dosage levels and in the control groups.

This implies that the presence of the extract in the animal and the various oral concentrations used during the test had little or no impact on the particular organs. For example, the OWI for the liver (4.34, 3.75, 3.81 and 3.35) and the adrenal glands (0.04, 0.03, 0.03 and 0.03) at the dosage levels 1000, 300, 100 mg/kg and the control group respectively remained almost at constant. This fact suggests that there could not have been any damage to the organ. Any alterations in liver weight may suggest treatment-related changes including hepatocellular hypertrophy either due to enzyme induction or peroxisome proliferation (Greaves, 2000). Liver weights may be elevated in studies of less than 7 days duration for potent hepatic enzyme-inducing compounds. Elevated heart weight may be the only evidence of myocardial hypertrophy that is often macroscopically and microscopically difficult to recognize. Changes in kidney weight may reflect renal toxicity, tubular hypertrophy or chronic progressive nephropathy (Greaves, 2000). It has been noted that 7 % of nephrotoxic conditions are related to medication toxicities because kidneys are routinely exposed to high concentrations of medications or metabolites because their

intrinsic function is to metabolize, concentrate and excrete compounds (Guo and Nzerue, 2002). The OWI values of the kidney in this study (0.57 to 0.61) are too close to warrant any fear of nephrotoxicity. The histopathology of the kidney during acute testing and the gross pathology at all dosage levels during sub-acute toxicity testing showed no signs of toxicity. Variations in adrenal gland weight which may indicate hypertrophy, hyperplasia, or atrophy of the organ associated with stress, endocrinopathies, or test article effects (Greaves, 2000), did not occur in this study.

The histopathological studies showed that the extract had no harmful effects on the wall of the gastrointestinal tract. Herbal remedies taken orally have been associated with gastrointestinal disturbances. Some herbs such as *Ginko biloba* and *Oenothera biennis* (Yoganandum *et al*., 2010) have been linked to adverse effects such as gastritis and diarrhea. The 1:1 mixture of methanol and dichloromethane extract of *Teclea trichocarpa* did not induce any notable side effects during both acute and sub-acute toxicity testing.

The pathological picture observed during toxicity studies is the best indicator of the harm done to a particular organ by the chemical. The liver is usually the first casualty during oral dosing because all chemicals pass through it once absorbed from the gut and undergo first pass metabolism (Frank and Robert, 2005). The importance of investigating the effects of new drugs in the liver cannot be over-emphasized because majority of drugs that cause hepatic injury during pre-clinical studies do not progress to clinical trials or are developed with substantial patient monitoring (Boone *et al*., 2005). Alanine aminotransferase (ALT) and Aspartate aminotranferase (AST) are the markers of liver function and are only released from cytosol and sub-cellular organelles during cell injury. Alanine aminotransferase is more hepatocellular

specific whereas creatinine is an indicator of muscle wasting. Alanine aminotransferase is a critical parameter for identification of potential drug-induced injury in both pre-clinical studies and human patients (Boone *et al.*, 2005 and Salawu *et al.,* 2010). It is apparent from the results that the ALT and AST values were actually declining and no increases in these parameters were noted. The decrease in enzymatic parameter was unexpected. It is traced to the fact that during the assays, freeze thawing of the sample was occasioned by workload exigencies. Notably, the non-enzymatic parameters, proteins, albumin and creatinine remained unchanged.

All organs that were sectioned and prepared showed no lesions when observed under the microscope. The 28 days oral toxicity test of the extract therefore had no effects on the cellular integrity of these organs. The fact that the non-enzyme parameters analyzed namely creatinine, protein and albumin was within normal limits corroborates the absence of pathological lesions observed grossly and microscopically. The haematological parameters tested did not show any significant variations associated with toxicity to haemopoietic organs. The modest variations noted in any parameter were well within the normal biological range since none of the parameter increased or decreased significantly in relation with the control values at all dosage levels. There was a small non-dose related increase in the mean haemoglobin levels and thrombocytes count with time in all treatment levels and the control. In a situation whereby the the haemopoietic organs are affected by the toxic agents, the blood film shows an elevated levels of immature neutrophils and nucleated RBC. Absence of such a phenomenal corroborates the fact that the extract was not toxic the rats.

The brine shrimp lethality test confirmed that the extract has cytotoxic effects. During the acute and sub-acute toxicity testing of the dichloromethane-methanol (1:1) extract of *Teclea*

trichocarpa root bark on rats, it was noted that the extract did not affect feed and water intake and had minimal effect on the overall body metabolism of rats as indicated by almost normal increase in weight in all treatment groups. The extract's acute oral dosage that can cause 50 % death of animals is well above 2000 mg/kg per body weight of rats and less than 5000 mg/kg body weight as confirmed during oral acute testing. The extract did not induce any pathological changes in tissue cells during the 28 days dosing with up to 2000 mg/kg of the extract daily for 28 days. The extract may therefore be considered safe when used orally, but further investigations are required to know it's effects if used via other routes.

RECOMMENDATION FOR FURTHER RESEARCH

Whereas death of laboratory rats that were administered intraperitoneal dose of 300 and 2000 mg/kg body weight occurred within minutes, no lesions were observed in any organ tested. Indeed, lower dosage of 50 mg/kg repeated daily (6 days) until a dose of 300 mg was achieved did not cause death or produce any overt lesion. The fact that deaths occurred after high dose of the extract (300 and 2000 mg/kg) were administered intraperitoneally suggests that some of the chemicals from the extract may not have been absorbed across the intestines and this warrants further investigation. There is therefore need to investigate effects via other routes of administration. Further work is also recommended on isolation and characterization of responsible phytochemicals followed by anthelmintic bioactivity profiling of isolated compounds.There is also need to carry out the toxicity test of the individual phytochemicals found in *Teclea trichocarpa* extracts. .

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APPENDICES

APPENDIX 1

The percentage yields of various plant parts extracts obtained from dichloromethane-methanol (1:1) extract

Plants	Family	Plants parts	% Yield
Albizia gummifera	Fabiaceae	Pods	9.9
		Roots	0.73
		Root barks	1.8
		Stem barks	2.9
Crotalaria auxillaris	Leguminoceae	Twigs	0.2
Manilakara discolor	Sapotaceae	Root barks	3.8
		Stem barks	11.3
Teclea	Rutaceaea	Roots	3.5
trichocapa		Stem barks	6.9
		Twigs	1.5
		Root barks	7.9
		Root wood	
Zanthoxylum usambarense	Rutaceae	Stem barks	4.3
		Root barks	7.9

APPENDIX 2: OECD guidelines for testing of chemicals (2001) No. 423, with a starting dose of 2000 mg/kg body weight.

start

| 5mg/kg 3 animals | 50mg/kg 3 animals | 300mg/kg 3 animals | 2000mg/kg 3 animals |

2-3 0-1   2-3 0-1   2-3 0-1   2-3 0-1

| 5mg/kg 3 animals | 50mg/kg 3 animals | 300mg/kg 3 animals | 2000mg/kg 3 animals |

2-3 0-1   2-3 0-1   2-3 0-1   2-3 0-1

0

0

| Category >0-5 | Category 2 >5-50 | \category 3 >50-300 | Category 4 >300-2000 | Category 5 >2000-5000 | Category 5 or unclassified |

| 3(at 50) at 1st step | other | 3(at 300) At 1st step | other | 3 | 2 | 1 | 0 |

| 5 | 25 | 30 | 50 | 200 | 300 | 500 | 1000 | 2000 | 2500 | 5000 | 00 |

Per step three animals of single sex (normally females) are used
0,2,3:numbers of moribund or dead animals at each step
GHS: Globally Harmonized classification System (mg/kg b.w.)

Unclassified
Testing at 5000 mg/kg b.w

94
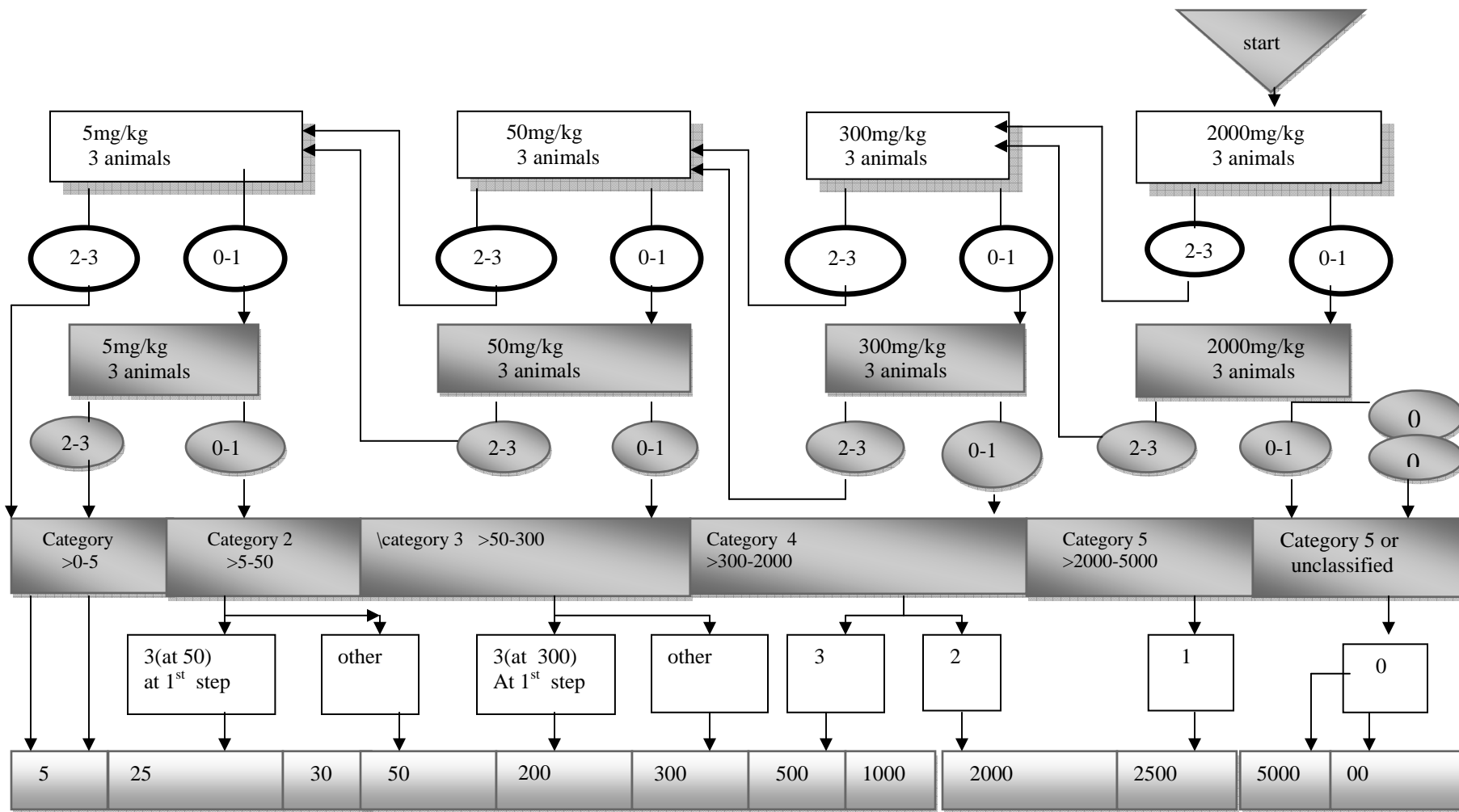
APPENDIX 3: OECD guidelines for testing of chemicals (2001) No. 423, with a starting dose of 300 mg/kg body weight.

start

| 5mg/kg 3 animals | 50mg/kg 3 animals | 300mg/kg 3 animals | 2000mg/kg 3 animals |

2-3   0-1   2-3   0-1   2-3   0-1   2-3   0-1

| 5mg/kg 3 animals | 50mg/kg 3 animals | 300mg/kg 3 animals | 2000mg/kg 3 animals |

2-3   0-1   2-3   0-1   2-3   0-1   2-3   0-1   0

0

| Category >0-5 | Category 2 >5-50 | \category 3 >50-300 | Category 4 >300-2000 | Category 5 >2000-5000 | Category 5 or unclassified |

| 3(at 50) at 1st step | other | 3(at 300) At 1st step | other | 3 | 2 | 1 | 0 |

| 5 | 25 | 30 | 50 | 200 | 300 | 500 | 1000 | 2000 | 2500 | 5000 | 00 |

Per step three animals of single sex (normally females) are used
0,2,3:numbers of moribund or dead animals at each step
GHS: Globally Harmonized classification System (mg/kg b.w.)
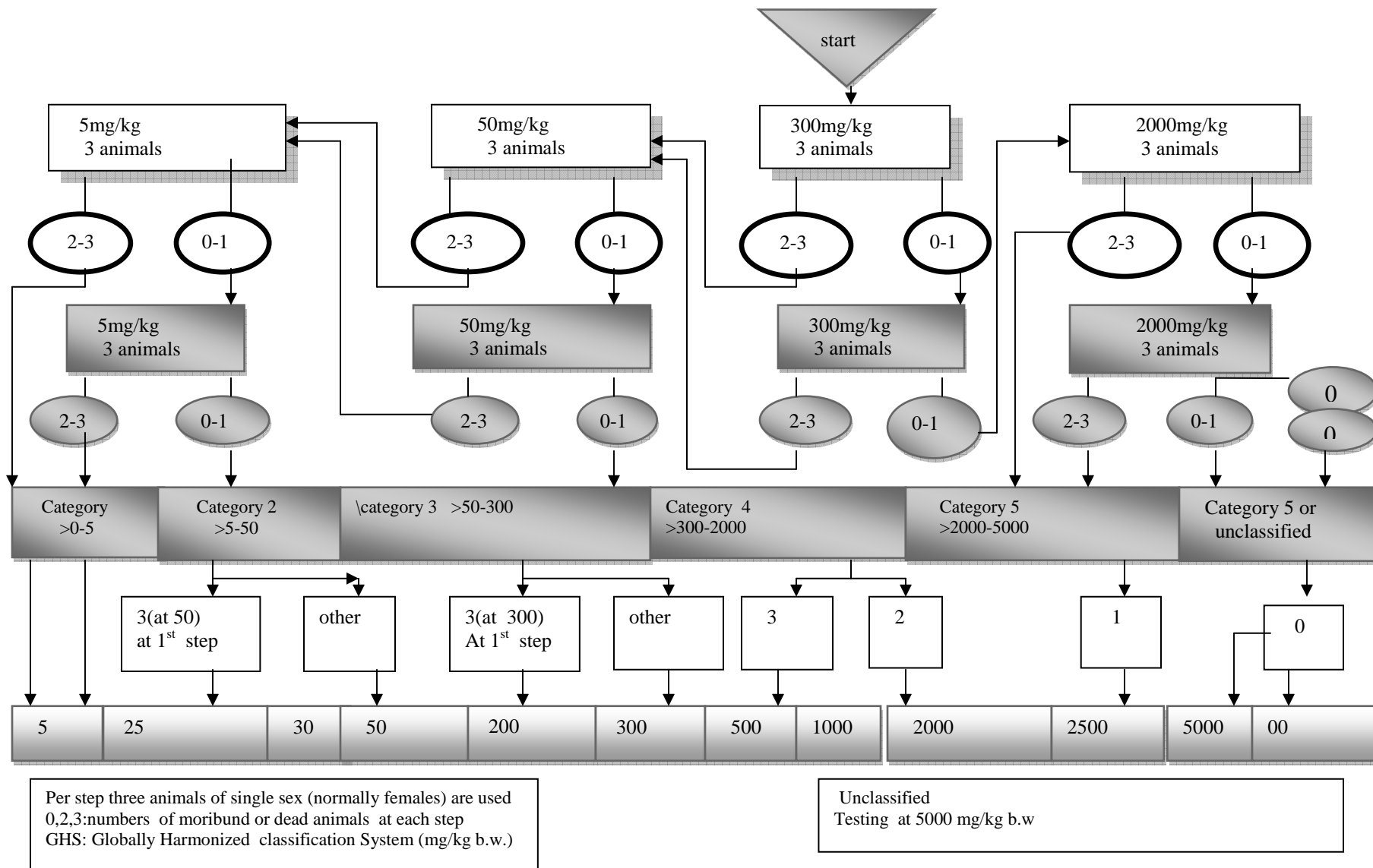
Unclassified
Testing at 5000 mg/kg b.w

95

APPENDIX 4: OECD guidelines for testing of chemicals (2001) No. 423, with a starting dose of 50 mg/kg body weight.

start

| 5mg/kg 3 animals | 50mg/kg 3 animals | 300mg/kg 3 animals | 2000mg/kg 3 animals |

2-3 0-1   2-3 0-1   2-3 0-1   2-3 0-1

| 5mg/kg 3 animals | 50mg/kg 3 animals | 300mg/kg 3 animals | 2000mg/kg 3 animals |

0

0

2-3 0-1   2-3 0-1   2-3 0-1   2-3 0-1   0

| Category >0-5 | Category 2 >5-50 | category 3 >50-300 | Category 4 >300-2000 | Category 5 >2000-5000 | Category 5 or unclassified |

| 3(at 50) at 1st step | other | | 3(at 2000) at 1st step | 2(at 2000) at 1st step | | 1 | 0 |

| 5 | 25 | 30 | 50 | 200 | 300 | 500 | 1000 | 2000 | 2500 | 5000 | 00 |

Per step three animals of single sex (normally females) are used
0,2,3:numbers of moribund or dead animals at each step
GHS: Globally Harmonized classification System (mg/kg b.w.)

Unclassified
Testing at 5000 mg/kg b.w

96