PHYTOCHEMICAL COMPOSITION, CYTOTOXICITY AND TOXICOLOGICAL STUDIES OF *ROSMARINUS OFFICINALIS, CATHARANTHUS ROSEUS* AND *MYRSINE AFRICANA* CRUDE EXTRACTS

Zelipha N Kabubii

A thesis submitted in partial fulfillment for award of Master of Science degree in Natural Products and Bioprospecting, Department of Public Health, Pharmacology and Toxicology, University of Nairobi

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Zelipha N Kabubii

Signature……..Date……

This work has been submitted for examination with our approval as University Supervisors.

Prof. James M. Mbaria, (BVM, MSc, Ph.D.)
Department of Public Health, Pharmacology and Toxicology
Faculty of Veterinary Medicine (University of Nairobi)

Signature…… Date…….

Dr. Mbaabu Mathiu (BVM, Ph.D.)
Department of Veterinary Anatomy and Physiology
Faculty of Veterinary Medicine (University of Nairobi)

Signature …… Date ……..……..

DEDICATION

To my entire family: My loving husband, Munene, dear children: Oscar, Moses and Abigail.

I also wish to dedicate this work to my yet to come grandchildren.

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ABBREVIATIONS AND ACRONYMS

ACS	-	American Chemical Society
ALT	-	Alanine aminotransferase
ANOVA	-	Analysis of variance
AST	-	Aspartate aminotransferase
Bwt	-	Body weight
EFSA	-	European Food Security Authority
HB	-	Hemoglobin Concentration,
IDRC	-	International Development Research Centre
LC	-	Lethal concentration
LD	-	Lethal dose
MCH	-	Mean cell hemoglobin
MCHC	-	Mean corpuscular hemoglobin concentration
MCV	-	Mean corpuscular volume
NCAPD	-	National Coordinating Agency for Population and Development
NP_s	-	Natural products
OECD	-	Organization of Economic Cooperation and Development
PCV	-	Packed cell volume
RBC	-	Red blood cells
RKRHCP	-	Republic of Kenya registration of herbal and complementary and natural products
Thromb.	-	Thrombocytes
WBC	-	White blood cells
WHO	-	World Health Organization

ABSTRACT

Natural medicinal products have gained recognition worldwide in the treatment and control of diseases. One of the major concerns is the lack of adequate pharmacological and toxicological data to support their use. *Catharanthus roseus* is a commonly used plant especially in the control of diabetes. *Myrsine africana* is traditionally used as veterinary and human anthelmintic while *Rosmarinus officinalis* is used as a spice for it has high antioxidant and other therapeutic properties. This study was carried out on the crude extracts of leaves of *C.roseus*, R. *officinalis* and dry seeds of *M. africana* to screen for their phytochemical composition. Oral acute toxicity and the effects on hematological and biochemical parameters of each of the plant extracts at doses of1000 and 5000 mg/kg body weight were determined. Male albino Wistar rats were used as the Organization for Economic Co-Operation and Development (OECD) guidance document 423 recommends use of one sex animals. A control group was given distilled water. A 28 day repeated oral administration of freeze dried aqueous extract of *R. officinalis* on four groups of Wistar rats at dosages 0, 500, 1500 and 3000 mg/kg body weight (bwt) was carried out to evaluate the plants sub-acute toxicity. The cytotoxicity and lethality effects on the brine shrimps (*Artemia* s*alin*a) in four organic and an aqueous extracts of each of the three plants was studied using concentrations 10, 100, 1000 µg/ml of each extract as described by Meyer *et al*., 1982. Brine shrimp median lethal concentration ($LC_{50)}$ for each extract was calculated using a regression line of probit against log concentration. The phytochemical analysis showed presence of 5 types of bioactive compounds namely terpenoids, tannins, anthraquinones, alkaloids and reducing sugar in *Catharanthus roseus.* Terpenoids, tannins, flavonoids, saponins and reducing sugars were also found present in *Myrsine africana* extract. *Rosmarinus officinalis extract* contained terpenoids, tannins, cardiac glycosides, flavonoids, reducing sugars and saponins. The median lethal dose (LD50) of the aqueous extracts for each of the three plant extracts in albino Wistar rats was estimated to be > 5000 mg/kg body weight. Alanine aminotransferase (ALAT), aspartate aminotransferase (AST), urea, White blood cells (WBC) and mean corpuscular volume (MCV) were significantly elevated in the groups treated with. *C. roseus* extract ($p<0.05$), but thrombocytes and percentage weight gain were significantly reduced in these groups. Red blood cells (RBC), packed cell volume, (PCV), mean corpuscular

hemoglobin concentration, (MCHC), AST and serum urea were significantly elevated in the groups given M. *africana* extract. ALAT both at 1000 and 5000mg/kg body weight of *R. officinalis* treated groups were significantly reduced at 48 hours but at 14 days they had normalized to baseline values. Sub-acute toxicity testing of *R. officinalis* aqueous extract showed no significant difference of hematological and biochemical parameters at 500 and1500 mg/kg body weight both at 14 and 28 days testing. Significant elevation of WBC, percentage lymphocytes and ALAT at 3000 mg/kg body weight when compared with the control was reported both at days 14 and 28 testing ($P < 0.05$). Methanolic extracts of *M. africana* and *C. roseus* showed very strong cytotoxicity to brine shrimps with LC_{50} of < 10 µg/ml. Aqueous extract of *M. africana* did not cause significant cytotoxicity against the brine shrimp, with LC_{50} > 1000 µg/ml. It was concluded that the phytochemicals present in each plant extracts may be responsible for bioactivity effects that were recorded. It was also concluded that Myrsine *africana* and *Catharanthus roseus* are likely to cause acute renal-hepatotoxicity and hematopoietic system toxicity at oral concentrations that were tested. This is explained by the elevated biochemical parameters (AST, ALAT, and urea) and the significantly altered hematological parameters. It was recommended that these plants should be used with care and at lower concentrations. Sub-acute and chronic toxicity testing of these plants is recommended in order to clearly establish the effects of repeated doses. Methanolic seed extract of *M. africana* is likely to have antitumor and insecticidal properties due to its high cytotoxicity and isolation of the constituent bringing this effect is recommended. The results of .*R. officinalis* toxicity studies indicate that the plant is not acutely toxic but prolong toxicity studies are recommended to confirm the safety of the plant.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Since antiquity, man has used plants to treat common infectious diseases even long before mankind discovered the existence of microbes. 80% of population in the non-developed countries depends on traditional medicine for their primary health care (WHO, 2002: WHO, 2008). According to International Development Research Centre (IDRC), 85% of Africans use herbal remedies in their routine health care in Sub-Sahara Africa (Stanley, 2004). Although herbal medicine has been perceived by the public as relatively low risk, there has been more recognition of the potential risks associated with them as the use of (NPs) increase. Potential harm can occur via inherent toxicity of herbs, as well as from contamination, plant misidentification, and interactions with other herbal products or pharmaceutical drugs. Regulatory safety assessment for NPs relies on both the assessment of cases of adverse reactions and the review of published toxicity information effects (De Smet, 1995). The regulation of herbal medicine practice in Africa is still a major challenge (Madiba, 2010)

Plant compounds exhibit enormous structural diversity, unfortunately only a small proportion of that diversity has been seriously explored for pharmacological potential. Traditional medicine has a great potential of promoting livelihood and the priority focus is on safety/efficacy, conservation, domestication, production and commercialization. The global increase in demand for ethnoherbals is attributed to dissatisfaction with conventional

medicine in terms of effectiveness, safety and accessibility. Phytochemical, pharmacological and toxicological standardization for medicinal plants need be instituted so that dosage levels can be described in an informed way.

Africa is home to an estimated 45,000 plant species, which is about one-fifth of the size of the world flora. About 4,500 (10%) of these plants are rare species, while 15,000 species are endemic to the continent. Madagascar, with about 13,000 plant species, has the highest (82%) endemicity. One-third of the 5,000 forest species in the continent are used in traditional medicine (Iwu, 1993). More than 4,000 plant species in tropical Africa are used for medicinal purposes, and research done in 2006 showed that 50,000 tons of medicinal plants were consumed annually in this region (Karki, 2006). The use and commercialization of non-timber forest products which include medicinal plants has been found to be an important livelihood strategy in developing countries especially for the rural people (Schackleton *et al*., 2009), hence enhancing their living standards (Mbuvi and Boon, 2008).

Out of 10,000 flora species in Kenya, about 1200 have been identified as medicinal (Kokwaro, 2009). More than two –thirds of Kenyans turn to medicinal plants for health care and this has been mostly due to lack of access of modern medicine (NCAPD, 2004). The current study comes up with scientific data on safety of three plants which are common in Kenyan ethnomedicine; *Catharanthus roseus* (Madagascar periwinkle), *Rosmarinus officinalis* (Rosemary), *Myrsine africana* (Cape Myrtle/ African boxwood). A prove of their use for medicinal purposes is investigated by assessing the composition of their phytochemicals, oral toxicity and their ability to cause cytotoxicity of brine shrimps.

1.2 Problem statement

Many people believe that natural products or traditional medicine are safer than the conventional modern medicine. Due to their easy access and low cost, these products have increasingly become popular. However, inappropriate use of herbal medicines can cause harmful, adverse reactions. These reactions could be acute and sometimes chronically manifested. Scientific evidence of tests done to evaluate safety of natural products is limited and hence these remedies are used in ignorance due to lack of appropriate information.

1.3 Justification

The safety of herbal medicine has continually been questioned due to reported illness and fatality of the test animals, (Park *et al.,* 2010). Toxicological studies are important in hazard identification stage of safety assessment of drugs. Consumer awareness about safe usage is also very crucial and also good for more training, collaboration and communication among providers of natural products medicine. The possibility of interspecies dosage conversion (WHO, 2000; Curry *et al*., 2011) make it possible to correlate safety doses of the natural products remedies used on the animal model to the human dosage levels.

Scientific proofs showed that rosemary is well tolerated and of very low toxicity (EFSA, 2008; (Anadón *et al.,* 2008), hence it has been regarded as consumer safe. *Myrsine africana,* commonly used as an anthelmintic, has been regarded as a moderately safe herb (Ahmad *et al.,* 2011a). *Catharanthus roseus,* a plant commonly used for the control of diabetes and other ailments is regarded as very toxic if consumed orally (Kevin *et al*., 2012). This study was able to validate these claims and also showed how the extracts affected the blood

parameters. Therefore the study was able to offer scientific information that will go hand in hand with ethnomedicinal use.

1.4 Objectives

1.4.1 General objective

To screen for the phytochemical composition, cytotoxicity and toxicological effects of *Rosmarinus officinalis*, *Catharanthus roseus* and *Myrsine africana* crude extracts.

1.4.2 Specific Objective

1. To determine the phytochemical composition of the crude extracts of *C. roseus*, *R. officinalis* (leaves) and *M. africana* (seeds).
2. To determine the cytotoxicity effects of the aqueous and four organic extracts of *C. roseus*, R. *officinalis* (leaves) and *M. africana* (seeds) on brine shrimp larvae.
3. To evaluate the hematological and biochemical effects of:

(a) Acute toxicity of *R.* o*fficinalis C. roseus* and *M. africana* aqueous extract in male albino Wistar rats.

(b) Sub-acute toxicity in a 28 day oral repeated dose of *R. officinalis aqueous* extract in albino Wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Natural Products

Compounds which have biological activities and are derived from natural sources, *e.g.*, plants, animals and microorganisms, are defined as natural products. Natural products have been used by human societies for millennia and have been the major sources of chemical diversity for starting materials for driving pharmaceutical discovery over the past century (Cowan, 1999). Approximately 200,000 natural products of plant origin are known and many more are being identified from higher plants and microorganisms (Lee, 1999**,** Strobes *et al*., 2004, Aly *et al*., 2010; Kinghorn *et al*., 2011). Over the past decade, biotechnology, pharmaceutical and human health care industries have increased their interest in natural products as sources of new biochemical compounds for drug, chemical and agro-products development. This interest has been stimulated by the importance of traditional knowledge as a lead in new product development (Kinghorn *et al.,* 2008). There are no alternative conventional drugs as up to date for some natural product based drugs like the cardiac glycosides, (WHO, 2011). Natural products of higher plants are probable sources of antimicrobial agents which have added advantages of being safe and biodegradable (Adenisa *et al*., 2000**).** Natural products from plants may serve as blue prints in the development of new drugs or as phyotomedicine to be used to treat disease (Abubakar *et al*., 2008**)**

Between 1981 and 2006, 1,184 new drugs were registered of which 28% were natural products or their derivatives and 24% of the new drugs had pharmacophores (functional

groups with pharmacological activity) derived from natural products (Newman and Cragg, 2008). Many natural products and synthetically modified natural product derivatives have been successfully developed for clinical use to treat human diseases in almost all therapeutic areas. These compounds may be derived from primary or rather secondary metabolism of these organisms (Bérdy, 2005)**.** Naturally occurring drugs that are part of the war against cancer include vinca alkaloids (vincristine, vinblastine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), podophyllotoxin and its derivative (etoposide, teniposide), camptothecin and its derivatives (topothecan, irinothecan), anthracyclines (doxorubicin, daunorubicin, epirubicin, idarubicin) and others (Bhanot *et al*., 2011) .Half of all anti-cancer drugs approved internationally were either natural products or their derivatives and were developed on the basis of knowledge gained from small molecules or macromolecules that exist in nature (Newman *et al*., 2007)

Modern chemistry has ushered in a new era for the study and use of natural products. Analytical and structural chemistry have provided the tools to purify various compounds and to determine their structures, which, in turn, has given insights into their action on the human body (Trigg, 1989**.** At least twenty one NP and NP-derived drugs were launched onto the market in the United States, Europe or Japan between 1992 and 1998 (Shu, 1998).

2.2 Natural products from the marine

Material sources from exotic natural environments such as the oceans deeps, or extreme ecosystems like the Polar Regions and taxa of microorganisms for fermentations are lately being exploited for Natural Products sources (Newman and Gragg, 2006). More than 15 000

structurally diverse natural products with different bioactivities have been discovered from marine microbes, algae and invertebrates between 1970 and 2004 (Salomon *et al.*, 2004). Studies have shown that marine invertebrates harbour high levels of microbial diversity that are logical sources for marine biodiversity (Webster, 2001). Many of the marine-derived natural products for treating cancers have gone through clinical and some are in the pre-clinical trials (Salomon *et al*., 2004).

2.3 Historical background of evolvement of natural products.

Nearly all civilization has accumulated experience and knowledge of the use of natural products due to their diverse bioactivities, the oldest medical text coming from ancient Mesopotamia, circa 2600 BC, and it describes approximately 1,000 plants and plant-derived substances, such as the oils of Cedrus species (Newman *et al.,* 2003). Natural products in medicines flourished in the Orient. Charaka Samhita devoted to the concepts and practice of Indian Ayurveda, which was written around 900 BC and contains 341 plant-derived medicines**.** (Dev, 1999). Traditional Chinese medicine (TCM) was compiled around 350 BC**,** **(**Jiao and Wang, 2005; Zhong and Wan, 1990.

Traditional medicinal practices forms basis of most of the early medicines which eventually undergo chemical, clinical and pharmacological evaluation (Butler, 2004). It is documented that the Sumerians and Ancient Greeks used poppy extracts medicinally since 1803, (Der Marderosian and Beutler, 2002). Several alkaloids including morphine, a commercially important drug, has been isolated from *Papaver somniferum* L. (opium poppy). Crude morphine was derived from *P. somniferum* in 1870 to yield a pain killer heroin which latter

was readily converted to codeine, a widely used pain killer (Der Marderosian and Beutler, 2002). *Digitalis purpurea* L. (foxglove) had its use in Europe in the 10th century but it was not until the 1700s that the active constituent digitoxin, (cardiotonic glycoside) was found to enhance cardiac conduction, thereby improving cardiac contractibility (Haefner, 2004).

The anti-inflammatory agent, acetylsalicylic acid (aspirin) derived from the natural product, salicin was isolated from the bark of the willow tree *Salix alba L*. (Mishra, 2011). Quinine was isolated from the bark of Cinchona for the treatment of malaria in 1800 (Kinghorn *et al*., 2011). Penicillin was discovered by Fleming in 1929,) from *Penicillium notatum* which is a fungus, happen to be the most famous of natural product discoveries (Mann, 1994).

2.4 Natural products industry in Kenya

Use of natural products is closely associated with indigenous knowledge (Kaluwa *et al*., 2014). Kenya has a rich cultural and natural heritage. This is reflected in the enormous indigenous knowledge, embedded in the vast biodiversity found in the country (NCAPD, 2008). Natural medicinal products in Kenya flourish unrecognized and unregulated by the government or other institutions and this is a major challenge in the entire natural product industry (Madiba, 2010). This has resulted in the proliferation of herbal practitioners dispensing various forms of herbal medicines that are touted as able to resolve just about any health problem (NCAPD, 2008).

The Kenyan Pharmacy and Poisons Board (PPB) is involved in the registration of the medicinal products that have been formulated in commercial manner as herbal and

complementally products (PPB, 2010). There are several publications with regards to the products obtained from plant extracts. A number of these are showing pharmaceutical activities and potential for the development of new pharmaceutical products (Irungu *et al*., 2012; Langat *et al*., 2012; Matheka *et al*., 2012.) Judging from these published laboratory results analysed in various institutions, it shows that Kenya definitely has a big potential in natural products (Kigen *et al*., 2013).

Some of the major challenges in this Industry include disappearance of species due to overexploitation (Rukangira, 2001; Stanley, 2004) and also the lack of a documentation of the traditional knowledge threatening its disappearance since most herbalist are old or have died, (Thairu, 1975).

2.5 Some Selected Kenyan natural products in the development pipeline.

Table 2.1: Natural products in development pipeline in Kenya

Product Name	Use / Benefits	Current status
MUPAL (University of Nairobi)	Duodenal stomach ulcers and hyperacidity	Reasonable testing in laboratory animals for safety, activity and some pharmaceutical aspects
Mondia tonic (KEFRI, KARI and KWS)	Anti-malaria	Laboratory testing done and products proved to be effective and safe
PROSGEM (University of Nairobi)	Benign prostate enlargement	Formulated as capsules ; pharmacological and clinical data available in public domain;
KV–1 (KEMRI)	Treatment of opportunistic viral infections (Herpes Simplex Cytomegavirus and Varicella zoster)	Laboratory testing done and product candidate proved to be effective and safe.
KT–4 (KEMRI)	Potential nutraceutical drug for treatment of mild Hypertension.	Safety studies show product is safe: Formulation and clinical trials are on-going.

Reference: **Republic of Kenya Ministry of sports, Culture and the Arts, the Natural Products Industry Policy 2012 (Draft), NPIP, 2012**

2.6 Therapeutic areas amenable to natural products

2.6.1 Infectious diseases

2.6.1.1 Fungal pathogens.

Currently marketed anti-fungal drugs target the cell membrane and the cell wall. Caspofungin, one of the most recent clinically available anti-fungal agents, is derived from pneumocandin, a natural product metabolite produced by *Glarea lozoyensis.* Micafungin and anidulafungin are FDA approved anti-fungal agents (Liu *et al.*, 2006).

2.6.1.2 Viral pathogens.

Most marketed anti-viral drugs inhibit viral replication (Westby and Ryst, 2005). A number of anti-viral lectins of algae origin are small proteins that bind carbohydrates found on viral envelopes and are currently in preclinical trials for prevention of transmission of human immunodeficiency virus (HIV), and other envelope viruses such as Ebola or the coronavirus responsible for severe acute respiratory syndrome, SARS, (Ziolkowska and Wlodawer, 2006).

2.6.2 Oncology

Majority of the chemotherapeutic agents currently in use are natural product derived drugs. Drugs that interfere with cell division and proliferation, including alkylating agents, alkaloids, tubulin polymerization agents and topoisomerase inhibitors, are examples of cytotoxic agents used in cancer therapy and include such natural product examples as taxol, vinblastine and anthracyclines **(**Newman *et al*., 2003). Genistein, a soy isoflavone, has been

found to regulate the genes that are critical for the control of cell proliferation, cell cycle, apoptosis, and cell signal transduction pathways (Sarkar *et al.,* 2006).

2. 6. 3 Other therapeutic areas

2.6.3.1 Cardiovascular and endocrinology

Fields of endocrinology and cardiovascular diseases have not benefited as broadly from natural product discovery programs. However, there are a few notable exceptions. Rapamycin has been found to have cardiovascular protective effects of ischemia disease with hypoxia–re-oxygenation injury is derived from a natural product (Ollivier, 2006). Exenatide, a natural product derived compound from the saliva of a lizard, has recently been approved by the Food and Drug Administration (FDA) for treatment of diabetes (Lee *et al*., 2008)

2.6.3.2 Inflammation.

Natural product derived drug such as Macrolides have been proven to have anti-inflammatory properties distinct from their anti-bacterial activity. Macrolides including azithromycin and the ketolide antibiotic telithromycin have been prescribed for alleviating symptoms of pulmonary pneumonia (Johnston, 2006). One suspected mechanisms for the observed anti-inflammatory activity of macrolides is their ability to suppress the over-abundance of neutrophils in lungs (Lotter *et al.,* 2006). The immunosuppressive agent, rapamycin, was reported to inhibit LPS-induced tissue factor expression and help alleviate inflammation in atherosclerosis (Ollivier *et al*., 2006)

2.7 Ethnomedicine

Ethnomedicine, the study of how people conceptualize disease and healing within the context of their culture (Fabfrega, 2004), has contributed a lot to the advancement of natural products development. Traditional healers play great roles in the primary healthcare systems of the local people since they attend to the poor people who have little access to modern medications **(**Gathuma *et al*., 2004). Medicinal plants that have been exploited for pharmaceuticals traces their origin from traditional medicine, artemisinin, a good example, is an antimalarial agent from sweet worm tree. *Arteminisia annua* has been used in Chinese medicine since 200BC is one drug used as part of combination therapy for multiresistant *plasmodium falciparum (*Trigg, 1989; Oketch-Rabah, 1996).

It is now estimated that the natural plants industry is the fastest growing sector in the entire agribusiness industry (Makunga *et al*., 2008). Many ethnobotanical studies have been accomplished in Kenya with traditional uses of plants from the various communities being documented (Morgan, 1981; Ochoki, 1982; Johns *et al*., 1990; Stilles and Kassam, 1991; Kokwaro, 1993, Sindiga *et al*., 1995; Bussmann *et al*., 2006; Jeruto *et al*., 2008; Jeruto *et al*., 2007; Keter and Mutiso., 2011; Lindsay and Hepper, 1978; Nanyingi *et al.*, 2008; Nguta *et al*., 2012; Mbaabu and Matu, 2013; Kaluwa *et al*., 2014 and many other ethnobotanical surveys. There is continued search for novel products from traditional medicinal plants (Makunga *et al*., 2008) particularly for development of effective new drugs that are non-toxic and inexpensive (Taylor *et al*., 2001).

Medicinal plants are the richest bio resources of folk medicine and food supplement, nutraceutical phymacenticals and chemical entities of synthetic drugs (Ncube *et al.,* 2008). Modern medicine has evolved from folk medicine and traditional system. This has happened after chemical and pharmaceutical screening (Boopathi and Kumar, 2011). Traditional systems of medicine from India Siddha, Ayurveda and Unani are prepared from a single or combination of a number of plants and the required quantities and nature of secondary metabolite in the raw drug determine the efficacy, (Vinoth *et al*., 2011; Savithramima *et al.,* 2011). The market and public demand of herbal medicine have been increasing due top belief that they are less toxic and this is increasing the risk of extinction or loss of genetic diversity (Misra, 2009).

Kenya has a rich plant heritage with very potent biochemicals, (Kokwaro, 1983, Riley and Brokensha, 1988), like many other developing countries the nation, has a wide spread use of herbal medicine especially in the rural area (WHO, 2008) .Most of ethnobotanical information of herbal medicine and healing procedures remain undocumented. This is because most communities in Kenya pass the information orally and only to very close relatives who may not necessary practice the art (WHO, 2008). Most communities categorize the diseases e.g. as pollutants, misfortune curse and the treatment may differ from case to another, (Kipkorir and Welbourn, 2008).

2.8 Phytochemicals

The term phytochemicals is generally used to components that may have biological significance for example antioxidant (Agarwar and Rao, 2001). There are over 10,000

different phytochemicals having the therapeutic effects against cancer, stroke or metabolic syndromes, (Higdon, 2007). Secondary metabolites are chemically and taxonomically extremely diverse. They have found themselves into different uses, human therapy, veterinary, agriculture and even scientific (Vasu *et al.*, 2009). To promote the ecological survival of plants, secondary metabolites have evolved to interact with molecular targets affecting the cells, tissues and physiological functions (Wink and Schimner, 1999). Some plants secondary metabolites' exert their actions by resembling the endogenous metabolites, Ligands hormones, signal transduction molecules or neural transmitted end hence they become beneficial to human medicine due to similarity in potential targets for example central nervous system, endocrine system and circulatory system (Kaufman *et al.,* 1999**).**

Unlike most conventional medicines, which are single chemical entities, (which are known a lot about since they were constructed synthetically), natural products often contain many active compounds (sometimes hundreds), thus making it challenging to figure out how the whole product affects the human body. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human or animal body (Edeoga *et al*., 2005). The most important of these bioactive constituents which are mainly secondary metabolites include alkaloids flavonoids, tannins and phenolic compounds. Recent work has indicated potential roles of secondary products at the cellular level as plant growth regulators, modulators of gene expression, and in signal transduction (Kaufman *et al*., 1999).

Phyotomedicine can be derived from barks, leaves, flowers, roots, fruits, seeds (Crigg and David, 2001). Knowledge of the chemical constituents of plants is desirable because such information will be of value in the synthesis of complex chemical substances (Parekh and Chanda, 2007). To promote the ecological survival of plants, Structures of secondary products have evolved to interact with molecular targets affecting the cells, tissues, and physiological functions in competing microorganisms, plants, and animals (Wink and Schimmer, 1999). In this respect, some plant secondary products may exert their action by resembling endogenous metabolites, ligands, hormones, signal transduction molecules, or neurotransmitters and thus have beneficial medicinal effects on humans due to similarities in their potential target sites example: central nervous system and endocrine system (Kaufman *et al*., 1999)

Phytochemicals are divided into primary metabolites such as fats and sugars and these are found in all plants. Secondary metabolites – these are not found in all plants and serve or move specific function (Meskin and Mark, 2002). Some secondary metabolites are toxins and are used to put off the predators. Others like the pollinators have pheromones and are used for attraction (Meskin Mark, 2002). These pigmented metabolites can have therapeutic properties while still capable of causing side effects (Lai and Roy, 2004).

2.8.1 Alkaloids

These are over 12, 000 cycling nitrogen containing compounds found in over 20% plant species (Zulak *et al.,* 2006). The recorded use of alkaloids for medical purposes goes back 5000 years (Goldman, 2001). Most traditional poisons such as atropine and phyoscyamine

from *Atropa belladonna*, some social drugs such as caffeine, nicotine cocaine and opiates are alkaloids (Zenk and Juenger, 2007). Most cholinesterase inhibitors used routinely in treatment of cholinergic dysregulation Alzheimer disease (hyperzine and galatamine) are alkaloids (Mukhejee *at al.,* 2007). Ecologically, alkaloids are toxic against insects and other herbivores (Harborne. 1993).

2.8.2 Terpenes

These are lipid soluble compounds and more than 30,000 diverse groups are known. Their structure includes 1 or 5 carbon isoprene units which are ubiquitously synthesized by all organisms through the merolate and deoxy – d- glucose pathways, (Rohmer, 1999)**.** Terpenoids are generally present in complex mixtures and have different ecological roles in a plant. Monoterpenoids are major components of many essential oils and are economically important as fragrances and perfumes. Common acyclic compounds of six isoprene units and are biosynthetically derived from squalene (Harborne, 1993)

2.8.3 Flavonoids

Flavonoid is a component that is present in normal human diet and is associated with therapeutic uses against stroke, cardiovascular diseases and cancer among. Different naturally occurring flavonoids have been described and subcategorized into flavones, flavans, flavanones, isoflavonoids, chalcones, aurones and anthocyanidines (Amanlo *et al*., 2005, Veitch *et al*., 2007). These flavonoids have remarkable biological activities, including inhibitory effects on enzymes, modulatory effect on some cell types, protection against

allergies, antiviral, anti-malarial, antioxidant, anti-inflammatory and anti-carcinogenic properties (Veitch *et al*., 2007) .

2.8.4 Saponins

Saponins are structurally complex amphiphatic glycosides of steroids and triterpenoids and are widely produced by plants (Sparg *et al*., 2004; Vincken *et al*., 2007). Saponins are also produced by certain marine organisms, such as starfish and sea cucumbers (Tang *et al*., 2009; Van Dyck *et al*., 2010). Chemically, the term saponins define a group of high molecular weight glycosides that consist of a glycan moiety linked to an aglycon (Hostettmann and Marston, 2005). The backbones of saponins are synthesized via the isoprenoids pathway through a largely unidentified number of sequential and/or parallel enzymatic steps (Misawa, 2011). Saponins are often present as complex mixtures and their composition may vary depending on the genetic background, the tissue type, the age and the physiological state of the plant and environmental factors (Szakiel *et al*., 2011a). These compounds have been found to cause hemolysis, enzyme inhibition, and alteration of gut surface tension in herbivores (Applebaum and Kirk, 1979).

2.8.5. Glycosides

In chemistry, glycosides are defined as compounds containing a carbohydrate and a noncarbohydrate residue in the same molecule; specifically, these compounds include a sugar molecule tied up with another chemical at the anomeric carbon via a glycosidic bond (Lindhorst, 2007). Glycosides may play a central role in different biological functions, presumably by regulating some specific plasticity-related proteins such as G proteins, which

may be crucial in the transduction of intracellular signals (Neves *et al*., 2002**).** Studies have suggested that cardiac glycosides target cancer cells selectively and have a significantly lower mortality rate (López-Lázaro, 2007).

2.8.6 Anthraquinones

Anthraquinones are a class of natural compounds that consists of several hundreds of compounds that differ in the nature and positions of substituent groups (Schripsema *et al*., 1999). This class of compounds contains derivatives that consist of the basic structure of 9, 10 anthraquinones (Baja *et al*., 1999). Anthraquinones are widely applied in medicine, food and the dye industry. In the pharmaceutical industry, the natural and synthetic derivatives of 9, 10 anthraquinone are beneficial to mammals and humans as they can display antibacterial, antitrypanosomal and antineoplastic activities (Heyman et *al*., 2009, .Tarus *et al*., 2002).

2.8.7 Tannins

Tannins encompasses some very diverse oligomers and polymers (Harborne *et al*.,1999).Tannins are a heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible complexes with proteins , alkaloids, nucleic acids and minerals (Mueller-harvey and Mcallan, 1992). On the basis of structural characteristics it is possible to divide the tannins into four major groups: gallotannins, ellagitannins, complex tannins, and condensed tannins (Mangan *et al*., 1988). Tannin-containing plant extracts are used as astringents, against diarrhoea, as diuretics, against stomach and duodenal tumours (De Bruyne et *al.,* 1999).

2.8.8 Reducing sugars

A sugar is classified as a reducing sugar only if it has an open-chain form with an aldehyde group or a free hemiacetal group (Campbell and Farrel, 2012). Knowledge of carbohydrate status in plants is very important in understanding many biological (Huber *et al*., 1985). The soluble sugar pool in most plants is formed mainly by sucrose, glucose, fructose and minor quantities of other substances such as aminosugars, sugar alcohols, sugar phosphates and uronic acids (Campbell and Farrel, 2012)

2.9 Toxicity of natural products

Excessive consumption of some of secondary metabolites brings toxic effects, for example; excessive glycosides, alkaloids and terpenoids results in lesions in nervous system (Conn, 1979; Mabry and Grill, 1979). Some types of tannins (condensed) are also responsible for mucosal toxicity and the consequence is a reduced nutrient absorption (Reed, 1995). Saponins in excess have been reported to impair growth and reduced food intakes (Milgates and Roberts, 1995).

Earliest report of the toxicity of herbs originated from Galen, a Greek pharmacist and physician. He showed that herbs do not contain only therapeutic constituents, but that they may also contain harmful substances (Cheng *et al.,* 2004). Toxicity in medicinal natural products may originate from: (1) accidents due to a mistake in botanical identification, (2) accidental ingestion of cardiotonic plants, (3) inappropriate combinations, including the use of potentially toxic plants, (4) plants that interfere with conventional pharmacological therapy (5) Contamination with heavy metals (Thomson *et al*., 2000).

The premise that traditional use of a plant for perhaps many hundreds of years establishes its safety does not necessarily hold true (De Smet, 1995). The more subtle and chronic forms of toxicity, such as carcinogenicity, mutagenicity, and hepatotoxicity, may well have been overlooked by previous generations and it is these types of toxicity that are of most concern when assessing the safety of herbal remedies (Shaw *et al*., 1995). The primary aim of toxicological assessment of any NP is to identify adverse effects and to determine limits of exposure level at which such effects occur (Sims *et al*., 2010).

2.10 Examples of natural products that have recorded potential toxicity

Table 2.2: Natural products with potential toxicity:

Common name and scientific name	**Potential toxicity**
Ginseng(*Panax ginseng*)	Central nervous system stimulation, hypertension, skin eruptions (Chan *and Fu*, 2007)
St. John's wort (*Hypericum perforatum*)	Highly potent cytochrome P450 enzyme inducer which affects drug metabolism, hepatotoxicity and nephrotoxicity in pregnancy and lactation (Gregoretti *et al*., 2004)
Kava kava (*Piper methysticum*)	Anxiolytic hepatotoxic, cytochrome P450 enzyme inhibitor (Gow *et al*.,2003)
Ginkgo(Ginkgo biloba)	Gastric irritability, spontaneous bleeding (Sierpina *et al*.,2003)
Aloe *(Aloe vera)*	Cytogenetic toxicity (Verma *et al*.,2012)

2.11 Acute toxicity testing

There several types of toxicity tests performed by pharmaceutical manufactures during new drug assessment and evaluation for toxic characteristics. These include; acute, sub-acute and chronic toxicity. Acute toxicity is a term used to describe the adverse effects that are caused by a single exposure to a toxic substance or brief multiple exposures over a very short span of time –usually less than 24 hours. The described acute toxicity should occur within 14 days of administration of the substance (OECD, 2000). Acute toxicity studies are usually done to establish the lethal dose (LD) of a substance (Robinson *et al.,* 2007). Exposure routes may be by oral, inhalation/mucosal, dermal or injection. Acute toxicity is involved in estimation of LD_{50}, the dose which has proved to be lethal to 50% of the tested group (OECD, 423).

2.11.1 Significance of acute toxicity tests

Regulatory requirements for health products in many countries are becoming stricter and demanding with the increased health consciousness (WHO, 2008). Pharmacotoxicological data which should include pharmacological activity, acute, sub-acute, chronic and sub-chronic tests is part of requirement when registering a herbal product (RKRHCP, 2010).

Determination of safety of natural products/herbal remedies is necessary as many people use them for self-medication and little data is available about the pharmacology and toxicology for most of the common herbal remedies (Fragoso *et al*., 2008). Acute toxicity is an initial screening step in the toxic assessment and evaluation characteristics of all biological compounds (Akhila *et al*., 2007) and studies in animals are usually necessary for any pharmaceutical intended for human use. Acute toxicity studies may also aid in the selection

of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans. The goals of acute lethal toxicity testing) include the following: (1) defining the degree of hazard that may result from exposure to a test substance; determining susceptible populations and species;(2) identifying target organs or systems; providing information that can be used in developing risk evaluations; (3) provide information to clinicians that will enable them to predict, diagnose, and/or provide treatment for acute exposures (Svendsen, 1994).

Long-term studies usually start with a dose-finding exercise under acute conditions. Furthermore, the information on acute systemic toxicity generated by the test is used in hazard identification and risk management in the context of production, handling, and use of chemicals (Leahy, 1997).

2.12 Sub-acute toxicity

These tests express the overall side effect observed after repeated administration of the test substance (2-6 weeks) and enable the determination of the principal behavioral changes as well as anatomical, physiological and biochemical manifestations of tissue damage provoked by the substance (WHO, 1993). Results of many sub-acute toxicity tests of various plant extracts showed that the major organs usually affected are the liver and kidneys. Hepatotoxic and nephrotoxic effects are mostly to be expected, as the liver acts as the main detoxifying organ for chemical substances, while the kidney is a principal route of excretion for many chemical substances in their active and/or inactive forms (Abdulrahman *et al*., 2007)

2.13 Cell-based cytotoxicity tests and significance of bioassays

Bioassay testing of plant extracts bioactivity in most cases correlates reasonably well with cytotoxic and anti-tumor properties (McLaughlin *et al.,* 1993). One basic premise is that toxicology is simply pharmacology at a higher dose, thus if we find toxic compounds, a lower dose might elicit a useful, pharmacological, perturbation on a physiologic system, (McLauglin, 1991).

Cell-based cytotoxicity tests are important in-vitro procedures performed for natural products. Cytotoxicity assays (CTAs) are performed to predict potential toxicity, using cultured cells which may be normal or transformed cells. (CTAs) involve short term exposure of cultured cells to test substances, to detect how basal or specialized cell functions may be affected by the substance, prior to performing safety studies in whole organisms. It can also provide insight towards the carcinogenic and genotoxic dispositions of natural products. The ability of a biological material to inhibit cellular growth and viability can also be ascertained as an indication of its toxicity. Assessment parameters for cytotoxic effects include inhibition of cell proliferation, cell viability markers (metabolic and membrane), morphologic and intracellular differentiation (O'Brien, 2006)

2.14 Effects of toxicity to an organism

Alteration in the biochemical indices will lead to impairment of normal functioning of the organs (Afolayan *et al*., 2009). Significant changes in the body weight increment alterations are indices of adverse effects of drugs and chemicals and it will be significant if the body weight loss occurred exceeds 10% from the initial body weight (Ekwall *et al*., 1994). Organ

weight changes are an important indicator of physiological and pathological damage. The heart, liver, kidney, spleen and lungs are the primary organs affected by metabolic reaction caused by toxicants. Very severe liver and kidney injury has been described after the ingestion of a large variety of different herbal preparations (Stickel *et al*., 2003). Liver assays give information about the state of the liver, describing its functionality (albumin), cellular integrity (transaminases) and its link with the biliary tract (alkaline phosphatase) (Agbaje *et al.*, 2009**).** Assessment of haematological parameters is usually used to determine the extent of harmful effect of plant extracts on the blood constituents of an animal model (Olson *et al*., 2000). Altered haematological parameters can explain altered blood relating functions of chemical compounds/plant extract (Ashafa *et al*., 2009). Hematological parameters provide information regarding the status of bone marrow activity and hemolysis (Sebastian, 2012).

2.15 *Catharanthus roseus*

2.15.1 Description of the plant

Catharanthus roseus is commonly known as "rosy periwinkle" and belongs to the family of Apocynaceae, a native of Madagascar. The plant is found commonly in tropical rainforests in other countries and is a tender, perennial plant which grows as a herb or a subshrub sprawling along the ground or standing erect (30 cm to 1 m in height) (Jaleel and Panneerselvam, 2007) Rosy periwinkle has attractive white or pink flowers comprising five petals. The leathery, dark green leaves are arranged in opposite pairs. The fruit of *C. roseus* is made up of two narrow, cylindrical follicles which house numerous grooved seeds. Like many other plants in the Apocynaceae family, the sap is a milky (latex) (Frode and Medeiros, 2008).

Figure 2.1: A photograph of aerial part of *Catharanthus roseus*

2.15.2 Uses of *C. roseus*

Catharanthus roseus is a renowned medicinal plant, and is a rich source of alkaloids, which are distributed in all parts of the plant (Sing *et al.*, 1997). It has traditionally been used to treat diverse elements such as eye inflammations, rheumatism and diabetes. Among the Luo community in Kenya, rosy periwinkle is used as an antimalarial and antidiabetic remedy (Kokwaro, 1976). Two of the dimeric alkaloids vinblastine and vincristine mainly present in the aerial parts, have found extensive application in the treatment of human neoplasm. Among the monomeric alkaloids ajmalicine (raubacine), found in the roots has been confirmed to have a broad application in the treatment of circulatory diseases, especially in the relief of obstruction of normal cerebral blood flow (Aslam *et al*., 2010). *C. roseus* exhibits high in-vitro antiplasmodial activity, which may be due to the presence of compounds such as alkaloids, terpenoids, flavonoids and sesquiterpenes that were previously isolated from the plant (Jaleel and Panneerselvam, 2007; Collu *et al*., 2001; Vimala, 2000; Hirose and Ashihara., 1994). It also possesses known antibacterial, antifungal, antibiotic, antioxidant, wound healing and antiviral activities (Prajakta *et al.,* 2010)

2.16 *Myrsine africana*

2.16.1 Plant description and distribution

M. africana is a *Myrsinaceae* and is an evergreen shrub growing to 2m at a slow rate and is native to Africa and Asia. The flowers are dioecious (individual flowers are either male or female, but only one sex is found on any one plant). *M. africana* (also called Cape Myrtle, African boxwood) typically has dense, dark-green to red foliage and produces tiny bright purple berries which are edible. *M. africana* grows well in dry areas and has a wide

distribution from the Himalayas, China, and southern Africa. It is common in the summer and winter rainfall areas (McClintock, 1994; Pooley, 2003).

Figure 2.2: A photograph showing an aerial part of *Myrsine africana*

2.16.2 Uses of *M. africana*

The seeds and roots of *M. africana* are widely used for livestock and human as an anthelmintic, especially in the treatment of tapeworms (Gathuma *et al*., 2004; Mbaabu and Matu, 2013). The crude extract of *M. africana* was found not efficacious against

Haemonchus contortus in sheep (Githiori *et al*., 2002). The plant is also used for the treatment of diarrhea, rheumatism, toothache pulmonary tuberculosis and relieving hemorrhage *(*Zhong, 1985). *M. africana* is traditionally used as a fragrance in tea, carminative, spice, appetizer and flavoring agent, (Kokwaro, 1993).

2.17 *Rosmarinus officinalis* (rosemary)

2.17.1 Plant description and distribution

R. officinalis commonly known as rosemary belongs to the *Lamiaeceae* family and is an aromatic, evergreen, shrubby herb. It grows to a height of up to 2m. *Rosmarinus officinalis* is a native Mediterranean plant which derives its name from its refreshing effects. Rosemary is a perennial plant that has great climatic adaptability. Its leaves are needle like evergreen and the flowers are white, pink, purple or blue according to the different cultivars. The plant is widely used and grown all over the world as a decorative plant gardens or even as a fence. (Lopez-Munoz *et al*., 2006)

Figure 2.3: A photograph of aerial part of *Rosmarinus Officinalis*

2.17.2 Uses of *R. officinalis*

R. officinalis is widely used as a spice when cooking, especially in Mediterranean dishes and it has naturally occurring antioxidants (Inatani *et al.*, 1983). The herb was used to strengthen memory and as a symbol of remembrance. Greek students twined rosemary in their hair when studying for exams in hope of aiding their memories according to Parkison (1567-1650). Other traditional medicinal use of rosemary includes: relieve muscle pain and spasm,

stimulate hair growth, stimulate circulatory and nervous system, increase urine flow, treat indigestion, relieve of respiratory disorders, as an analgesic, antirheumatic and antiepileptic, (Blumenthal *et al.*, 2000).

Four main categories of compounds found in Rosemary include flavonoids, phenols, volatile oil, and terpenoids (Barnes *et al.,* 2007). This plant's antioxidant properties are due to the presence of carnosic, carnosol, rosmanol, epirosmenol, and rosmarinic acids (Haraguchi *et al., 1*995). Studies have shown that rosemary plant, due to its dilatory properties, can increase blood flow and its external use have vasodilatory effects on the skin (Frishman *et al*, 2004). In addition, this plant has antispasmodic properties due to its alpha-and beta- pinenes (Taddei *et al*., 1988; Hosseinzadeh and Nourbakhsh, 1989. Rosemary has been found to have food preservative qualities (Oiye *et al.,* 2013). *Rosmarinus officinalis* essential oils or some, of their components are commonly used in make-ups, sanitary products and food preservatives (Bakirel *et al.*, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection and identification of plant materials

Rosemarinus officinalis was kindly provided by "Tarcit Energies", a farm in Meru area of Kenya which produces and sells rosemary .The fresh leaves of the *Catharanthus roseus* were collected from University of Nairobi flower gardens while *Myrsine africana seeds* were from the Samburu area of Kenya. Identification and authentication of the plants was done at the herbarium, School of Biological Sciences, University of Nairobi.

3.2 Extraction of plants materials: aqueous extracts

The plant materials (leaves of *C. roseus* and *R. officinalis* and *M. africana* seeds) were cleaned with tap water and rinsed with distilled water, air-dried at room temperature (22-26°C) to a constant weight after which they were ground to a uniform powder using an electric mill. For each plant powder, 100g was soaked each in 1L distilled water for 72 hours. The mixtures were then filtered through cotton wool and then with filter paper (125mm). These filtrates were frozen at -20^{o} C for 24 hours followed by freeze drying. The powdered extracts were weighed into sealable air tight polythene bags and stored sealed in the refrigerator at 4^{o} C. Percentage yield of extracts was assessed on w/w basis.

3.3 Acute toxicity test for *R. officinalis, M. africana* and *C. roseus* aqueous extracts

3.3.1 Laboratory animals

A total of 35 male Wistar albino rats (195 – 225 g) were required for this study. They were obtained from the animal house of the department of Biochemistry, University of Nairobi. The animals were housed in the research room in the Biochemistry department and the temperature of this room was maintained by a vanned heater at 27– 30^0C. This research room was well ventilated and maintained on light for12 hours and 12 hour darkness. The rats were provided with the standard rat pellets and clean water. They were allowed to acclimatize for 10 days prior to starting of the experiment. The initial weights of the rats were taken and they were also marked with a permanent marker on the tail.

The animal studies were in compliance to the ethical procedure for the care and use of laboratory animals approved by the "Animal care and use committee (ACUC)" of the Faculty of Veterinary Medicine University of Nairobi.

3.3.2 Baseline parameters assessment

After the 10 day acclimatization period, the rats were randomly assigned into 7 groups, 5 rats per cage. Each animal was bled aseptically from the tail vein when the animal was under restrain and 1.8ml blood was collected. About 1.3 ml of the blood was collected into EDTA tubes, mixed thoroughly for hematological measurements and the rest about 0.5ml was put into plain tubes for biochemical parameters measurements. The blood was stored at 4^0C awaiting further treatment. Hemoglobin concentration (Hb), packed cell volume (PCV), red blood cells (RBCs), white blood cells (WBCs), mean corpuscular hemoglobin concentration

(MHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and thrombocytes were analysed in an automatic hematological analyzer within six hours. The blood in the plain tubes was immediately centrifuged at 3000 revolutions per minute (r p m) for 10 minutes to extract serum which was stored at -20^{o} C for biochemical assays. Aspartate aminotransferase (AST), alanine aminotransferase (ALAT), total proteins, creatinine and serum urea were assayed for each rat using "Human" commercial kits by a UV mini spectrophotometer according to the manufacturer's protocol.

3.3.3 Oral acute toxicity testing

This was done by modifying the, OECD, 423 guidelines. The acute toxic class method set out in this guideline is a stepwise procedure with the use of 3 animals of a single sex per step. However for the sake of this study 5 animals were used per doses. The administered doses were pre-determined using 1 animal per category and found not to cause mortality or morbidity.

3.3.4 Experimental design for oral acute toxicity testing

The acute testing procedure was done 15 days after the initial bleeding. The animals were kept overnight fasting prior to extract administration of different concentrations of the aqueous plants which was constituted in 2ml distilled water. A gavage was used for the oral administration of the extracts. All rats in groups 2 and, 3 were given 1000 and 5000mg/kg body weight, respectively of *Catharanthus roseus* aqueous extract. Groups 4 and 5 were administered with 1000 and 5000mg/kg body weight of the *Myrsine africana* aqueous extract respectively. Group 6 and 7 were also administered with 1000 and 5000mg/kg body weight

of *Rosmarinus officinalis* aqueous extract respectively. Group 1 served as the control and the rats were administered with 2 ml distilled water. Food was withheld for further 3 hours post administration of the extracts. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during first 24 hours after which the number of dead rats was recorded. The live animals were observed up to the 14^{th}day for any abnormal behavior.

The animals were weighed at 48 hours, 7th and 14^{th} days using an electronic balance. Daily observations on the changes in fur, eyes and mucus membrane (nasal), respiratory rate, circulatory signs, salivation, lacrimation, defecation ptosis, drowsiness, tremor and convulsion changes were noted.

3.3.5. Animal bleeding, hematological and biochemical assays

Blood was collected again at 48 hours and 14 days post extract administration from each of the rats. Just as the first procedure, 1.8ml was collected via the tail lateral vein using a 2 ml hypodermic syringe and needle when the animal was restrained. A blood aliquot (1.3 ml) was put into the EDTA tubes and thoroughly mixed for hematological analysis. The remaining 0.5 ml of the blood was put in the plain tubes for biochemical analysis. The blood was treated and analysed as had been done initially during baseline assessment. At the end of study, day 14, the animals were anesthetized heavily with diethyl ether in the anesthesia chamber to kill them after which they were incinerated.

3.4 Sub-acute toxicity studies of *R. officinalis*

3.4.1 Experimental design in sub-acute toxicity studies of *R. officinalis*

The study was done with 20 male albino Wistar rats (175-180g). They were grouped by complete randomized design into 4 groups with 5 rats per group and each rat was housed in its own cage. The rats were fasted for 6 hours before extract administration. Group 1, the control, received 2 ml of distilled water orally using a gavage, once daily for 28 days. The second, third and fourth groups were orally given with rosemary aqueous extract (500, 1500 and 3000 mg per kg body weight) respectively once daily for 28 days. The body weight of the animals were measured weekly and recorded using an electronic balance. The animals were observed daily for any abnormal changes.

3.4.2 Blood collection and subsequent blood parameter measurements

As in acute testing, 1.8 ml blood was collected when the animals were under restrain via lateral tail vein 2 weeks prior drug administration, at days 14 and 28 using 2 ml hypodermic syringes. An aliquot of 1.3 ml was put into EDTA tube for hematological assay and 0.5 ml in plain tubes for biochemical assay. Serum for biochemical assays was collected after centrifugation at 3000 rvm for 10 minutes and stored at -20^{o} C for ALT, AST, creatinine urea and total proteins measurements. The following hematological parameters were measured using an automatic hematology analyzer: PCV, Hb, RBC, WBC, MCV MCH, MCHC and thrombocytes.

3.4.3 Procedure for differential white blood cells count.

Differential white blood cells were determined by making a one drop blood smear on a microscopic slide which was air dried and fixed with alcohol for 0.5 to 1 minute in order to prevent hemolysis. The slide was stained with Giemsa stain at pH 7.2 for 30 minutes. Differential leucocyte counts were done in an area where the morphology of the cells was clearly visible. Counting was done under the oil immersion objective by moving the slide in area including the central and peripheral of the smear. A total of 100 cells were counted in which every white cell seen was recorded in a table under the following heading: neutrophil, basophil, eosinophil, monocyte and lymphocyte. The percentage count of each type was calculated.

3.4.4 Organs harvesting

The rats were anaesthetized in a chamber using diethyl ether. Each animal was mounted on a dissecting board for organs collection. The liver, kidney, spleen, lungs and the heart in each animal were carefully removed. These organs were washed in physiological saline, blotted out, observed macroscopically and weight recorded. Percentage organ weight ratios were calculated. The organs were stored in 10% buffered formalin solution.

$$\text{Relative organ weight} = 100 \times \frac{\text{absolute organ weight}}{\text{Weight of animal at sacrificing}}$$

3.4.5 Statistical analysis

The hematological, biochemical and weight measurements results were expressed as mean ± standard deviation of the mean. One-way analysis of variance (ANOVA) was employed for

between and within group comparison. 95 % level of significance, $p \geq 0.05$, was used for the statistical analysis in acute and sub-acute toxicity studies.

3.5 Brine shrimp *(Artemia salina*) cytotoxicity test of the 3 plant extracts.

The procedure of (Meyer *et al*., 1982) was adopted to determine the lethality of plant extracts to brine shrimp.

3.5.1 Extract preparations for brine shrimp assay

Four organic and one aqueous extracts were used for this study. The organic solvents used were; hexane, DCM (dichloromethane), dichloromethane: methanol (1:1), methanol: water (95:5). 50g of each of the plants powder was soaked each in 500ml of the solvent for 48 hours, filtered using cotton wool and No.1 Watman filter paper. The filtrates were evaporated in a rotor evaporator at 40^{o}C to complete dryness. The dry powder was weighed for yield calculations. The aqueous extract was freeze dried. These extracts were stored in plastic air tight containers at 4^{0} C.

3.5.2 Culturing and harvesting of *A. salina*

A. salina (2g) cysts were incubated for hatching in a shallow rectangular dish with a plastic divider with several 2 mm holes making two unequal compartments. The container was filled with 3.3% solution of artificial sea water and 50 mg yeast sprinkled into the larger compartment which was darkened. The smaller compartment was illuminated by a tungsten filament light and gently sparged with air. After 24 hours, hatched *A. salina* cysts were

transferred to fresh artificial seawater and incubated for a further 24 hours under artificial light. The phototropic nauplii were collected by pipette from the lighted side.

3.5.3 Preparation of test extracts and the controls

Stock solutions of each of the organic and aqueous extracts (10 000 µg/ml) were made in dimethyl sulphoxide (DMSO) and deionized water respectively. The test extracts 5 µl, 50 µl, and 500 µl for 10 µg/ml, 100 µg/ml, and 1000 µg/ml respectively, were transferred into 15 ml tubes and made to 4.5 ml with the brine solution. Negative controls with 5 µl, 50 µl, and 500 µl DMSO for the organic extracts and a similar set up of de-ionized water were set alongside tests. A positive control of stock solution of etoposide (10,000 µg/ml) was prepared in deionized water and aliquots of 5 µl, 50 µl, and 500 µl for 10 µg/ml, 100 µg/ml, and 1000 µg/ml, respectively were transferred into 15 ml tubes and made up to 4.5 with brine solution. The tests and the controls were done in triplicates.

3.5.4 Brine shrimps bioassay.

A. salina nauplii (10*)* were counted macroscopically in the stem of a Pasteur pipette against a lighted background and transferred into each sample vial and the solutions were made to 5ml with brine solution. A drop of dry yeast suspension was added as food to each vial. All the vials were maintained under illumination. The surviving nauplii were counted with the aid of a 3x magnifying glass after 24 hours. Percentage mortality at the three dose levels and control were determined. The surviving nauplii were killed by the addition of 100 µl of 5% (v/v) phenol to each vial.

3.6 Statistical analysis to determine brine shrimps LC_{50} of the extracts

A regression line equation was derived for each extract from a trend line of probit against Log_{10} concentrations. This was respectively used to calculate the LC_{50} value (Finney, 1971).

3.7 Phytochemical screening

Phytochemical screening was performed as described by (Harborne, 1998; Evans, 2002).

3.7.1 Test for anthraquinones

A sample (0.5g) of the aqueous extract was boiled with 10 ml of sulphuric acid, H_2SO_4, and filtered while hot. The filtrate was shaken with 5 ml of chloroform and the chloroform layer pipetted into another test tube after which 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes. A yellow colour indicated presence of anthraquinones (Evans, 2002).

3.7.2 Test for terpenoids (Salkowski test)

To a 0.5g sample of the extract was added 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids (Harborne, 1998)

3.7.3 Test for flavonoids

Dilute ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 ml) was also added. A yellow colouration that disappeared on standing indicated the presence of flavonoids. A second method entailed adding a few drops

of 1% Aluminum a portion of the filtrate. A yellow colouration indicated the presence of flavonoids. A third a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 minutes. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution and observed for a yellow colouration (Harborne, 1998).

3.7.4 Test for reducing sugars (Fehling's test)

Equal volume of Fehling A and Fehling B reagents were mixed together. A 2 ml portion of the mixture was added 1ml of crude extract solution of each plant and gently boiled. A brick red precipitate appeared at the bottom of the test tube that indicated the presence of reducing sugars (Harborne, 1998).

3.7.5 Test for saponins

To a 0.5 g of extract for each plant was added 5 ml of distilled water in a test tube. The solution was shaken and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion. (Harborne, 1998).

3.7.6 Test for tannins

About 0.5 g of the extract was boiled in 10 ml of water in a test tube and then filtered.

A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration which was positive for tannins (Evans, 2002)

3.7.7 Test for alkaloids

About 0.5 g of extract was diluted to 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate (2 ml) of dilute ammonia was added. Chloroform (5ml) was then added and shaken to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids (Evans, 2002).

3.7.8 Test for cardiac glycosides (Keller-Killiani test)

A sample of the extract (0.5g) was mixed with 5 ml distilled water and 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring in some cases appeared below the brown ring, while in the acetic acid layer a greenish ring formed just above the brown ring and gradually spread throughout these layers. This indicated presence of cardiac glycosides (Harborne, 1998).

CHAPTER FOUR

4.0 RESULTS

Table 4.1: Aqueous extract yields

plant	Weight of powder (g)	Amount after freeze drying	% yield
C. roseus	300	27.6	9.2
M. africana	300	17.4	5.8
R. officinalis	1000	62	6.2

4.1 Cage side observations:

No mortality or clinical signs observed in rats administered with the aqueous extracts of *C. roseus, M. africana or R. officinalis* at 10000 and 5000 mg/kg bwt up to the 14^{th} day. General behavior of the rats was found to be normal throughout the study period.

4.2 Hematological and biochemical results in acute toxicity

Reference for treatment groups in acute toxicity testing

Groups

2 and 3 -*C. roseus* 1000 and 5000 mg/kg bwt respectively

4 and 5- *M. africana* 1000 and 5000 mg/kg bwt respectively

6 and 7- *R. officinalis* 1000 and 5000 mg/kg bwt respectively

4.2.1 Hematological results in acute toxicity

At 48 hours testing the, mean WBC in group 2, rats treated with *C. roseus* (1000 mg/kg bwt), was significantly elevated than the control group, $P < 0.5$. This group had the highest mean WBC of 29.0 ± 12.2 m/mm^3, figure 4.1. Group 5, fed with *R. officinaisl*, 5000 mg/kg bwt, had the lowest mean WBC of 7.1 m/mm^3 but at day 14, this level had gone to baseline. There was no significant difference in WBC in the groups treated with *M. africana*, $P > 0.05$, (figure 4.1).

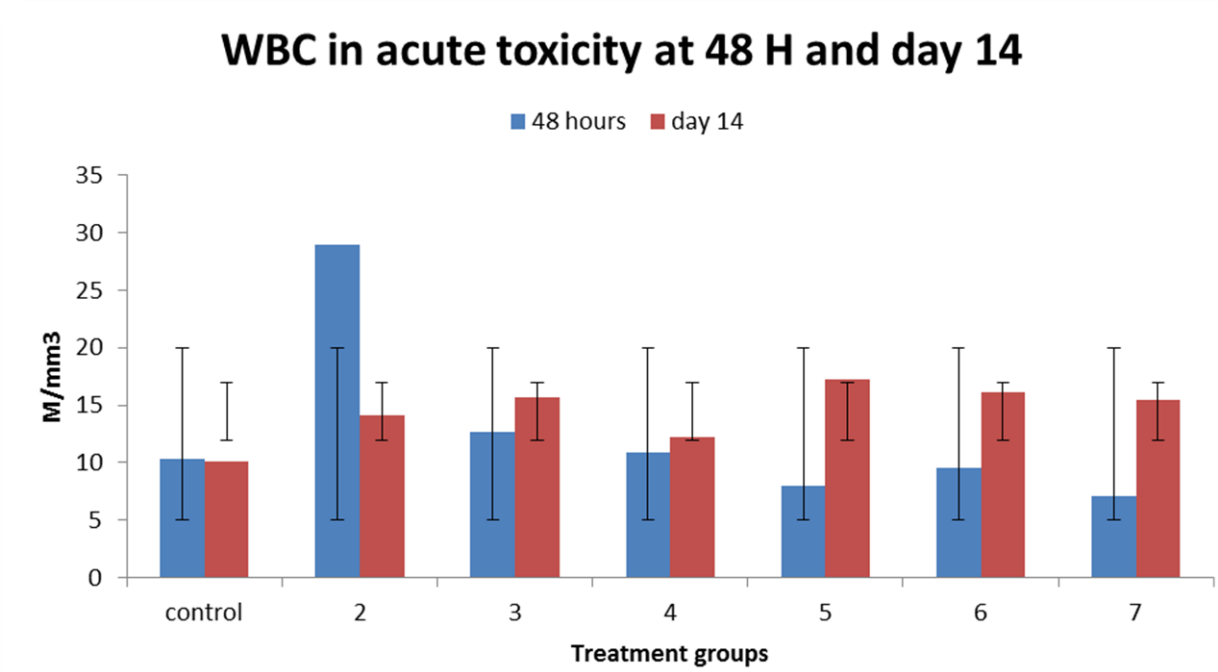

Figure 1.1: Effects of white blood cells counts by the 3 plants extracts

The mean red blood cells (RBC) in group 5 (*M. africana*-5000mg) at 48 hours was significantly higher than the control and all the other treatment groups ($p < 0.05$) with a mean of 7.6 m/mm^3 while the control had 5.8 m/mm^3, figure 4.2. This value had normalised at day 14 testing.

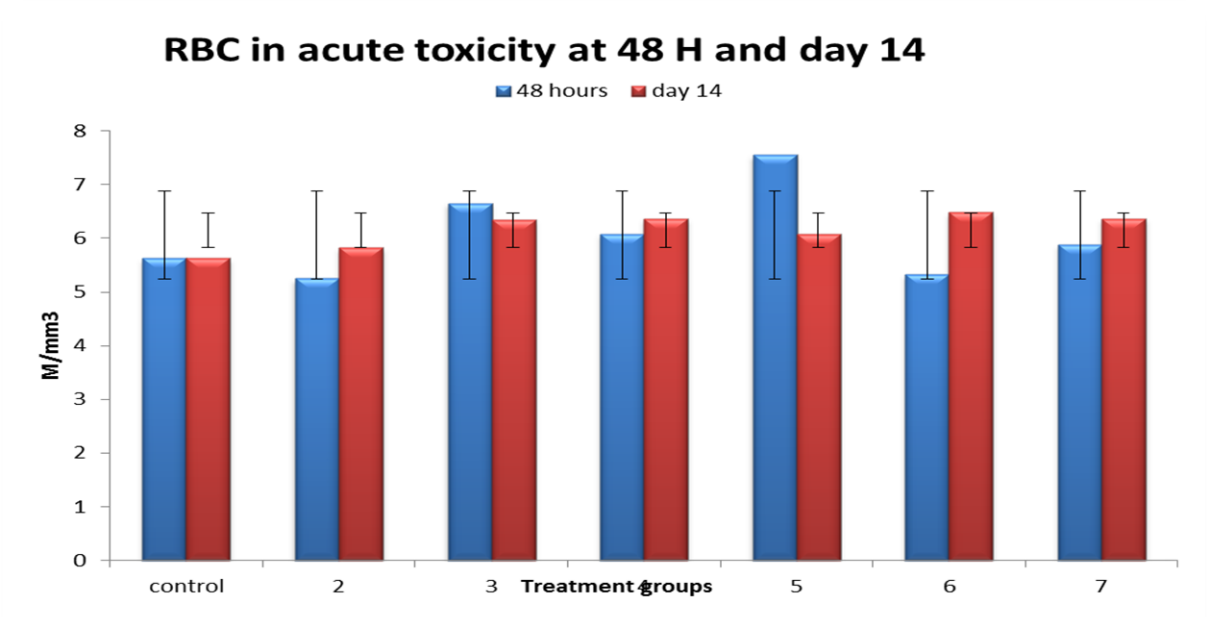

Figure 4.2: Effects of red blood cells counts by the 3 plants extracts

At 48 hours, group 5, treated with *M. africana* 5000 mg/kg bwt, had a significant increased packed cells volume, PCV, $P < 0.05$ (figure 4.3), with the highest mean of 44.08 % when the untreated group had a mean of 34.4 %. The rest of the groups both at 48 hours and day 14 had no significant difference with the control.

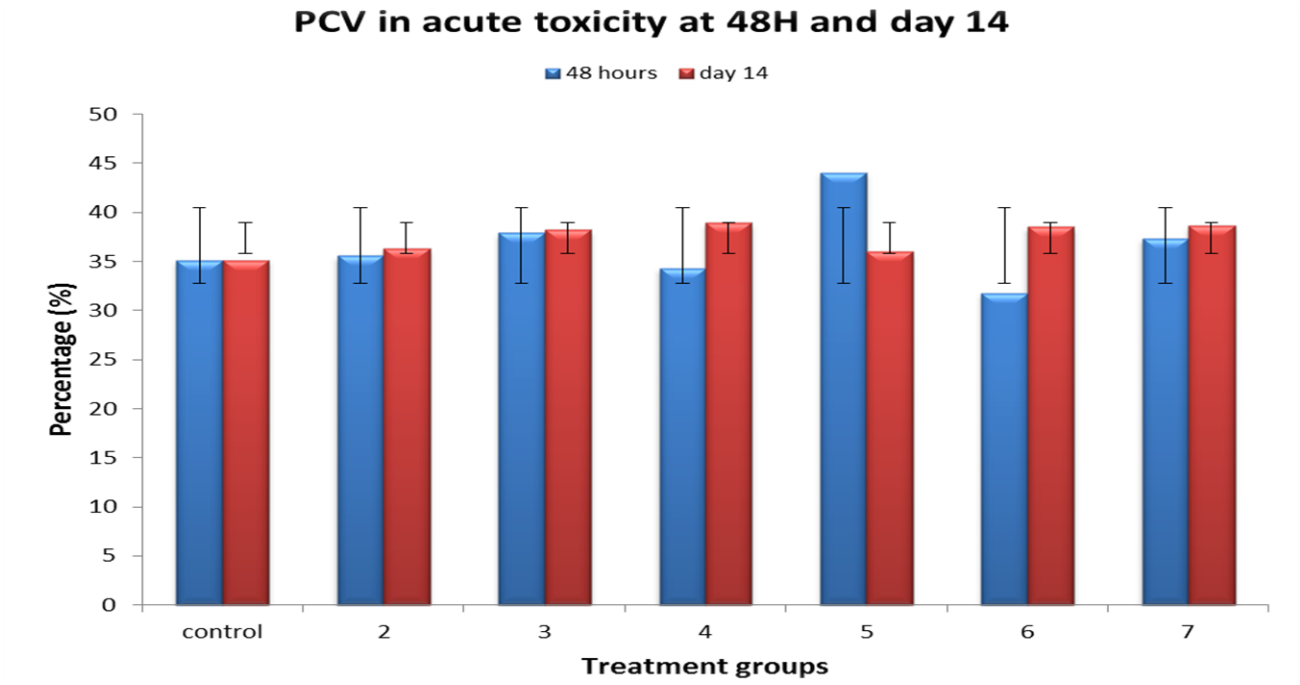

Figure 4.3: Effects of packed cells volume by the 3 plants extracts:

At 48 hours, group 3 (5000 mg/kg bwt *C. roseus)* had significantly low mean corpuscular volume (MCV) than all the other treatment groups with 22.9 ±18.3 g/l while the control group had 63.8 ±0.6 g/l. Other groups had no significant difference with the control at day 14, figure 4.4.

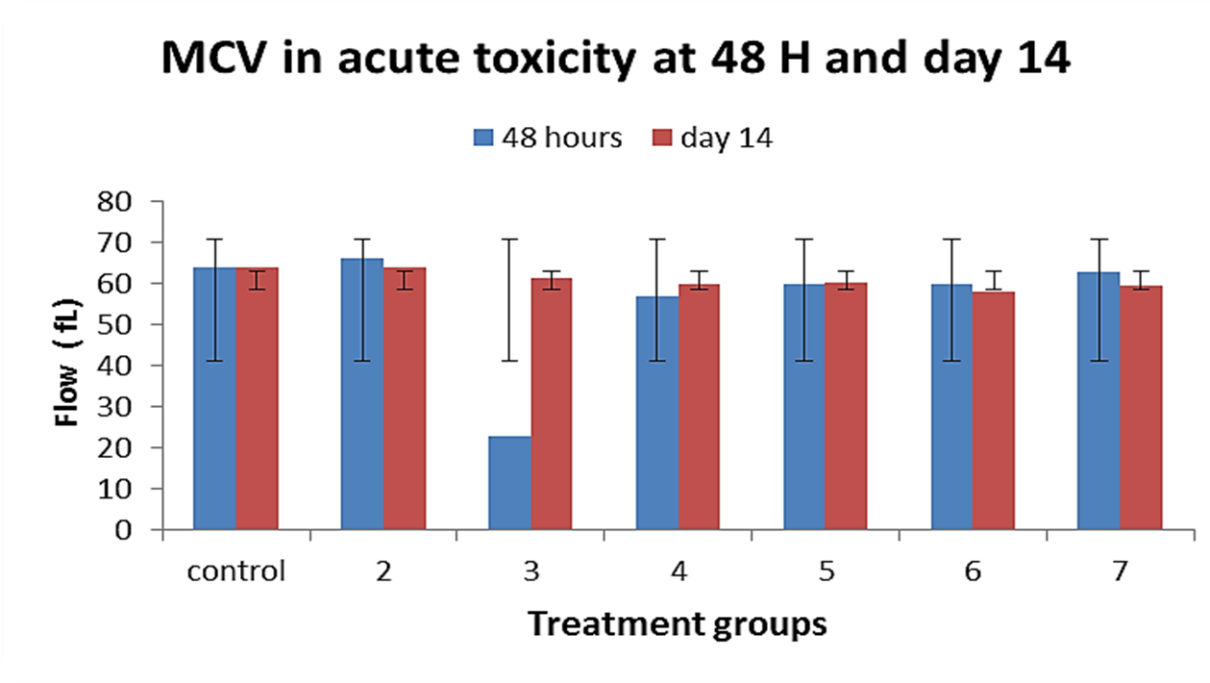

Figure 4.4: Effect of the mean corpuscular volume by plants extracts in acute toxicity

The mean corpuscular hemoglobin concentration (MCHC) values were significantly increased in group 4, treated with *M. africana* 1000 mg/kg bwt, with a mean of 43.5% at 48 hours. This value reduced at day 14 to 39.4%. The rest of the groups had no significant difference with the control at 48 hours or at day 14 (figure 4.5).

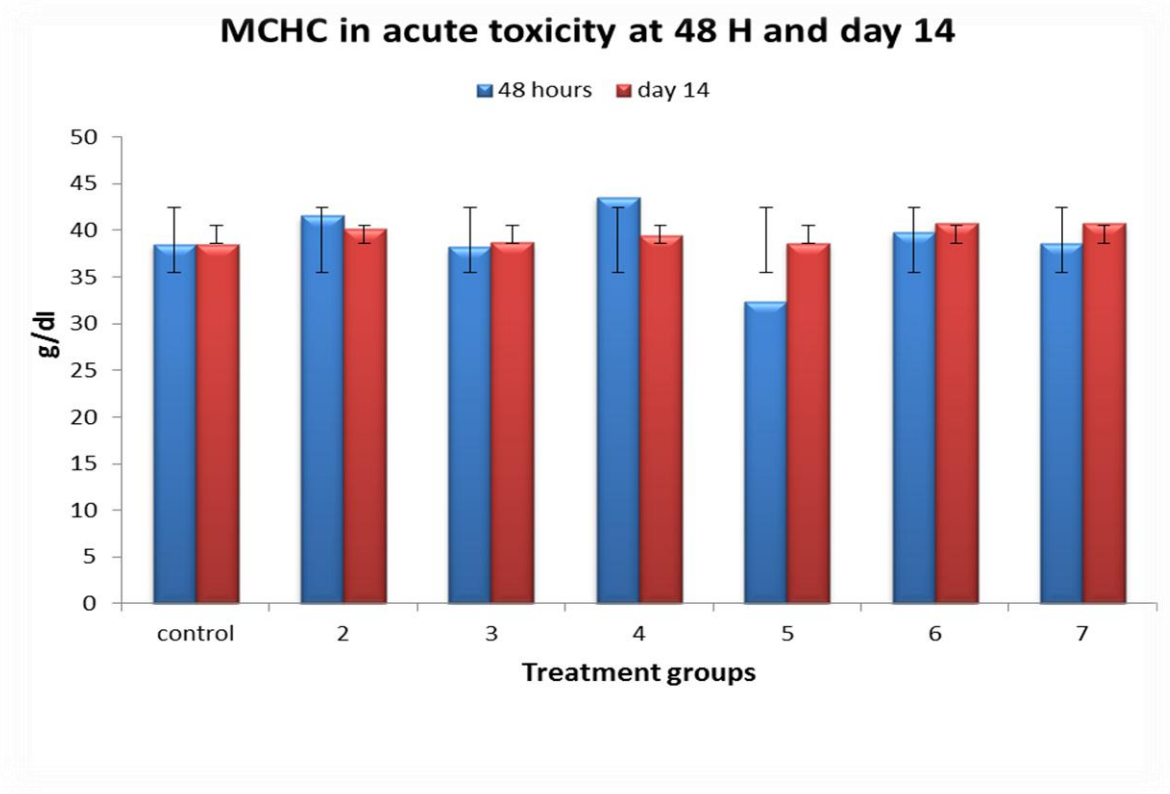

Figure 4.5: Effects of the mean corpuscular hemoglobin concentration by the plants extracts in acute toxicity

At 48 hours, the group treated with *C. roseus*, 1000 m*g*/kg bwt (group 2), had a significantly decreased thrombocyte level compared with the other treatment groups, with a mean of 188 m/mm^3 and a p value of 0.03. This value had normalized at day 14. A significant increase of thrombocytes, $P < 0.05$, was observed in groups 5 and 6 *(M. africana* 5000 and. R. *officinalis* 1000 mg /kg bwt respectively), with mean values of 589 and 529 respectively (figure 4.6).

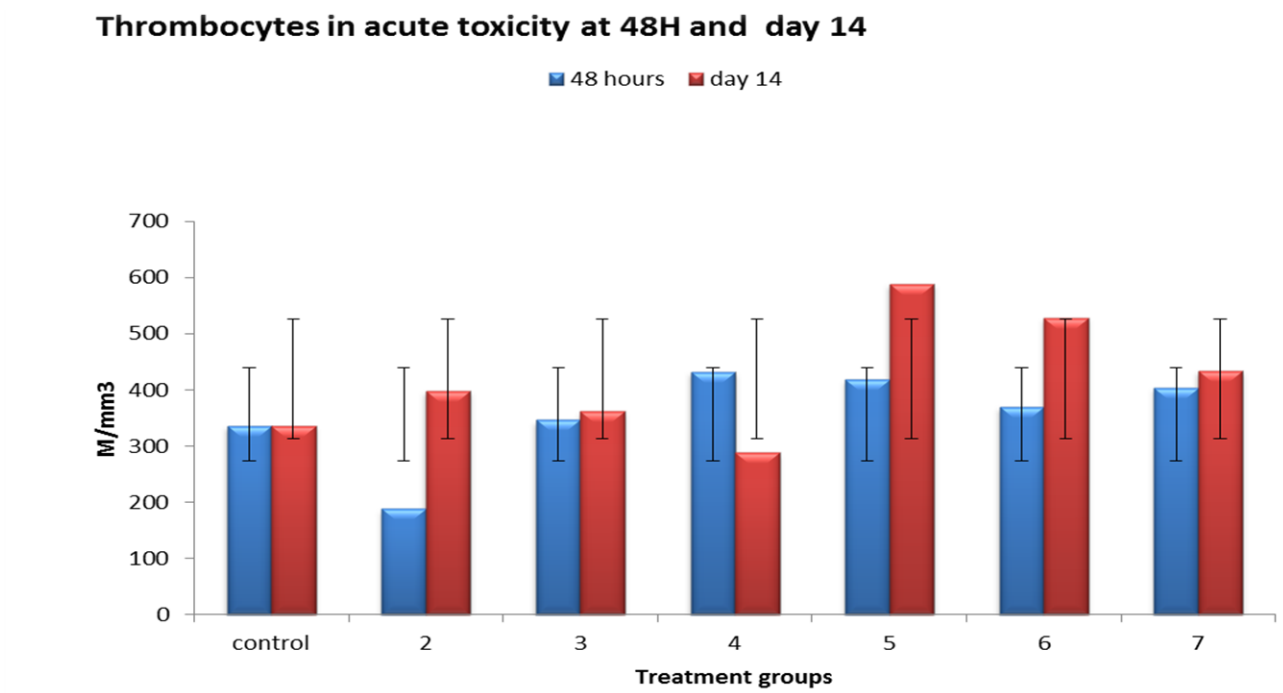

Figure 4.6: Effects of thrombocytes count by the 3 plants extracts in acute toxicity

Hemoglobin (HB) and mean cell hemoglobin (MCH) were not significantly affected by the extracts in all groups both at 48 hours and 14 ^{the} day testing, $P > 0.05$, tables 4.2 and 4.3.

Table 4.2: showing the non-significant effects of hemoglobin by the plants extracts.

Group	Extract	HB at 48 H **(g/l)**	p value	HB at day 14 (g/)l	p value
1	control	14.0 ± 0.8		13.9 ± 1.0	
2	*C. roseus* 1000mg	14.354± 0.4	1.0	14.76 ± 0.8	0.9
3	*C. roseus* 5000mg	14.54 ± 0.5	1.0	14.86 ± 0.5	1.0
4	*M. africana* 1000mg	13.86 ± 2.5	1.0	15.24 ± 0.9	1.0
5	*M. africana* 5000mg	14.48 ± 0.3	1.0	14.48 ± 1.6	1.0
6	*R. officinalis* 1000mg	12.74 ± 2.0	1.0	15.26 ± 0.4	0.9
7	*R. officinalis 5000g*	14.42 ± 0.5	1.0	15.96 ± 0.9	0.9

Table 4.3: showing the non-significant effects of MCH by the plants extracts

Group		MCH at 48 H **(p/g)**	p value	MHC at day **14 (p/g)**	p value
1	control	23.92 ± 1.2		24.02± 1.4	
2	*C.roseus* 1000mg	27.34 ± 0.6	0.9	25.7 ± 1.4	0.8
3	*C. roseus* 5000mg	22.12 ± 0.2	0.9	24.2 ± 0.9	0.8
4	*M.africana* 1000mg	24. 3 ± 1.2	0.9	23.24 ± 0.6	0.8
5	*M.africana* 5000mg	22.48 ± 3.0	0.9	22.5 ± 0.4	0.8
6	*R.officinalis* 1000mg	23.78 ± 1.5	0.9	23.02 ± 0.7	0.8
7	*R.officinalis* 5000mg	24.16 ± 0.4	0.9	24.05 ± 0.6	0.8

4.2.2 Biochemical results in acute toxicity of albino Wistar Rats.

At 48 hours, the groups treated with *C. roseus* and *R. officinalis (*5000 mg/kg bwt) (groups 3 and 7) showed significantly low ALAT, $p < 0.05$, compared with the control group, (figure 4.7).

At day 14, these levels had gone up and were as baseline levels. Group 3 (*C. roseus* 5000 mg/kg) showed significantly high ALAT, $p = 0.01$ at day14. Groups treated with *M. africana (*groups 4 and 5) had no ALAT significant difference, $p > 0.05$, (figure 4.7).

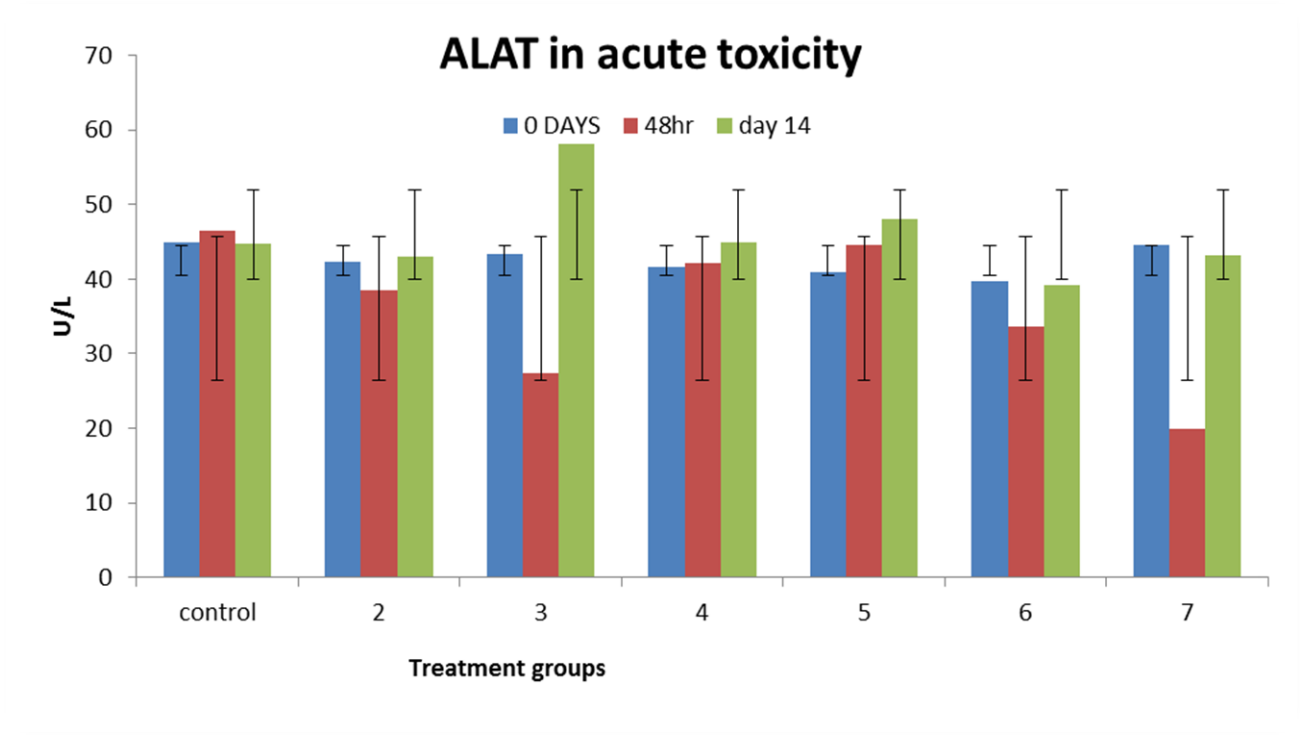

Figure 4.7: Effects of alanine aminotransferase by the 3 plants extracts in acute toxicity

Figure 4.8 shows that the three plants extracts did not have any significant difference of AST at 48 hours, $p < 0.05$, but at day 14 there was significant increase in the levels of AST in groups 3, 4 and 5 (*C. roseus* -1000 mg, *M. africana* 1000 and 5000 mg/kg bwt respectively). *R. officinalis* did not affect the rat AST significantly

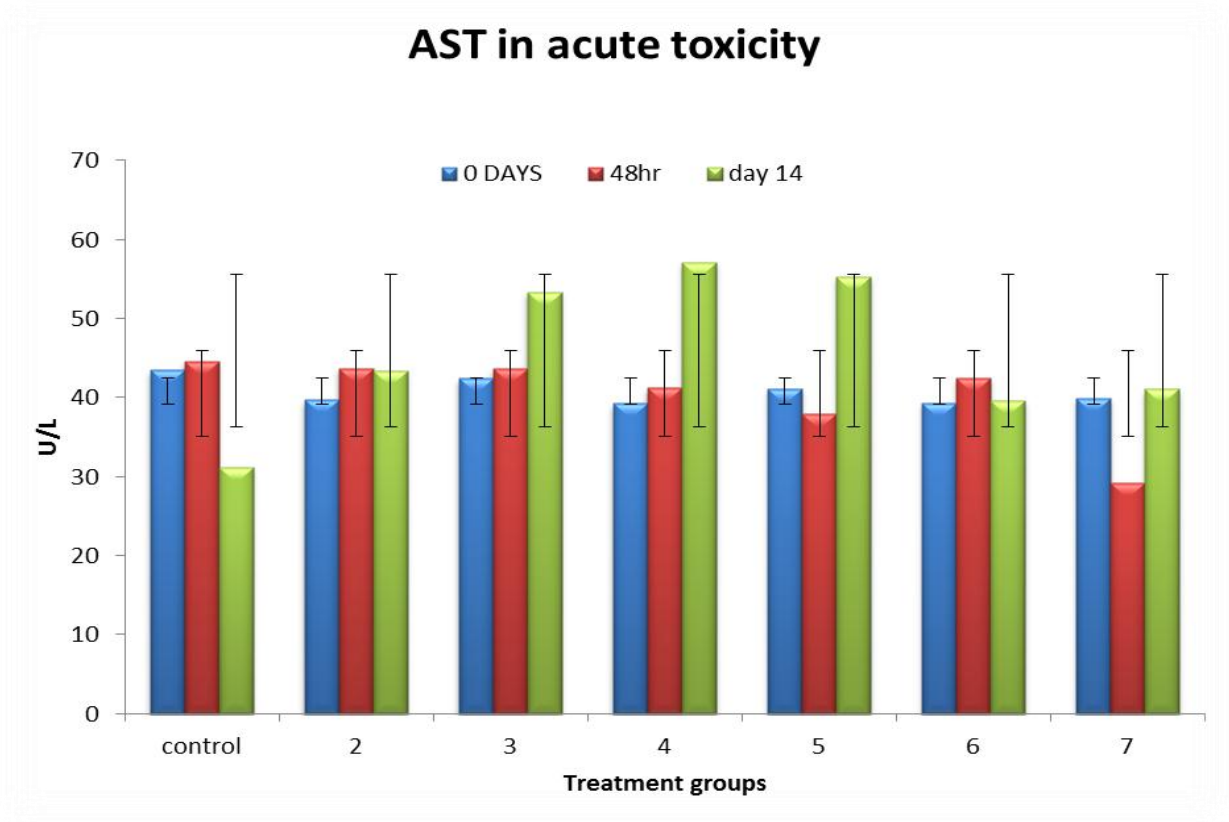

Figure 4.8: effects of aspartate aminotransferase by the 3 plants extracts in acute toxicity

At 48 hours, there was significant increase in the mean creatinine, $P < 0.05$ in groups 3, treated with *C. roseus* and group 5, *M. africana* 5000 mg /kg bwt, figure 4.9. These groups had means of 16.6 and 15.6 mg/ dl respectively. The creatinine levels eventually became low at day 14 and showed no significant difference with the control $p > 0.05$, figure 4.9.

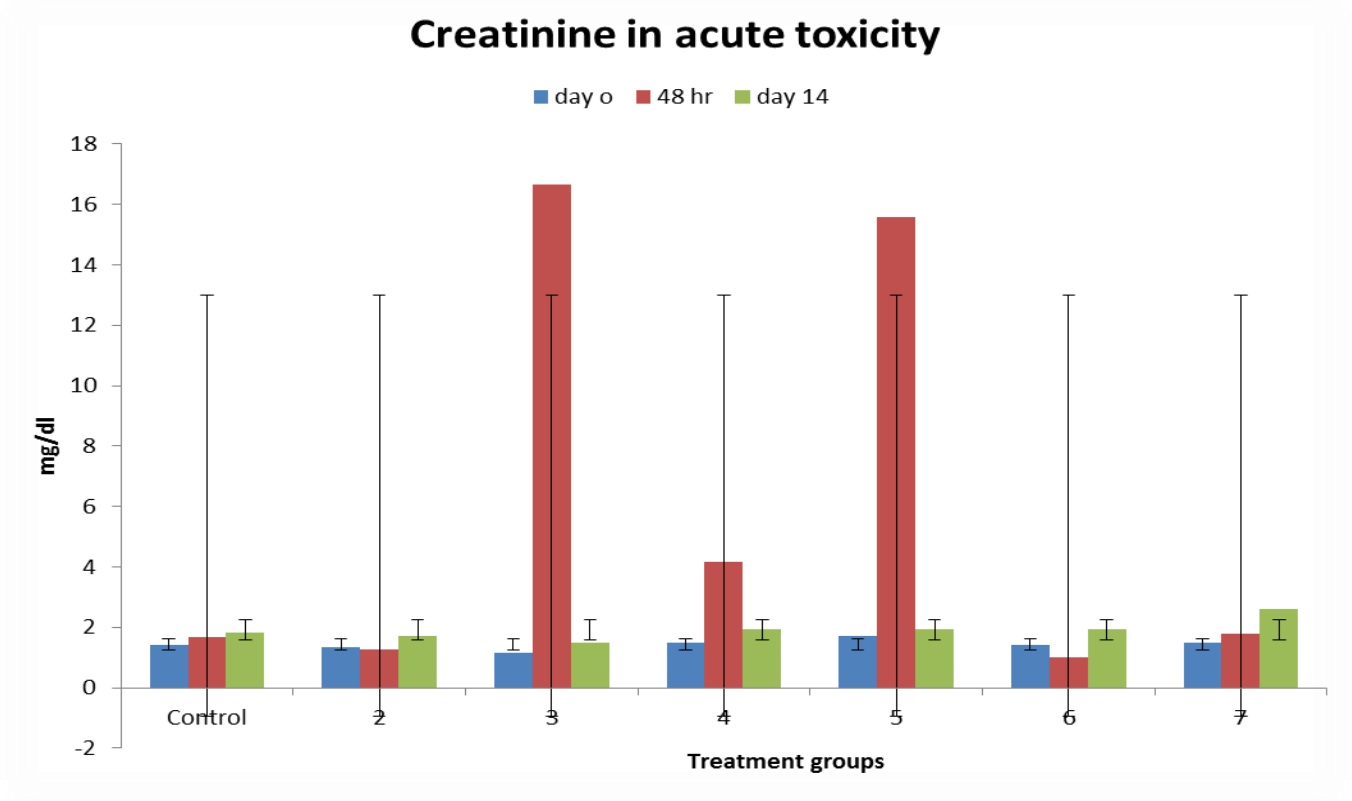

Figure 4.9: Effects of serum creatinine by the 3 plants extracts in acute toxicity

Figure 4.10 shows that serum urea in the group 3 (treated with *C. roseus* 5000mg/kg bwt) was the only significantly elevated at 48 hours among the treated groups with a p value of 0.04 and a mean of 56.1 mg/dl while control had a mean of 42.9 mg/dl. At day 14 the mean urea for this group had gone to normal values. Groups 2, treated with C. *roseus* 1000 mg/kg bwt had the highest mean urea at day 14 with, 70.6mg/dl which was significantly different from the control,

P = 0. Groups 4 and 5 (*M. africana* 1000 and 5000 mg/kg bwt) had 21.8 and 27.8 mg/dl respectively, $p \leq 0.05$, being significantly decreased than the control, figure 4.10.

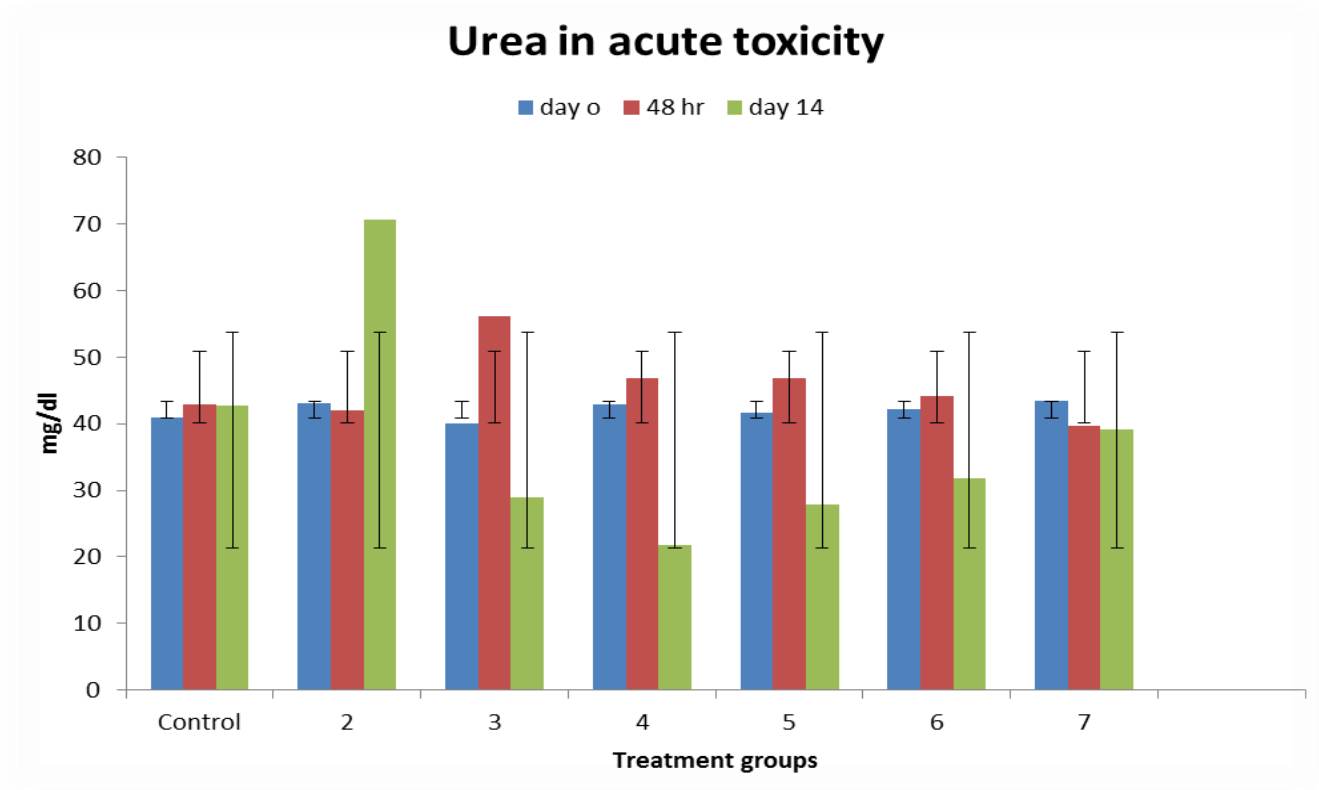

Figure 4.10: Effects of serum urea by the 3 plants extracts in acute toxicity

There was no significant difference in the mean total proteins in all the treatment groups, p > 0.05 (table 4.4)

Table 4.4: showing the non-significant effects of total proteins by the 3 plants extracts in acute toxicity.

Group	Extract	Total proteins 48 H **(g/dl)**		P value	Total proteins day 14 **(g/dl)**		p value
1	control	7.7 ±	0.5		8.02 ±	1.5	
2	*C .roseus* 1000mg	7.98 ±	1.1	1	5.86 ±	1.6	0.3
3	*C. roseus* 5000mg	7.84 ±	1.0	1	6.2 ±	1.1	0.5
4	*M. africana* 1000mg	8.18 ±	0.9	1.00	8.64 ±	1.8	1.0
5	*M. africana* 5000mg	7.5 ±	0.9	1.00	6.48 ±	0.8	0.7
6	*R. officinalis* 1000mg	8.72 ±	2.1	0.9	8.72 ±	2.2	1.0
7	*R. officinalis* 5000g	7.94 ±	2.1	1	7.48 ±	1.0	1.0

4.2.3 Total weight gain

Percentage weight gain was significantly low in groups 2 and 3 (treated with C. *roseus,* 1000 and 5000 mg/kg bwt), with means of 3.7 and 4.7% respectively at P < 0.05 testing. The highest weight gain was in group 6, treated with *R. officinalis* 1000 mg/kg bwt with 8.9%, figure 4.11.

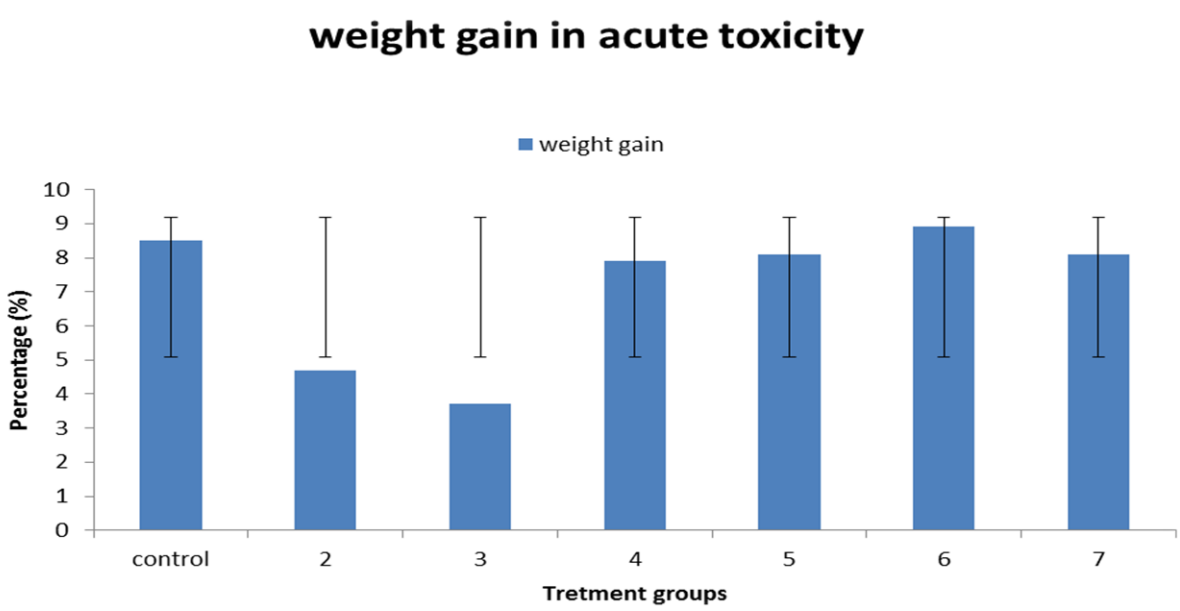

Figure 4.11: percentage weight gain of rats treated with the 3 plants extracts in acute toxicity.

4.3 Sub-Acute results

4.3.1 Observations in sub-acute testing of *R. officinalis* in Wistar rats.

The general behavior of the rats was found to be normal throughout the study period**.** Macroscopic examination did not show any changes in the colour of organs of the treated animals compared with the untreated animals. There was no change observed in the feeding behavior.

4.3.2 Hematological analysis in sub-acute toxicity.

One way ANOVA on hematological measurement showed no significant difference between the non-treated groups (baseline) and the control group, $p > 0.05$.

At the 14th day measurements, mean WBC of group 4 (3000mg/kg bwt) was found to be significantly different from the control group with a p value of 0.01 and a mean of 24.0 m/mm^3 while the control had 15 m/mm^3, table 4.5. At day 28, there was still a significant elevation of the WBC, p =0.08, table 8. The mean WBC was also found to increase as the dose increased both at day 14 and day 28, tables 4.6 and 8. HB, MCV, MCH, MCHC, PCV, RBC means showed no significant difference P > 0.05 in sub-acute oral toxicity testing of *R. officinalis* neither at 14 or 28 day testing, table 4.5 and 466.

Table 4.5: Hematological parameters at 14th day in sub-acute toxicity

	Control		500 mg/kg bwt		1500 mg/kg bwt		3000mg/kg bwt	
WBC	15.6 ±	2.5	13.2 ±	5.6	20.3 ±		*27.4 ±	2.1
RBC	5.9 ±	0.6	6.0 ±	0.3	5.8 ±	0.6	5.3 ±	0.7
PCV	36.6 ±	4.4	37.0 ±	2.4	36.5 ±	3.7	39 ±	1.9
Hb	14.3 ±	1.0	15 ±	0.5	14.0 ±	1.3	13.9 ±	1.0
MCV	62.2 ±	2.0	62.2 ±	1.6	63.6 ±	1.7	63.7 ±	2.0
MCH	24.0 ±	1.6	24 ±	0.8	24 ±	1.1	24 ±	0.7
MCHC	39.2 ±	2.4	39.4 ±	1.1	38.4 ±	1.3	38.6 ±	0.6
Thromb	235 ±	32.1	277 ±	24.6	257 ±	31.2	221.2 ±	23.8

***Showing parameter with significant difference**

Table 4.6: hematological parameters at 28th day in sub-acute toxicity

	Control		500 mg/kg bwt		1500 mg/kg bwt		3000 mg/kg bwt	
WBC	17.5 ±	5.8	17.9 ±	4.2	17.1 ±	5.0	*24.1	2.1
RBC	6.0 ±	0.3	5.6 ±	0.4	5.9 ±	0.2	5.7 ±	0.3
PCV	38.64 ±	1.5	36.78 ±	0.6	37.04 ±	1.5	38.7 ±	0.8
Hb	15.4 ±	0.4	22.2 ±	16.1	14.5 ±	0.7	14.6 ±	1.1
MCV	64.3 ±	0.9	64.4 ±	1.3	63.9 ±	3.2	62.4 ±	0.9
MCH	25 ±	0.8	26 ±	0.4	25 ±	1.2	24 ±	1.2
MCHC	39.7 ±	0.9	40.7 ±	0.5	39 ±	0.7	39.1 ±	0.8
Thromb	233 ±	49.7	239 ±	39.0	209 ±	57.4	213 ±	23.3

***Showing parameter with significant difference**

Analysis of differential cells namely: total neutrophils, eosophils, lymphocytes and monocytes a showed no significant difference with the control at both 14 and 28 days, P > 0.0.5, except at day 14, the lymphocytes in the group 4, treated with 3000 mg/kg bwt were significantly elevated, table 4.7.

Table 4.7: Differential cells count in sub-acute toxicity of *R. officinalis* of the 3 doses

%	Neutrophils		Lymphocytes		Eosinophils		Monocytes	
1-Control-day 14	18.2 ±	2.8	80.4 ±	3.9	1.2 ±	1.8	0.2±	0.4
2-500mg-14 days	19.6 ±	2.3	79.0 ±	1.6	1.2 ±	1.3	0.4±	0.5
3-1500mg-14 days	14.6 ±	5.5	84.6 ±	5.9	1.4 ±	3.1	0.8±	1.3
4-3000mg-14 1-days	16.4 ±	4.0	*91.8 ±	4.3	2.0 ±	2.3	1.0±	1.2
1-Control-day 28	20.0 ±	9.9	80.4 ±	3.9	1.2 ±	1.1	0.0	0.0
2-500mg-day 28	20.8 ±	19.9	74.2 ±	15.4	0.4 ±	0.5	0.6±	0.9
3-1500mg-day 28	18.2 ±	9.8	82.8 ±	6.3	1.2 ±	1.3	0.4±	0.9
4-3000mg-28 days	20.8 ±	2.8	85.8 ±	4.3	1.2±	1.3	1.0±	1.2

*** showing significantly different from control**

4.3.3 Biochemical parameter analysis in Sub-acute toxicity of *R. officinalis.*

At day 28, the ALAT levels were significantly different in 1500 and 3000 mg/kg bwt groups from the control, $p < 0.05$. There was no significant difference of ALAT with the control in groups treated 500 mg/kg bwt groups, figure 4.12.

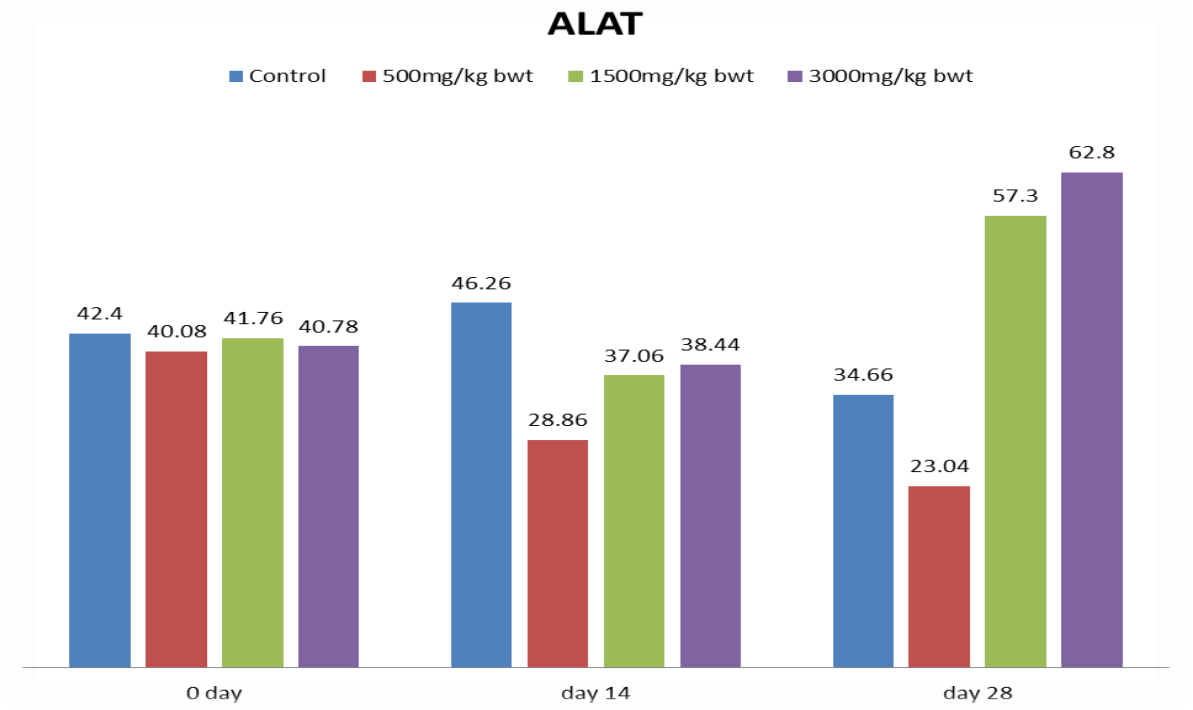

Figure 4.12: Alanine aminotransferase: in sub-acute toxicity of *R. officinalis* of the three doses

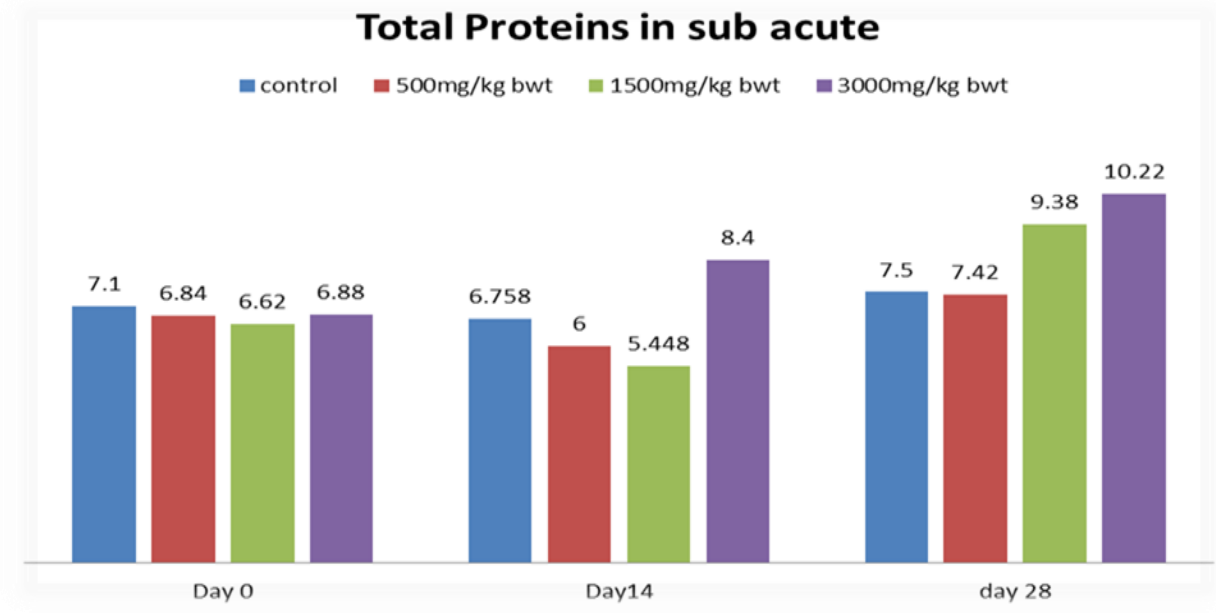

Figure 4.13: Total proteins in sub-acute toxicity testing of *R. officinalis* of the three dose

AST, urea and creatinine were not significant different from the control $p > 0.05$ in all groups, table 4.8. At 14^{th} and 28^{th} days testing, the total proteins in group 4, was significantly different from the control p =.021 and .019 respectively, table figure 4.13.

Table 4.8: showing non-significantly different mean AST, creatinine and urea levels in sub-acute toxicity

groups	AST		creatinine		urea	
1 -Control-day 14	57.7±	3.2	1.2 ±	0.1	39.9 ±	7.8
2-500mg/kg bwt-day 14	49.8 ±	8.8	1.3 ±	0.2	40.8 ±	8.4
3-1500mg/kg bwt-day 14	45.1 ±	12.7	1.0 ±	0.2	43.6 ±	4.9
4-3000mg/kg bwt-day 14	45.5 ±	6.9	1.4 ±	0.2	44.9 ±	3.3
1-Control-day 28	52.6 ±	3.4	1.2 ±	0.2	42.3 ±	6.7
2-500mg/kg bwt-day28	50.7 ±	3.5	1.4 ±	0.1	45.4 ±	14.8
3-1500mg/kg bwt-day 28	50.9 ±	5.7	1.5 ±	0.4	46.0 ±	14.6
4-3000mg/kg bwt-day 28	50.2 ±	4.3	1.7 ±	0.1	47.5 ±	2.1

4.3.4. Mean weight change in sub-acute toxicity testing of *R. officinalis.*

There was no significant difference in mean weight change of the treated groups with the control in sub-acute testing, $P > 0.05$, figure 4.14.

4.3.4.1 Organ weights

There was no treatment group that showed significant difference in absolute organ ratio in any of the organs compared to those of the control, $p > 0.05$ (figure 4.15).

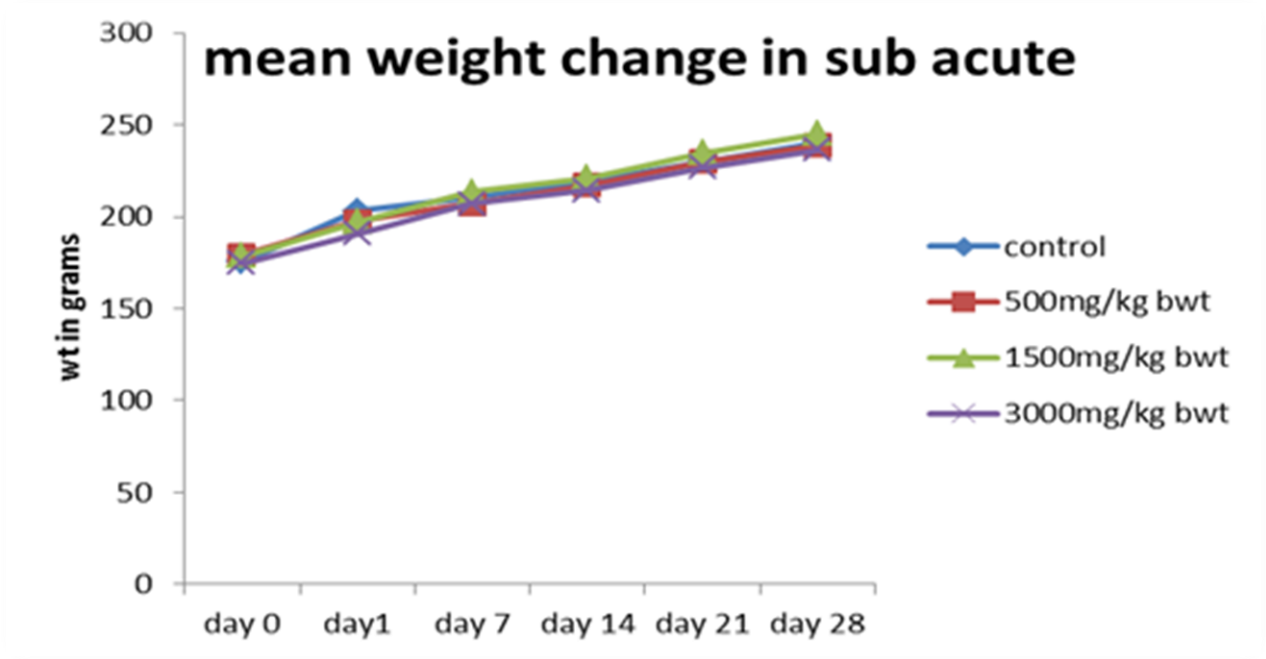

Figure 4.14: Weight profile in sub-acute toxicity testing of *R.officinalis* of the three doses.

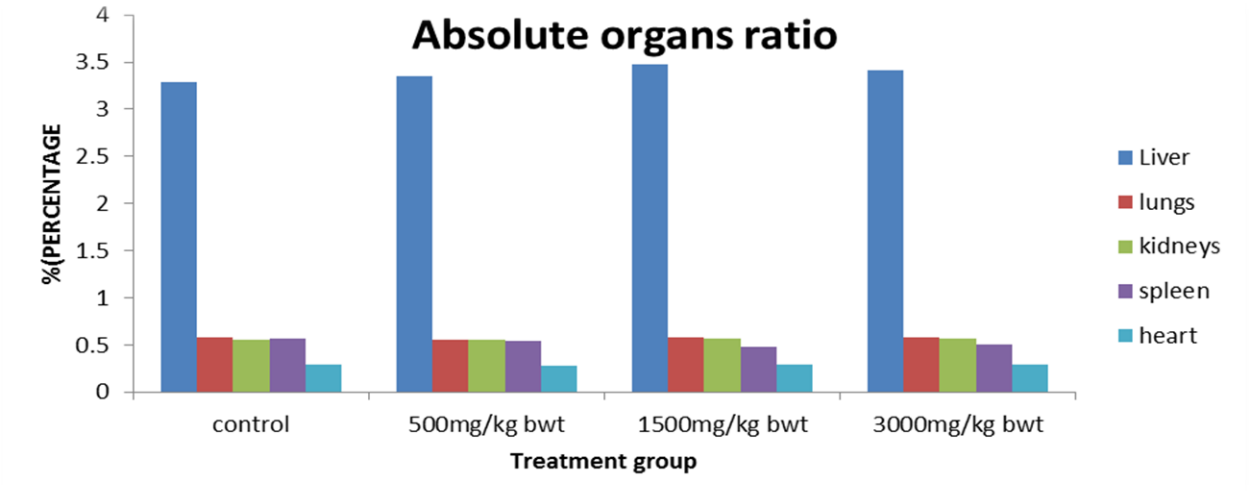

Figure 4.15: Effects on absolute organ weight ratios in different treatment groups in sub-acute toxicity testing of *R. officinalis*.

4.4 Results of brine shrimp assay for the 3 plants organic and the aqueous extracts.

Table 4.9: Percentage extraction yields for organic extraction:

% yield of Extract	n-hexane	DCM	DCM:methanol(1:1)	methanol: water (95:5)
C. roseus	2	8	14	22
M. africana	2.2	10.4	10	14
R .officinalis	4	12	20	18

Table 4.10: LC_{50} of different extracts of *C. roseus,M. africana* and *R. officinalis* (Appendix 1)

Extract/LC_{50}	hexane	DCM	DCM:methanol	methanol:water	aqueous
C. roseus	159.8	498.5	498.5	3.05	268.5
M. africana	498.5	119.5	498.5	0.34	1820
R. officinalis	498.5	168.8	227.3	498.5	498.5

4.4.1Analysis of brine shrimp assay

4.4.1.1 Controls

The negative controls did not show any mortality of the brine shrimps. The positive controls (etoposides) showed 100, 86.6 and 73.3% mortality corresponding to 1000, 100 and 10 μg/ ml respectively.

4.4.1.2 Brine shrimp cytotoxicity in n-hexane extract

C. roseus hexane extract was the most cytotoxicity with an LC_{50} value 159.78 μg/ ml while *M. africana* and *R. officinalis* each had an LC_{50} of 498.52, table 4.10. All the plants extracts gave 100% mortality at 1000 μg/ml concentration,figures 4.16 ,4.17 and 4.18.

4.4.1.3 Brine shrimp cytotoxicity in dicloromethane (DCM) extracts

Highest cytotoxicity (lowest LC_{50}) value was at the *M. africana* extract (119 μg/ml) followed by *R. officinalis,* 168 μg/ml and *C. roseus* μg/ml 498.5 μg/ml,table 4.10. (100%) mortality was found to be in all extracts at 1000 μg/ml**.**

4.4.1.4 Brine shrimp cytotoxicity in DCM: methanol (1:1)

Highest cytotoxicity was found to be in *R. officinalis* extract LC_{50} of 227.27 μg/ml with 10 % mortality at 100 μg/ml, figure 4.18.

4.4.1.5 Brine shrimp cytotoxicity in methanol:water extract (95:5)

This solvent extract recorded the lowest LC_{50} value among the five different extracts, signifying the strongest cytotoxicity which was with *M. africana* at less than 1 μg/ml followed by *C. roseus* at 3 μg/ml, table 4.10.4.4.1.6 Brine shrimp cytotoxicity in aqueous extract *C. roseus* had the lowest LC_{50}, 265.86 μg/ml in this group of extract followed by *R. officinalis* at 498.5 μg/ml and M. africana being the least cytotoxicity with over 1820.5, table 4.10.

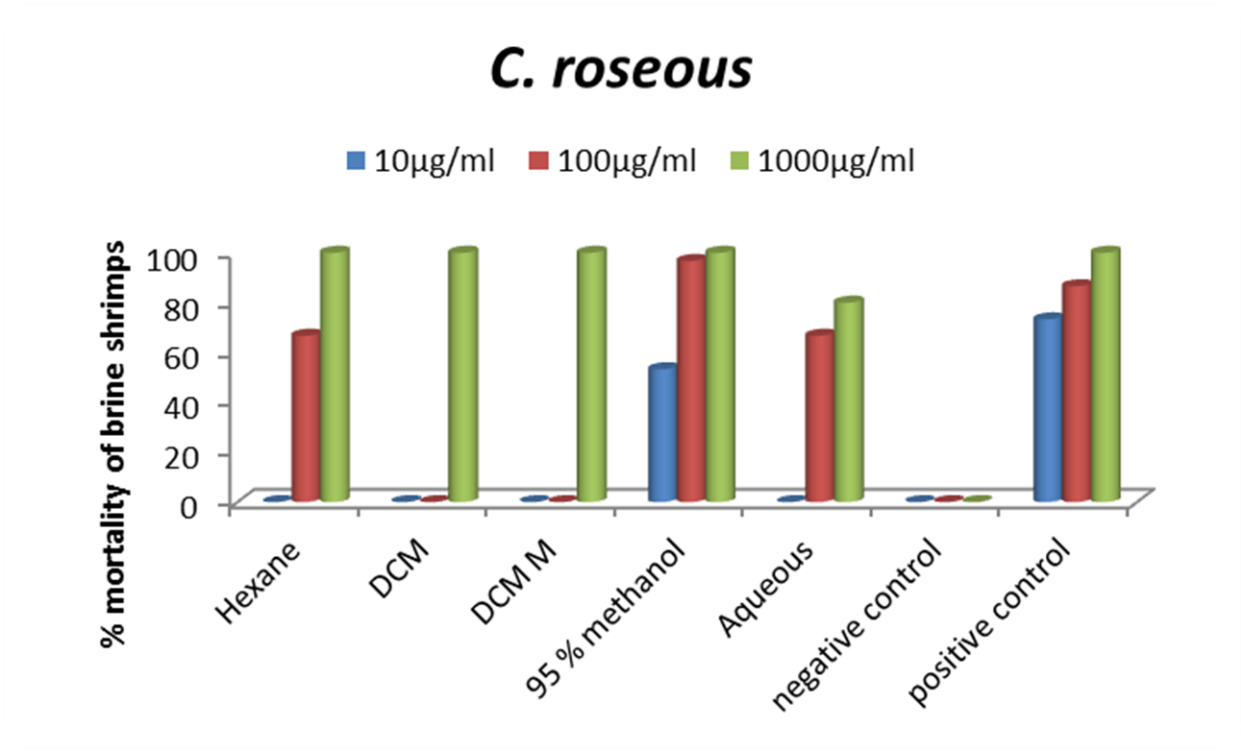

Figure 4.16: Mortality of brine shrimps in different extraction solvents of the *C. roseus*

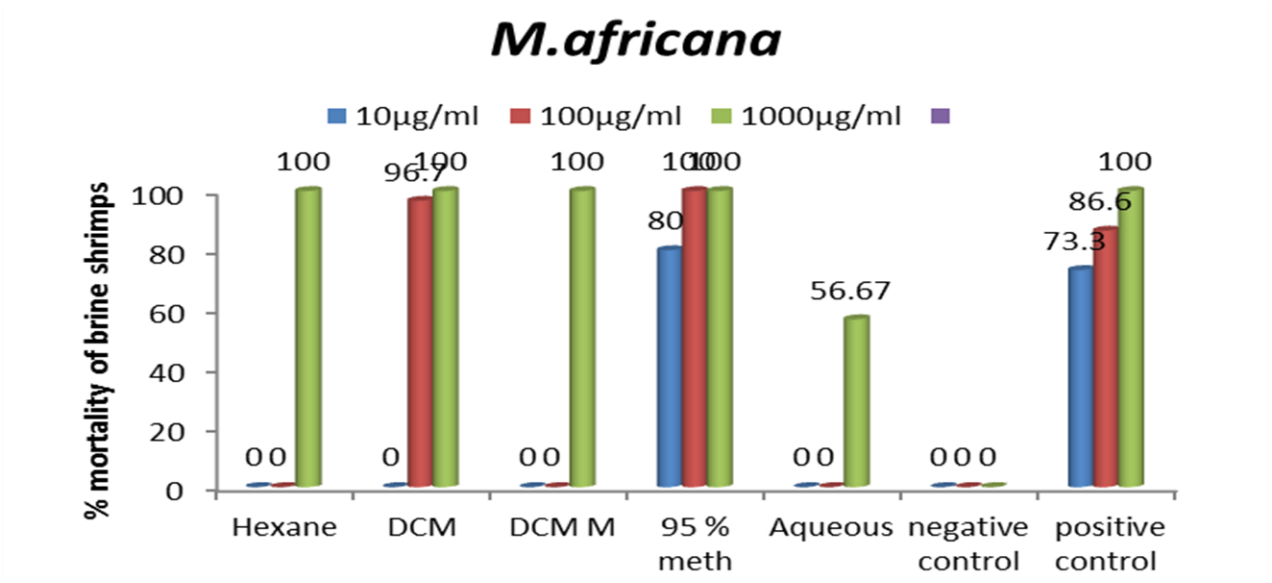

Figure 4.17: Mortality of brine shrimps in different extraction solvents of the *M. africana* .

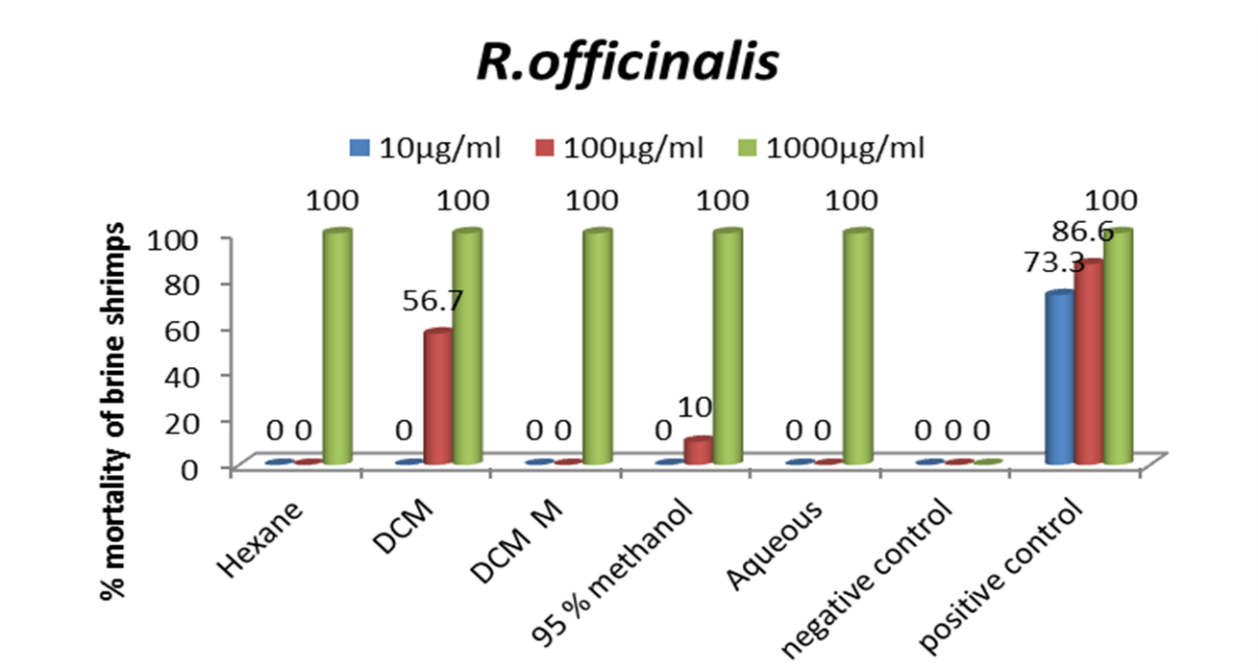

Figure 4.18: Mortality of brine shrimps in different extraction solvent of the *R. officinalis*

4.5 Phytochemical Results

Table 4.11: showing phytochemical results of *C. roseus, M. africana* and *R. officinalis* crude extracts

Test for	***R. officinalis***	***C. roseus***	***M. africana***
Terpenoids (Salkowish Test	+ve	+ve	+ve
Tannins			
1)Lead sub acetate	+ve	+ve	+ve
2)Ferric chloride	+ve	+ve	+ve
Anthraquinones	+ve	-ve	-ve
Glycocides	-ve	+ve	-ve
Flavonoids			
1)Ammonium test	+ve	-ve	+ve
2)Aluminium chloride test	+ve	-ve	+ve
Saponins			
Frothing test	+ve	-ve	+ve
Reducing sugars	+ve	+ve	+ve
Alkaloids			
1)Dragerndroff test	-ve	+ve	-ve
2)Mayers test	-ve	+ve	-ve
3)Wagner test	-ve	+ve	-ve

-ve sign and +ve sign indicate absence and presence of phytochemical respectively.

CHAPTER FIVE

5. DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

During the evaluation of the toxic characteristics of medicinal plants, the determination of LD_{50} is usually an initial step to be conducted. Data from the acute toxicity study may (a) serve as the basis for classification and labeling; (b) provide initial information on the mode of toxic action of a substance; (c) help arrive at a dose of a new compound; (d) help in dose determination in animal studies; and (e) help determine LD_{50} values that provide many indices of potential types of drug activity. Acute toxicity is usually defined as the adverse change(s) occurring immediately or a short time following a single or short period of exposure to a substance or substances, (Rhodes *et a.l.*,1993**)**.

Acute toxicity studies in animals are conducted using the route intended for human administration,for the administration of the compounds At present the following chemical labelling and classification of acute systemic toxicity based on oral LD_{50} values are recommended by the Organisation of Economic Co-operation and Development ;very toxic, $\leq$ 5 mg/kg; toxic, > 5 $\leq$ 50 mg/kg; harmful, > 50 $\leq$ 500 mg/kg; and no label, > 500 $\leq$ 2000 mg/kg OECD,423. Rat models are superior to mouse models for testing the pharmacodynamics and toxicity of potential therapeutic compounds, partially because the number and type of many of their detoxifying enzymes are very similar to those in humans (Lindblad, 2004).

Analysis of blood parameters in animal studies is relevant to evaluate the risk of alterations of the hematopoietic system in toxicity studies, for necessary application to humans. The assessment of hematological parameters could be used to reveal the deleterious effect of foreign compounds including plant extracts on the blood constituents of animals. They are also used to determine possible alterations in the levels of biomolecules such as enzymes, metabolic products, haematology, normal functioning and histomorphology of the organs (Olson e*t al.,* 2000)

The Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), are red blood cells (RBC) indices used in classifying types of anaemia. Disease or response to toxic substances is indicated by alterations in the key biochemical parameters which are the sensitive indicators of organ function or metabolic defects. The liver plays a major role in the metabolism and detoxification of compounds . It is therefore the prime target organ for drugs and toxic substances (Shah *et al*., 2011). Biochemical evaluation is important since there are several reports of liver and kidney toxicity related to the use of phytotherapeutic.Alanine aminotransferase (ALAT) , aspartate aminotransferase (AST) and alkaline phosphatase (ALP) are three most important and common liver enzyme key indicators of liver damage (Pieme *et al.*, 2006); Shashi., 2007).

In preclinical toxicity studies, renal changes are particularly liable to occur because of the fact that the kidneys eliminate many drugs and their metabolites (Obidah *et al*., 2009). In this study, creatinine and urea determinations were critical as markers of kidney function. Total

protein measurements can reflect nutritional status and may be used to screen for and help diagnose kidney and liver diseases and many other conditions (Thierry *et al*., 2011).

Catharanthus roseus is a well known medicinal plant and widely used in folk medicine/ ayurvedic system. The plant is also used in the Kenya ethnomedicine, (Kokwaro, 1976). In the present study, water solvent for crude extraction of *Catharanthus roseus* leaves was used justifying the ethnomedical where the traditional healers use hot water for extraction (Johns *et al.,* 1990). *C. roseus* aqueous extract showed no mortality even when an oral dose of 5000 mg per kg body weight in the male Wistar rats was administered. No changes in the behaviour of rats were observed. No adverse gastrointestinal effects were observed after administered with extract up to 5000/mg/kg/bwt. Therefore, the median acute lethal dose value (LD_{50}) was estimated to be more than 5000 mg/kg/bwt (OECD,423). Previous studies found out that 4000 mg/kg bwt of aqueous extract of *C. roseus* did not cause adverse effects in mice (Chattopadhyay *et al***.,** 1991). The current studies showed a 4.7 and 3.7 percentage weight gain in groups treated with 1000 and 5000 mg/kg respectively (figure 4.11). This was significantly lower than for the rats treated with the vehicle and is an indication that though the LD_{50} is within the safety range , the margin of safety is very low. It can be stated that both 1000 mg and 5000 mg/kg bwt *C. roseus* extracts interfered with the normal metabolism of the animals as corroborated with significant difference ($p = .04$ and .004 respectively). Chokshi, (2007) and Kevin *et al.*,(2012) also found significant weight decrease after giving rats 5000 mg/kgbwt of *C. roseus* oral methanolic extract.

The effect of *C. roseus* extract on some hematological parameters in the male rats is indicated in WBC,MCV and the platelets (thrombocytes), figures 4.1 and 4.4, MCV is an index of the size of the RBCs. Smaller MCV indicates that the RBCs will be smaller than normal and this is described as microcytic. Elevated MCV, indicates that RBCs will be larger than normal and are termed as macrocytic. RBCs of normal size are termed as normocytic. These size categories are used to classify anaemia types. (Chernecky, 2001). In this study the toxic effect was produced only at a higher dose 5000 mg/kg bwt at 48 hours but not at 14 days. It was suggested that *C. roseus* could interfere with platelets formation as seen in the decreased platelets level , figure 4.6. This may have been attributed by . reduced production and secretion of thrombopoetin, the primary regulator of platelet production (Kaushansky, 1995).and this is an indication that the extract might have a hemostatic property.

ALAT and AST were affected by the *C.roseus* extract,figures 4.7 and 4.8 and this report agrees with (James *et al*., 2007) where these enzyme were significantly elevated in rabbits fed with aqueous extract of *C. roseus.* Alanine amino transferase is a cytoplasmic enzyme found in very low concentration in the liver and is released into the plasma following hepatocellular damage.

The increase in concentration of this enzyme, as observed in this study suggests the hepatotoxic effect of the aqueous extract of *C. roseus*. Similar observation was made by (Pinkerton *et al.,* 1988) who tested a continuous infusion of *C. roseus* alkaloid Vincristine and found a transient increase in liver enzymes.

Elevated creatinine and urea levels at 48 hours and also at day 14 figures 4.9 and 4.10 suggested that *C.roseus* extract caused a dyfunctioning of the kidneys. This was in agreement with (James *et al*., 2007) who found an increasing tread of creatinine in mice treated with leaves of *C. roseus*. Creatinine and urea tests are critical and sensitive indicators of kidney function (Obidah *et al.*, 2009). Hepatorenal toxicity is particularly liable to occur because these organs are involved in drug metabolism and elimination **(**Olson, 2000).

The non significance difference of serum total protein table 4.4, suggests that the renal functioning of the rats may not have been critically impaired (**Kachmar and Grant, 1982)**. These results are agreeing with the findings of (Pinkerson *et al*., 1988; James *et al*., 2007), who found non significant protein increase in animals feed with aqueous extract of *C. roseus*.

There are no available published conventional safety studies on *Myrsine africana* despite widespread use as an anthelmintic (Zabta *et al*., 2003; Kokwaro, 1993; Beentje, 2004). The oral acute toxicity testing of aqueous extract of M*yrsine africana* showed that no animal died at the doses of 1000 or 5000 mg/kg bwt within 24 hours. There was no delayed toxicity since even at day 14 post extract administration the animals showed no physiological impairment. This suggests that, this extract has an LD_{50} > 5000 mg/kg bwt in rats. According to (Schorderet,1992), substances with LD_{50} values greater than 5000 mg/kg body weight are classified as substances with low toxicity. The fact that there was no significant total weight loss in rats treated with *M. africana* supports the low toxicity implied by the LD_{50} classiffication (Schorderet, 1992**.** This justifies why the plant has a widespread use via oral route by traditional healers in Kenya (Nanyingi,*et al*., 2008)

The non significant change of WBC level by *Myrsine africana* aqueous seed extract, figure 4.4 suggested that no inflammation triggering the immune system was caused by this extract (Imaga *et al.,* 2009)**.** Mean RBC, PCV and MCHC were significantly different at 48 hours but normalized at day 14, figures 4.2, 4.3 and 4.4, suggesting that there may be a correlation between these responses and dosage (Banerjee *et al*., 2002).

Non- significant levels of HB, MCH MCV and thrombocytes (tables 4.2 & 4.3, figures 4.4 & 4.6) in *M. africana* treated groups suggested that *M. africana* at these doses did not induce anaemia in the rats.

Increased PCV at a dose of 5000 mg/kg bwt of animals treated with *M. africana* at 48 hours but went back to baseline values at day 14, figure 4.3, suggested that this extract could trigger toxicity in a dose related manner. (Githiori *et al*., 2002) tested the efficacy of *M. africana* extract in sheep innoculated with nematodes and found a significant reduction of PCV. Increase in the RBC count in the treated animals with 5000 mg/kg bwt,figure 4.2 displays the blood promoting action of the *M. africana* extract as explained by (Zhong,1985). An increased RBC, unaltered WBC and Platelets (thrombocytes) suggests a strong immuno-modulatory, antioxidant and endothelial protection and repair activity of *Myrsine africana* extracts (Imaga *et al*,. 2009). This is explained by the fact that WBC protect the body from infection by foreign organisms, the RBC boost the immune system and the platelets protect blood vessels from endothelial damage as well as initiate repair of these vessels (Olson, 2000). The current finding agrees with previous findings that the *M. africana* plant extract possess radical scavenging ability (Ahmad *et al.,* 2011).

Reduced MCHC, figure 4.5 , may be an indicator of hypochromia in early iron deficiency (Rose and Bentley, 1986).

The significantly high *M. african* induced elevated AST at days 14 for both the low and the high dose, and not at 48 hours, figure 11, is an indication of the presence of delayed liver toxicity (OECD,423) suggesting possible cellular damage to the liver by the extract (Cavanaugh, 2003).

Elevated creatinine by *M. africana* at 48 hours ,figure 4.9, indicating possible renal dyfunction. At day 14, there was no significant difference and hence it is dose-dependent extract which might not be very indicative of the glomerular filtration rate (GFR). The increased serum urea at day 14 whereas this parameter had low values at 48 hours, figure 4.10 for both high and low dose, might be as a result of tissue damage indicative of tissue necrosis (Eaton and Pooler, 2009). However this condition was not so severe since the total protein in this group did not have significant difference from that of the untreated group, table 4.4.

The results of this study indicated that *R. officinalis* aqueous extract on rats had an LD_{50} >5000 mg/kg/bwt since there was no death at the tested doses (1000 and 5000 mg/kg bwt) neither no signs of toxicity recorded. These results are agreement with (Arturo *et al*., 2008**)** where oral LC_{50} for rosemary aqueous leaf extract was established to be > 2000 mg/kg bwt in Wistar rats and >8.5mg/kg bwt for ethanolic extract in rats (EFSA, 2008). Rosemary aqueous extract is therefore a low toxic substance, (OECD, 423). This was also established by,

Haloui *et al., (*2000); Sancheti *et al., (*2006). Most common human exposure to rosemary occurs through ingestion of this plant as a food additive. The extract did not show an obvious alteration to the metabolic process seeing that there was no significant change of weight profile compared with the control. This low toxicity was validated by the fact that all hematological parametres in the rats did not show any significant difference at both the low and the high dose; 1000 and 5000 mg/kg bwt at both 48 hours and 14 days testing. Analysis of blood parameters in animal studies is relevant to evaluate the risk of alterations of the hematopoietic system in toxicity studies, for necessary application to humans and the study suggests that *R. officinalis* is safe (Olson *et al.*, 2000).

In the group treated with 1000 and 5000 mg/kg bwt of *R. officinalis*, ALAT was significantly reduced at 48 hours but it became reversible to normal at day 14, figure 4.13. Liver enzymes (ALAT and AST) are liberated into the blood whenever liver cells are damaged and enzyme activity in the plasma is increased (Edwards, *et al.,* 1995).

Acute renal failure (ARF), as a clinical syndrome characterized by a rapid decline in the ability of kidney to remove waste products, disturbance in acid-base balance, water homeostasis, and rapid reduction in glomerular filtration rate (GFR) (Bagshaw and Bellomo, 2007). This condition was not induced by the *R. officinalis* aqueous extract since creatinine, urea and total proteins values (figures 4.9, 4.10, and 4.11) were not significantly altered in the treated rats with *R. officinalis* groups compared with the control group. These biochemical results are supported by studies done by (Zohrabi *et al*., 2012**)** which showed that rosemary aqueous extract protects the rats against histological injury and functional

impairmet. Anado´ n *et al., (*2007) established that oral extract of *R .officinalis* in the rats did not affect the hematological and the biochemical parameter at dose of 2000 mg/kg bwt.

The need to evaluate the sub-acute toxicity profile of *R. officinalis* leaf aqueous extract was prompted by the increasing awareness and interest in natural product medicinal. This plant is widely used in Kenya and the whole world but little information on toxicity of the aqueous extract (which is more commonly administered in the ethnomedicine practice), is provided. Plant extracts are good source of biologically active substances but knowing the side effects before therapeutic application is essential.

Differential WBC count is usually done in order to provide information on the proportion of the different white blood cells present in circulating blood (Cheesbrough, 2000; Tatfeng and Enitan, 2012). In this study the significantly increased lymphocytes at day 14 sub acute toxicity, table 4.7, suggests that the extract induced lymphocytosis, confirming the protective nature of the rosemary extract. One of the major functions of lymphocyte is their response to antigen (foreign bodies) by forming antibodies that circulate in the blood or in the development of cellular immunity. These results are in agreement with earlier reported by (Fawzi *et al*., 2012) where the immunomodulatory activity of aqueous extract of *R. officinalis* was evaluated in mice and showed that IgM (Immunoglobulin M) and IgG (Immunoglobulin G) response increased significantly when the mice were fed with the extract.

Elevated alanine aminotransferase (ALAT) in the repeated dose toxicity study at day 28 in 1500 and 3000mg/kg bwt groups, figure 4.12 showed that at doses of above 1500 mg/kg bwt,

R. officinalis could be toxic to the liver. European Food Safety Authority has established that rosemary non-observed-adverse-effects-level (NOAEL) values are in the range of 180 to 400 mg extract/kg bwt per day. This was based on 90-days feeding studies in rats, (EFESA, 2008). In the current study, the high and low doses did not induce any alteration to the urea and creatinine, table10, which are used as kidney toxicity indicator.

Both low and high doses of *R. officinalis* extract did not show significant change in body and organs weight as former studies showed (Dimech *et al*., 2006). *R. officinalis* extract was shown to exhibit low toxicity in the animal model (Haloui *et al.*, 2000 and Sancheti *et al.*, 2006). Oral administration of *R. officinalis* should hence be taken at doses $\leq$ 1500 mg/kg.

The medicinal action of the plants is unique to a particular plant species, consistent with the concept that the combination of secondary metabolites in a particular plant is taxonomically distinct for each medicinal plants and their description and uses respectively. Some of the most important bioactive phytochemical constituents are alkaloids, essential oils,flavonoids, tannins, terpenoid, saponins, phenolic compounds and many more (Edeoga *et al*., 2005).
Saponins are plant-based anti-inflammatory compounds that may lower blood cholesterol and prevent heart disease as well as some cancers (Xu *et al*., 1996). Both *M. africana* and *R. officinalis* were found to contain flavonoids. Tannins have shown potential antiviral, (*Lu et al.*,2004)' antibacterial' (Akiyama *et al*., 2011) and antiparasitic effects (Kolodziej *et al*., 2005). All the 3 plants were found to contain tannins.

Flavonoids are capable of modulating the activity of enzymes and affect the behaviour of many cell systems, suggesting that these compounds may possess significant antihepatotoxic, antiallergic, anti-inflammatory, antiosteoporotic and even antitumor activities, (Carlo *et al*., 1999; Cushnie and Lamb, 2005). *C. roseus* was found not to contain this phytochemical but the other two plants had it. Pure isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesic, antispasmodic and bactericidal effects. Alkaloids are also kwown for their cytotoxicity (Nobori *et al*., 1969**)**. Glycosides are known to lower the bloodpressure according to many reports (Stray, 1998**).** *C. roseus* has been used for cancer treatment, anti-diabetic, reduce high blood pressure, externally against nose bleeding, sore throat and mouth ulcer (Kokwaro,1976). Traditional use of *C. roseus* as an anthelminthic agent has been recorded, (Hoskeri *et al*., 2011).

The Phytochemical investigations on the crude aqueous and methanolic extract of dry fruits of *M. africana* showed presence of terpenoids, tannins, flavonoids, saponins, reducing sugars as the active compounds,table4.11. The present study shows the absence of cardiac glycosides, anthraquinones and alkaloids. The presence of the above phytochemicals supported the following studied pharmacological activity of *M. aficana:* anti-tumor activity of aerial part, (Kupchan *et al*.,1969), purgative (Kakrani *et al*.,1983)' anti-fertility, (Belachew, 1994), anthelmintic and phytotoxic, (Gathuma *et al*., 2004), haemagglunitation activity (Ahmad *et al*., 2011) and antimicrobial, (Habtamu *et al.*, 2004).

Rosemary (*Rosmarinus officinalis* L.) is a spice and medicinal herb widely used around the world. Of the natural antioxidants, rosemary has been widely accepted as one of the spices

with very high antioxidant activity and anticancer agent (Leal *et al*., 2003), preservative qualities (Oiye *et al.*,2013). These reports agree with the current finding of the presence of terpenoids, tannins, cardiac glycosides, flavonoids, reducing sugars, saponins in extract.

Cytotoxicity studies play an important role in identification and isolation of new compounds from crude extracts (Sasidharan, 2008). Brine shrimp larva are highly sensitive to a variety of chemical substances. The assay is considered a useful tool for preliminary toxicity assessment of plant extract (Sol´y's *et al*.,1993, McLauglin *et al*.,1991). Plant parts such as roots, leaves, stems, rhizomes and fruits possess a myriad of chemical constituents that are biologically active against various disease conditions (Van and Wink, 2004). In this study, solvents of varying polarity were used in the extraction procedure in an attempt to study the range cytotoxicity of compounds obtained at different polarities from the leaves of *C. roseus*, *R. officinalis* and of *M. africana*. Brine shrimp lethality assay is a rapid inexpensive and simple bioassay for testing plant extracts bioactivity, the result in most cases correlate with cytotoxic and antitumor properties of the plant (McLaughlin *et al*., 1991). The activities of the extracts are manifested as toxicity to shrimps by bioactive components present in the extracts. A substance is considered to be cytotoxic if it inhibits vital metabolic processes or it causes disorders in living organisms resulting in perversion of behavior or death (Fatope, 1995). Standard brine shrimp lethality bioassay stipulates that an LC_{50} value < 1000 µg/ ml is considered bioactive in toxicity evaluation of plant extracts (Meyer *et al.*, 1982)

The current study showed that the degree of lethality was found to be directly proportional to the concentration of the extracts. Maximum mortalities took place at a concentration of 1000 µg/ ml whereas least mortalities was found at 10 µg/ ml , figures 4.16, 4.17 and 4.18. The

positive control had an LC_{50} of 3.0 µg/ ml which is slightly different from earlier calculations of (Nguta *et al*., 2012) which found etoposide to have an LC_{50} of 6 µg/ ml. This difference is within a reasonable margin owing to the fact that different bioassay environments and calculation method may differ.

Different solvents, depending on their polarity, extract varying quantities of components in crude plant material that may be beneficial or harmful to biological systems. Hexane, for instance, extracts waxes, fats, and fixed oils (Cowan, 1999). *C. roseus* had the highest toxicity in this extraction solvent showing that most cryptogenic compounds were extracted. This report validates the study done by (Usia *et al*., 2005; Murata *et al.,* 2008) that found triterpenoids, ursolic acid and oleanolic acid in considerable amounts in the leaf cuticular wax layer of *C. roseus.*

An LC_{50} greater than 500 exhibited by hexane extracts of both *M. africana* and *R. officinalis* was indicative of high cytotocity and possible presence of wax ,fats and fixed oils. This justifies the reason for these plants therapeutic uses in many areas. Earlier study had shown that *M. africana* hexane extract had a highest mortality of brine shrimps compared to other solvents and it indicated an LC_{50} of 33 µg/ ml which is a big discrepancy with the current report (Ahmad *et al*., 2011). This can be explained by the fact that different extraction methods yield different amounts of the bioactive compounds (Cowan,1999). In the previous findings, partitioning of a methanolic extract with n-hexane was done yielding much more cytotogenic components than what was gotten by maceration extraction in the current study.

In another study **(**Amara *et al.*, 2008**),** *C. roseus* hexane extract gave an brine shrimp LC_{50} of 107µg/ ml which is close to the value of the current study of 159 µg/ ml.

Dicloromethane which is a more polar solvent than hexane, commonly extracts alkaloids, aglycones, and volatile oils (Cowan,1999). The LC_{50} for *C. roseus, M. africana* and *R. officinalis* in this solvent were 498.5, 119.5 and 168 µg/ ml, respectively, table 4.10, showing that lesser of cytogenic compounds of *C. roseus,* more polar cytogenic component in *M. africana* and *R. officinalis,* were extracted by this solvent than in the hexane.

DCM: methanol solvent extracted more potent copounds in *R. officinalis* than in *C. roseus* and *M. africana*.This is indicated by the brine shrimp mortality caused by these extracts. Water has been found to improve the efficiency of less polar solvents particularly pure organic solvent. This implies that 80% acetone would extract more components compared to pure acetone (Keinanen, 1993). More study of structure elucidation can be able to identify each compound extracted by different solvents.

Methanol: water solvent extract yielded extremely high cytogenic secondary metabolites of *C. roseus* and *Myrsine africana.* These extracts had LC_{50} of 3.5 and < 1 µg/ ml respectively, table 11. Low LC_{50} value indicates possibility of the presence of antitumor and insecticidal compounds in the extract (Krishnaraju *et al*., 2005). The flower of *C. roseus* extract was found to have a brine shrimp LC_{50} of less than 1 µg/ ml (Rahmatullah *et al*., 2010). This is a demonstration that these compounds could be important in pharmaceutical industry (Laird and Kate, 1999). Previous studies found methanolic *M. africana* bark to have brine shrimp

LC_{50} greater than 1000 µg/ ml (Gakuya *et al*., 2004) and hence the seed of *M. afrcana* is suggested to be more potent than the bark.

M. africana aqueous extract was found to be non- toxic to the brine shrimps recording LC_{50} of >1000 µg/ ml. This can possibly explain why the leaves and fruits extracts against *Haemonchus contortus* in sheep was uneffective as it was demonstrated by (Githiori *et al*., 2002). This currentst study results agree with (Ahmad *et al*., 2011) who found non significant brine shrimp toxicity of the aqueous extract of the aerial part *M. africana.*

C. roseus extract was the most cytogenic of the aqueous extrcts with an LC_{50} of 256.7 µg/ ml. Earlier studies showed an aqueous extract of the leaves of *C. roseus* to have an LC_{50} of 170µg/ ml (Krishnaraju *et al*., 2005), a figure close to the one reported in this study.

R. officinalis aqueous extract had moderate cytotoxicity. LC_{50} 498 µg/ ml. Rosemary hot water infusion is commonly taken as herbal tea (Blumenthal and Brinckmann) and the brine shrimp moderate cytotoxicity validates this plants therapeutic property.

5.2 Conclusions

Today, a resurgence of the use of natural products remedies is being observed worldwide. This trend has been partly due to concerns over the serious adverse effects of conventional drugs and the movement towards a more natural life. There is need therefore, to provide information on the pharmacology as well as the toxic effects of these remedies to validate their use. The following were the conclusions drawn from the study:

1) Acute toxicity studies established that the aqueous extract of *Catharanthus roseus,* Myrsine *africana* and *Rosmarinus officinalis* did not cause any death or adverse effect in the

rats. The LD_{50} was established to above 5000mg/kg/ bwt in a Wistar rats. However, from the blood investigation, it showed that consumption *C. roseus* and *M. africana* extracts may bring about acute renal-hepato and hemapoietic toxicity. This was demonstrated by the significant elevation a number of parameters. Consequently, safety measures need to be taken in the administration of these extracts and these may include monitoring of the vital serum enzyme and hematological levels.

Rats treated with *C. roseus* had significantly low weight gain and therefore this plant proved to be the more toxic than *M. africana* and *R. officinalis* if consumed orally. *R. officinalis* showed very low toxicity since only ALAT was elevated in acute testing.

2) The current findings provide the basis for the selection of doses for use in long-term toxicity studies, which are important in order to provide safety data for repeated dose effects of these plants.

3) An oral sub-acute toxicity testing of *R. officinalis* for 28 days showed that this extract could be of non-toxic at low doses up to 1500 mg/kg bwt since none of the blood parameters was significantly elevated but at higher doses, toxicity was recorded being demonstrated by elevated WBC, percentage lymphocytes and ALAT by the 1500 and 3000 mg/kg bwt extracts.

4) This study reported the presence of various phytochemicals terpenoids, tannins, anthraquinones, alkaloids and reducing sugar in *C. roseus.* Terpenoids, tannins, flavonoids, saponins and reducing sugars were found in *Myrsine africana.* Terpenoids, tannins, cardiac glycosides, flavonoids, reducing sugars and saponins were present in *R. officinalis.* It was concluded that these bioactive compounds were responsible for the biochemical, hematological and the brine shrimp effects that were recorded. The same ones and others not

explored are responsible for the medicinal properties used in the treatment of different ailments using these plants. Therefore, extracts from these plants could be seen as a good source for useful drugs.

5) Different bioactive components were extracted by different extraction solvents. This was demonstrated by the brine shrimp assay results whereby different extracts of the same plant yielded a different mortality effects on the brine shrimps.

6) Low LC_{50}, < 10 μg/ml, demonstrated by the methanolic extract of *M. africana* indicated the possibility of the presence of potent antitumor and insecticidal compounds **(**Krishnaraju *et al*., 2005**).** *C. roseus,* methanolic extract also exhibited low LC_{50} and confirms its wide use as source of anticancer drugs.

5.3 Recommedations

The current study showed that *C. roseus, M. africana and R. officinalis* contained several metabolites and may contain much more than was studied. There is therefore need for further investigations purified chemical components. Isolation of the active principle of these extracts is key to drug discovery and it is highly recommeded.

It is also important to perform long term toxicity testing (sub-acute and chronic) for all of the studied plants inorder to provide data that will encourage good use, monitor any adverse effects and form a background basis for the development of these plants.

Monitoring of vital enzymes and hematological parametres for someone who is on long term use of natural products even though the products may be esteemed as non-toxic, is of essence as a safety precaution. It therefore recommeded that persons taking rosemary for a long time should have blood parmetres monitored.

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APPENDICES

Appendix 1: Approval Letter of Biosafety, Animal use and Ethics Committee

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,
00100 Nairobi,
Kenya

Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line: 4448648

Ms Zelipha N. Kabubii
c/o Dept of PHPT

12.05.2014

Dear Ms Kabubii,

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Phytochemical screening, bioactivity and toxicological studies of *Catharanthus roseus*, *Myrsine africana* and *Rosemarinus officinalis*

By Zelipha N. Kabubii J56/79712/2012

We refer to the above proposal that you re-submitted to our committee on 5.05.2014. We have now reviewed your proposal and note the following:

We are satisfied that the number of animals proposed are actually required for the experiments and that there will be humane treatment of the animals in the course of the experiments. Furthermore, that there will be sufficient time between blood sampling for the animals to recover without stressing them. This approval is given subject to the principal investigator seeking veterinary supervision, particularly during euthanasia of the animals.

We hereby approve your study as per your revised proposal of 5.05.2014.

Rodi O. Ojoo BVM, M.Sc, Ph.D.
Chairman,
Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine.

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APPENDIX 2: Brine shrimp assay for 4 organic extracts and one aqueous extract

	HEXANE EXTRACTS	Surviving shrimps after 24 hr out of 10							
	Conc.(µg/ ml).	1	2	3	Mortality	% mortality	probit	liner regression	LC 50
C. roseus	1000	0	0	0	30	100	7.33	y=3.67x-3.076	159.78
	100	4	3	3	20	66.7	5.43		
	10	10	10	10	0	0	0		
m.africana	1000	0	0	0	30	100	7.33	y=3.67x-4.887	498.52
	100	10	10	10	0	0	0		
	10	10	10	10	0	0	0		
R.officinalis	1000	0	0	0	30	100	7.33	y=3.67x-4.887	498.52
	100	10	10	10	0	0	0		
	10	10	10	10	0	0	0		
		DCM Extracts							
C Roseus	1000	0	0	0	30	100	7.33	y=3.67x-4.887	498.52
	100	10	10	10	0	0	0		
	10	10	10	10	0	0	0		
M.africana	1000	0	0	0	30	100	7.33	y=3.67x-2.613	119.46
	100	9	10	10	29	96.7	6.82		
	10	10	10	10	0	0	0		
R.officinalis	1000	0	0	0	30	100	7.33	y=3.67x-3.163	168.8
	100	5	4	4	17	56.7	5.17		
	10	10	10	10	0	0	0		

	DCM: Methanol								
C Roseus	1000	0	0	0	30	100	7.33	y=3.67x-4.887	498.52
	100	10	10	10	0	0	0		
	10	10	10	10	0	0	0		
m.africana	1000	0	0	0	30	100	7.33	y=3.665x-4.887	498.52
	100	10	10	10	0	0	0		
	10	10	10	10	0	0	0		
R.officinalis	1000	0	0	0	30	100	7.33	y=3.67x-3.6367	227.27
	100	9	8	10	3	10	3.75		
	10	10	10	10	0	0	0		
	METHANOL:WATER		**(95:5)**						
C Roseus	1000	0	0	0	30	100	7.33	y=.99x+4.52	3.05
	100	0	0	1	29	96.7	6.82		
	10	5	4	5	16	53.3	5.35		
m.africana	1000	0	0	0	30	100	7.33	y=.74x+5.34	0.34
	100	0	0	0	30	100	7.33		
	10	1	3	2	24	80	5.84		
R.officinalis	1000	0	0	0	30	100	7.33	y=3.67x-4.887	498.52
	100	10	10	10	0	0	0		
	10	10	10	10	0	0	0		
	Water Extract								
C Roseus	1000	2	3	1	24	80	5.84	y=2.92x-2.08	265.86
	100	3	3	4	20	66.7	5.44		
	10	10	10	10	0	0	0		
M.africana	1000	5	4	4	17	56.67	5.17	y=2.59x-3.4467	18205.O5

	100	10	10	10	0	0	0		
	10	10	10	10	0	0	0		
R.officinalis	1000	0	0	0	30	100	7.33	y=3.665x-4.887	498.52
	100	10	10	10	0	0	0		
	10	10	10	10	0	0	0		
	Positive control								
	1000	10	10	10	30	100	7.33	y=.865x+4.967	3.1
	100	2	3	3	22	86.6	5.60		
	10	2	1	1	26	73.3	6.05		
	Control(Water)								
	1000	10	10	10	0	0	-		
	100	10	10	10	0	0			
	10	10	10	10	0	0			

Table showing the mortality, liner regression line and calculated LC_{50} of the 5 types of extracts of the 3 plants at concentration of 10,100 and 1000 µg/ ml µg/ ml .

Appendix 3: Baseline Parameters

Baseline Hematological parametres for sub-acute Testing

	WBC		RBC	SD	PCV	SD	Hb	SD	MCV	SD	MCHC		MCHCH		Thromb.	SD
Control	13.132±	0.8	5.69±	0.2	34.84±	1.2	14.096±	0.2	63.8±	1.5	23.44±	1.9	38.66±	0.9	279.8±	30.9
500mg	13.82±	0.7	5.596±	0.3	37±	2.4	14.9±	0.3	62.62±	2.6	22.2±	1.3	39.1±	0.7	234.8±	19.3
1500mg	13.44±4	2.3	5.554±	0.5	36.4±	3.1	13.566±	0.7	63.2±	2.5	22.22±	1.1	37.5±	1.4	254.4±	15.2
3000m	13.398±	0.6	5.306±	0.5	36.64±	2.4	14.05±	1.3	62.38±	1.5	23.8±	1.3	38.38±	0.8	231.8±	6.1

Differential WBC

	Neutrophil	Sd	Lymphocytes	Sd	Eosophils	SD	Monocytes	SD
Control	20.4±	2.5	79.6±	1.82	1.2±	1.30	0.2±	0.45
500mg	19.±2	1.6	78.8±	3.42	1±	1.00	0.6±	0.55
1500mg	18.±2	9.8	76.6±	1.95	1±	1.23	0.8±	1.30
3000mg	19.4±	1.5	79.±8	3.42	1.4±	1.14	0.8±	1.30

Baseline Hematological parameters for acute Testing

	control		C.roseus 1000mg		C.roseus 5000mg		M.africana 1000mg		M.africana 5000mg		R.officinalis 1000mg		R.officinalis 5000mg	
WBC	9.36±	1.31	13.942±	9.31	10.91±	1.98	10.16±	1.51	9.18±	1.30	10.276±	1.37	11.1±	1.74
RBC	5.4±	0.23	5.57±	0.37	5.52±	0.26	5.5±	0.38	5.5±	0.40	5.46±	0.38	5.4±	0.31
PCV	35.2±	1.48	36.26±	0.95	36.28±	0.82	34.98±	1.66	35.8±	0.96	37.34±	0.83	37.46±	0.88
HB	13.28±	0.78	13.7±	0.83	13.24±	0.78	13.38±	0.89	13.22±	0.82	13.48±	0.87	13.62±	0.52
MCV	63.84±	0.85	63.88±	1.41	61.06±	3.98	62.44±	2.21	62.78±	1.20	63.54±	1.16	63.2±	0.45
MCH	23.28±	1.17	23.04±	1.61	22.54±	0.87	24.3±	1.18	23.92±	2.66	23.7±	1.53	23.42±	1.09
MCHC	37.3±	0.62	38.3±	0.87	38.54±	0.68	37.26±	1.44	36.04±	1.85	37.18±	1.09	37.46±	0.50
Thromb.	341.4±	27.75	343.8±	17.34	333.8±	19.31	337.6±	20.89	376.6±	16.52	346.8±	30.87	389.4±	34.78

Baseline Biochemical parameters before acute toxicity testing

		ALAT	SD	AST	SD	Creatinine	SD	Urea	S D	Protein	S D
Group 1-	Control	45 ±	2.5	43.5±	1.1	1.4±	0.3	40.92±	2.6	7.24±	0.5
Group 2-	C.roseus-1000mg	42.36±	4.4	39.78±	3.7	1.34±	0.3	43.12±	1.7	7.42±	0.9
Group 3-	C.roseus-5000mg	43.34±	1.1	42.44±	3.8	1.14±	0.2	39.96±	3.6	7.28±	0.3
Group4-	M.africana-1000mg	41.6±2	2.0	39.3±	1.7	1.4±8	0.2	42.94±	4.6	7.26±	0.2
Group5-	M.africana-5000mg	40.8±	2.5	41.18±	1.0	1.72±	0.1	41.74±	2.1	7.24±	0.6
Group6-	R.officinalis-1000mg	39.66±	3.9	39.4±	5.7	1.42±	0.1	42.222±	2.7	7.68±	0.4
Group 7-	R.officinalis-5000mg	44.642±	6.4	40.0±2	4.1	1.5±	0.3	43.38±	4.6	6.94±	0.7

Baseline Biochemicals before sub –acute testing.

	ALAT	SD	AST	SD	Creatinine	SD	Urea	SD	Proteins	SD
Control	42.4 ±	2.1	53.66±	4.2	0.8±	0.1	40.16±	1.5	7.1±	0.4
500mg/kg bwt	40.08±	2.8	55.74±	4.3	0.96±	0.2	39.34±	1.6	6.84±	0.2
1500mg/kg bwt	41.76±	3.7	55.32±	4.0	0.86±	0.2	40.26±	2.2	6.62±	0.3
3000mg/kg bwt	40.78±	3.2	57.88±	2.3	0.9±	0.2	39.34±	3.2	6.88±	0.2