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Evaluation of acute and sub-chronic toxicity of aqueous leaves extracts of *maytenus gracilipes celastraciae* (kombolcha) on some blood parameters and histopathology of liver and kidney in Swiss albino mice.

A thesis submitted to Department of Anatomy School of Medicine College of Health Sciences Addis Ababa University in partial fulfilment of the degree of Master of Science (MSc) in anatomy

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Declaration

This is to certify that the thesis prepared by Mengistu Ayele, entitled: Evaluation of the acute and sub-chronic toxicity of aqueous leaves extract of *Maytenus gracilipes* on some Blood parameters and histopathology of liver and kidney in Swiss albino mice at Addis Ababa University on year 2014/2015 and submitted in partial fulfillment of the requirements for the degree of Master of science in Anatomy complies with the regulations of the University and meets the accepted standards with respect to originality and quality. This thesis has not been presented for a degree in any other university, and that all sources of materials used for the thesis have been duly acknowledged. The Thesis has passed with----------remark.

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List of Abbreviations

^{0}C: …….Degree Celsius

AAU: ……... Addis Ababa University

ANOVA: ……..Analysis of variance

ALP……...Alkalin phosphates

AST ……..Aspartate aminotranspherase

ALT……...Alanine Aminotranspherase

CAM: ……Complementary and alternative medicine

Cm: …….....Centimeter

EDM: …….Department of Essential drugs and Medicinal policy

EPHI: …….Ethiopian Public health institute

GACP…….Good Agricultural Collection Practice

GIT: ……...Gastro Intestinal Tract

GMP: …….....Good Manufacturing Practice

Hrs: ……hours

HGF: ……..Hepatocytes growth factor

LD_{50}: ……..Lethal doses of fifty percent

µm: ……Micrometer

mm^2 …….....Millimeter square

SE: …….Standard Error

SPSS: ……....Statistical package for social sciences

T^0: ……..Temperature

TGF: ……..Transforming growth factor

TM/TRM: ……Traditional medicine

WHO: ……....World Health Organization

WBCs: ……..….White blood cells

RBCs: ……Red blood cells

OECD: ……Organization for Economic Cooperation and Development

HGB: …….. .Hemoglobin count

HCT: ……....Hematocrit

MCV: ……...Mean corpuscular volume

MCH: ……...Mean corpuscular hemoglobin

MCHC: …….. ..Mean corpuscular hemoglobin concentration

PLC: ……. Platelate count

NBF: …….Neutral buffered formalin

TB: ……....Total bilirubin

EDHS.....Ethiopian demographic and health survey

GFR.....Glomerular filtration rate

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Abstract

Human beings use plants for the purpose of disease control and prevention. *Maytenus gracilipes celastraciae* is a plant having vital traditional medicinal values for treating several human ailments such as for headache, epilepsy, allergic, peptic ulcer, cancer and immflamations. The present study was carried out to evaluate the acute and subchronic toxic effects of aqueous leaves extracts of *maytenus gracilipes celastraciae* (kombolcha) on some blood parameters and histopathology of liver and kidney in mice. LD_{50} was determined.

The thesis was carried out in Addis Abeba University, College of Health Science, School of Medicine, Department of Anatomy (Histology Laboratory).The study was conducted from May, 2014 -July, 2015.The aqueous leaves extracts of *M.gracilipes* were employed in swiss albino mice in single oral dose for acute study and repeated oral dose for subchronic study.

The $LD_{50,}$ of aqueous extracts of *Maytenus gracilipes* was found to be 10,000 mg/kg body weight. In the Subchronic study, the extract was administered orally at doses of 700 and 2100 mg/kg/day for 90 days . Body weight, biochemical and hematological parameters were determined at the end of the 90 days of daily extract administration.

In sub chronic toxicity study daily oral administration of aqueous extract at 700 and 2100 mg/kg body weight/day did not result in death or significant changes in body weight, hematological and biochemical parameters. Studies on Histopathological examination of selected organs (liver and kidney) showed normal architecture suggesting absence of morphological disturbances.

However, at 700 mg/kg body weight liver showed pyknotic nuclei and at 2100mg/kg some cellular infiltrates near the central vein and portal region, where as, in kidney peritubular infiltration and focal mono nuclear leukocytic infiltration was observed at higher dose.

Key words: *Maytenus gracilipes*, Aqueous extract, acute toxicity, subchronic toxicity, Histopathological studies.

1. INTRODUCTION

1.1. Traditional Medicine

The World Health Organization (WHO) defines traditional medicine as health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses and maintain well-being (WHO, 2001). It is known that many countries in African, Asia and Latin America use traditional medicine (TM)/complementary and alternative medicine (CAM) to meet some of their primary health care needs. In Africa, up to 80% of the population uses traditional medicine for primary health care (WHO, 2003).

Traditional medicine has maintained its popularity in all regions of the developing world and its use is rapidly spreading in the industrialized countries. In China, for example, traditional herbal preparations account for 30-50% of the total medicinal consumption (WHO, 2003). In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with high fever resulting from malaria is the use of herbal medicines at home (WHO, 2003). WHO estimates that in several Africa countries traditional birth attendants assist in a majority of births (WHO, 2003 and Bannerman *et al*., 1993).

Over one-third of the population in developing countries lack access to essential medicines (WHO, 2003). The provision of safe and effective TM/ CAM therapies could, thus, become a critical tool to increase access to health care (WHO, 2003). In Ethiopia up to 80% of the population uses traditional medicine due to the cultural acceptability of healers and local pharmacopeia, the relatively low cost of traditional medicine and difficult access to modern health facilities (EDHS, 2000). The vast majority of Ethiopia's population live in rural areas where the health care coverage is low and where existing public sector resources are being stretched to the limits. One of the greatest challenge facing the country is determining how best to narrow the gap between the existing services and the population whose access to them is very limited.

Ethiopia has a long history of traditional medicine and has developed ways to combat disease through traditional medicine (Negussie, 1988). The ways are also as diverse as the different cultures.

Healing in Ethiopian traditional medicine is not only concerned with curing of diseases but also with the protection and promotion of human physical, spiritual, social, mental and material wellbeing (Beshaw, 1991).

In Ethiopia it is widely believed that the skill of traditional health practitioners is 'given by God' and knowledge on traditional medicines is passed orally from father to a favourite child, usually a son or is acquired by some spiritual procedures. Traditional healing knowledge is guarded by certain families or social groups (WHO, 1990).

Healers obtain their drugs mainly from natural substances and in descending order of frequency these constitute plants, animals and minerals. Drugs are prepared in various dosage forms including liquids, ointments, powders and pills. Drugs are also prescribed in a non-formulated form, and additives are usually incorporated and more than one drug is used in a single dosage form. Drugs are administered using different routes, the main ones being, topical, oral and respiratory. When side-effects become severe, antidotes are claimed to be used. The healers impose restriction when certain types of drugs are taken by patients. The knowledge on medicinal plants is largely oral. In addition, Ethiopia‟s ancient church practices have documented some of the knowledge as inscribed in Parchments which partly characterize the traditional medical system usually described as medico religious written in Geez manuscripts of the 15th century (Dawit and Ahadu, 1993, Gelahun, 1989,).

Other ancient written sources include the book of remedy (Metsehafe Fews) of the 17th century which contains a wide range of medicinal plants prescription (Fekadu, 2001). These are the medical traditions of the followers of Coptic Christianity. Other cultural groups in the country have their own written or oral traditions that could be associated with individual clans or groups as partly stated by (Abbink, 1995 and Amare, 1976).

About 1000 identified medicinal plant species are reported in the Ethiopian Flora. However, many others are not yet identified (Edwards, 2001). Approximately 300 of these species are frequently mentioned in many sources. The greater concentration of medicinal plants are found in the South and South Western Ethiopian parts of the country following the concentration of biological and cultural diversity (Edwards, 2001). The various citations made from written

records of medicinal plants from Central, North and Northwestern part of Ethiopia are thus small fractions of medicinal plants present in Ethiopia. Studies on the Bale Mountains National Park in the South East Ethiopia revealed that the area, as much as it is a biodiversity hotspot, also turned out to be a medicinal plant hotspot with 337 identified medicinal species of which 24 are endemic (National Herbarium, 2004; Ermias, 2005; Haile, 2005). Out of the 283 species used as human medicine, 47 are used as livestock medicine and 76 are used for both human and livestock by the community healers, harvesters, traders and users (Edwards, 2001).

1.1.2. Herbal Medicine

Herbalogy or herbalism can be described as any type of medication that uses plant products for the treatment process (Wiseman, 2004). Herb can be defined as any form of a plant or plant product, including leaves, stems, flowers, roots, and seeds. These plants can either be used raw or as extracts, the resulting products contain dozens of chemicals, including fatty acids, sterols, alkaloids, flavonoids, glycosides, saponins, complex carbohydrate, glycopeptides, tannins, cyanogens, peptides, amines, sulphur compounds and others (Rotblatt and Ziment, 2007).

Herbs produce and contain a variety of chemical substances with varied pharmacological effects. They are huge reservoir of various chemical substances with potential therapeutic properties (Lewis and Elvin-Lewis, 1995). As the result herbal plants are being increasingly utilized to treat a wide variety of clinical diseases (Gupta *et al*., 2004).

Herbs have been used by all cultures throughout history and thus, herbal medicine is the oldest form of health care known to mankind. It was an integral part of the development of modern medication. Many drugs commonly used today are of herbal origin. Examples include aspirin (willow bark), digoxin (from foxglove), quinine (from cinchonabark), and morphine (from the opium poppy) (Vickers and Zollman, 1999). Higher plants as source of medicinal compound continue to play a dominant role in maintenance of human health since antiquities (Suffness and Dowos,1982). The primary benefit of using plant derived-medicine is that they are relatively safer than synthetic drugs, profound therapeutic benefit and affordable treatments (Iwu,1994). However, it must be noted that not all medicinal plants are safe for consumption in the crude form. Some level of toxicity may arise as a result of potential toxic compounds they contain and pesticide application during cultivation (Amdur *et*

al., 1991 and Evans 1999). The therapeutic properties of medicinal plants used by traditional medical practitioners may be due to one or more of the many compounds of the plant material. The growing interest in herbal medicine therefore demands toxicity risk assessment of the indigenous preparations used in the treatment of diseases (Yakubu *et al*., 2005).

The African continent is endowed with the richest biodiversity in the world with food plants used as herbs, food and for therapeutic purposes (Iwu, 1994). In sub Saharan Africa, thousands of kilogram of medicinal plants are collected and used by healers for treatments of different human and live stock diseases (WHO, 2001). Ethiopia has an enormous resources of plant species that are used in traditional herbal medicine .Among the 7000 higher plant species that are known to exist, about 800 of them are employed in traditional health care and 60% of the plants are said to be indigenous with their healing potential (Wolde and Gebre mariam,2002, Mesfin *et al.*,2005). Ethiopian plants have shown very effective medicinal value for some ailments of human and domestic animals.

The major reasons why medicinal plants are demanded in Ethiopia are due to culturally linked traditions and high trust of the community in medicinal values of traditional medicine is relatively low cost in using them.

1.1.2. Role of Traditional Medicines

Plant derived drugs remain an important resource, especially in developing countries, to treat different diseases. Traditional medicine has been the back bone of the health care system of developing countries where the majority of the population is either not able to afford modern medicine or has no adequate information concerning the use of modern medicine. This is due to various reasons, including limitation of conventional or modern medicine and lack of formal health care facilities (WHO, 2002).

The use of traditional medicine (TM) and complementary and alternative medicine (CAM) is increasing throughout the world. It accounts for a major part of the health care provided worldwide. In low and middle-income countries, up to 80% of the population rely on TM for their primary health care needs (WHO, 2002 & WHO/EDM/TRM/2002).Further more in many high-income countries CAM utilization is becoming increasingly popular, with up to 65% of the population reporting that they have used this form of medicine (WHO, 2000 & WHO, 2002). The desire to capture the wisdom of traditional healing systems has also led to a resurgence of

interest in herbal medicines particularly in Europe and North America, where herbal products have been incorporated into so-called „alternative", „complementary", „holistic" or „integrative" medical systems(Tyler, 2000).

The advantages of TM/CAM include its diversity and flexibility; its availability and affordability in many parts of the world; its widespread acceptance in low- and middle- income countries; it's comparatively low cost; and the relatively low level of technological input required. As a result, TM/CAM therapies have the potential to contribute to a better health care system in many countries (WHO/EDM/TRM/2002).

1.1.3. Harmful Effects of Traditional Medicine

The safety of several commercially available herbs has recently come into question due to reports of adverse effects and potential interactions with prescribed drugs (Popata *et al*., 2001). Nowadays, one of the most severe adverse effects examples associated with herbal medication is development of renal failure and hepatic dysfunction (Kefale, 2005). Moreover, there are numerous examples of potential side effects associated with the most commonly used herbal and other types of complementary and alternative medicine (Kefale, 2005). Some medicinal herbs contain toxic compounds which may cause liver toxicity and cancer; others may cause adverse reactions including allergic, cardiac and irritant effects (Mudiwa, 2000). For example, the Chinese herb ma huang (*Ephedra sinica*), which contains ephedrine used for breathing problems, caused heart attack and stroke among some Americans using it as a dietary supplement (Ang-Lee *et al.,* 2001); long-term use of kava (*Piper Methysticum*) which is used relieve anxiety can cause serious liver damage (Stevinson *et al*.,2002); and the use of ginkgo (gingko biloba), which stimulates peripheral circulation can result in bleeding during surgery (Ang-Lee *et al.,* 2001).

1.2. *Maytenus gracilipes*

1.2.1. Botanical Distribution of *Maytenus gracilipes*

Celastraceae is the large family of trees and shrubs. It is comprised about approximately 89 genera and 1300 species (Stevens, 2001). *Maytenus* is one of the largest genera include about 270 species distributed throughout Brazilian territory (Stevens, 2001). Most of which are used as traditional medicine in different countries of the world. Among various species, *M. gracilipes* is an ever green shrub widely distributed almost throughout the world specially Brazil, Paraguay,

Argentina, Cameron, Ivory Coast, Nigeria, Liberia and Ethiopia (Mossi *et al.,* 2002). In Ethiopia, *M. gracilipes* is widely distributed in different regions. It is commonly called Kombolcha in "Afaan Oromoo" and "Atat, Kamu, Kurava, Telalo, and Talo," alternatively in Amharic; Dubobeis, in Somali, Degmut and Hatchat in Tigrigna. It is a shrub or bushy tree up to 5 -10 m high. Leaves are simple and alternate, oblong, lanceolate or obovate (Fig.1) Flowers are sweet-scented, creamy white to pink in panicles. It is frequently found in humid mountainous woodland as an understorey tree at altitudes between 2200 and 3200 m. flowers shortly after the rains have stopped.

Figure 1.Photograph showing the leaves of *M. gracilipes* growing in Ankober woreda, North Shewa Amhara region as collected during the month of May, 2014.

1.2.2. Major Chemical Constituents of *Maytenus Gracilipes*

In phytochemical studies on *Maytenus* spp., triterpenes, flavonoids, sesquiterpene b-agarofurans and sesquiterpene evoninoate alkaloids have been isolated (Gonzalez *et al*., 1996; Chavez *et al*., 1998). Studies on biological activity have revealed that have antitumor (Gonzalez *et al*., 1996; Chavez *et al*., 1998), antimicrobial (Orrabi *et al*., 2001).However it also has shown insecticidal activities (Nunez *et al*., 2004) and cytotoxic activity (Kuo *et al*., 1990 & 1994).

1.2.3. Medicinal Use of *Maytenus gracilipes*

Plant extracts of the Celastraceae have been used for centuries as insecticide in traditional agriculture, and also for the treatment of many stomach complications, fever, rheumatoid arthritis and cancer. Many biological activities of this genus were determined experimentally as antiulcerogenic and analgesic (Gonzalez *et al*., 2001; Silva *et al*., 2005), antiulcer (Souza-Formigoni *et al*., 1991; Tabach and Oliveira, 2003; Ferreira *et al*., 2004; Jorge *et al*.,2004), antinociceptive, antiinflammatory (Jorge *et al*., 2004), and antioxidant (Vellosa *et al*., 2006; Melo *et al*., 2001). Furthermore the medicinal activity of *Maytenus gracilipes* is shown to have antioxidant, anti inflammatory, anti ulcer, anti cancer and for the treatments of many stomach complications (Gonzalez *et al.,* 2001*). Maytenus gracilipes* is also used to treat Epilepsy (Dhibe Qabana) (Yineger *et al*, 2007).

1.3. The Blood and Its compositions

Blood is fluid connective tissue which is denser and more viscous than water and feels
Sticky. It is composed of plasma, red blood cells, white blood cells, and platelets. It functions similarly in mice and humans to deliver oxygen and nutrients to various sites and to carry waste for disposal or breakdown. In addition, blood carries leukocytes, platelets, and clotting factors to sites for action. Erythrocytes and leukocytes, as well as platelets, originate in the bone marrow and usually migrate as mature cells to the peripheral blood. Plasma proteins are made in the bone marrow, the liver, and other cells in the reticuloendothelial system.

1.3.1. Formed elements of the Blood

The formed elements of the blood include three principal components: red blood cells (RBCs), white blood cells (WBCs), and platelets. RBCs and WBCs are whole cells; while platelets are cell fragments. RBCs and platelets have just a few roles, but WBCs have a number of specialized functions. Several distinct types of WBCs are; neutrophils, eosinophils, basophils, lymphocytes and monocytes.

Red blood cells typically appear as biconcave discs. On stained smears, they are circular with distinct, smooth margins. Mouse erythrocytes are 4–7 μm in diameter and appear similar in morphology to those in humans (Piper and Suzanne, 2012). Human erythrocytes are 6–8 μm in diameter (similar to the size of the nucleus of a resting lymphocyte) and 1.5–2.5 μm thick, with

an area of central pallor area comprising approximately one-third of the diameter of the cell (McGarry *et al*., 2010). Erythrocyte morphology is best appreciated where cells are well distributed, inside the feathered edge. Red blood cells are highly specialized for their oxygen and carbon dioxide transport function. Because mature RBCs have no nucleus, all their internal space is available for oxygen and carbon dioxide transport. Because RBCs lack mitochondria and generate ATP anaerobically (without oxygen), they do not use up any of the oxygen they transport.

White blood cells unlike red blood cells , have nuclei and do not contain hemoglobin. WBCs are classified as either granular (polymorphonuclear) leukocytes; neutrophils, eosinophils, and basophils and agranular (mononuclear) leukocytes; lymphocytes and monocytes.

Neutrophil, also known as polymorphonuclear neutrophilic granulocytes, function in the innate immune system in responses to tissue and cell injury, especially in inflammation and bacterial infections. Neutrophils in both mice and humans have cytoplasmic granules containing cytotoxic lysosomal enzymes and a nucleus that is separated into distinct lobes with a thin strand (filament) connecting them. In mice, only 20–25% of peripheral blood leukocytes are neutrophils, compared to 50–70% in humans (Piper and Suzanne, 2012). When ideally stained, the cytoplasm is light pink, and the numerous, evenly distributed fine granules have a light pink-to-purple color. Human neutrophils typically have three to five nuclear lobes, with three-lobe forming the most abundant (41%) (Piper and Suzanne, 2012). In females of both species, a small nuclear drumstick-shaped appendage representing an inactivated X chromosome (Barr body) can be seen. Barr bodies are much more common in female mouse blood smears. When activated, neutrophils can develop dark coarse granules; this is termed toxic granulation. In addition, activated neutrophils often contain vacuoles (Piper and Suzanne, 2012).

Basophils are granulocytes that have round, indented, band, or segmented nuclei. In mice, less than 1% of the leukocytes are basophils. In humans, basophils are roughly the same size as Neutrophils. They are rarely found in the peripheral blood, representing 0 or 1% of leukocytes (McGarry *et al*., 2010). They have round-to-oval shapes with smooth edges. Basophils get their name from their prominent (0.2–1 μm) cytoplasmic granules that have an affinity for basic dyes. These granules are numerous and unevenly distributed, with colors varying from deep purplish-

blue to dark purple-red. They contain heparin, histamine, and other chemicals. Basophils are not phagocytic; rather, when stimulated, the cells eject the chemicals contained in their granules.

Eosinophils are granulocytes that have band or bilobed nuclei. In mice, 0–3% of the Leukocytes are eosinophils (McGarry and Protheroe, 2010). In humans, eosinophils are roughly the same size (12-15μm) as neutrophils and represent between 0-7% of white blood cells in the periphery. Eosinophils get their name from their relatively large, spherical granules that have a particular affinity for stains containing acid eosin. The cells stain reddish-orange, sometimes with brownish tints. The granules contain histamine, enzymes, and other proteins. Eosinophils are rarely phagocytic. Typically, they migrate to peripheral sites and degranulate upon stimulation, releasing cytotoxins that are toxic to both parasites and host tissue.

Lymphocytes are most commonly found in the lymphoid organs (on spleen, thymus, and lymph nodes), but they also circulate in the peripheral blood. In mice, 70–75% of the peripheral blood leukocytes are lymphocytes (Piper and Suzanne, 2012). In humans, lymphocytes make up 30–70% of the peripheral leukocytes in children; in adults, this number decreases to 20–40% (Piper and Suzanne, 2012). The majority of lymphocytes in the periphery are small, ranging from 7 to 10 μ m in diameter. Their nuclear chromatin is clumped and darkly stained. The cells have high nuclear: cytoplasmic ratios and the cytoplasm is blue with a variable intensity. Large granular lymphocytes can be seen in both mice and humans.

Monocytes are phagocytic leukocytes of the blood that, in conjunction with tissue macrophages and neutrophils are important cells involved in first-line defence against pathogenic organisms or foreign cells. In mice, monocytes comprise 2–6% of the peripheral blood leukocytes. In humans, monocytes comprise 1–6% of human blood cells (Piper and Suzanne, 2012). Generally, monocytes are three or four times the size of adjacent red blood cells. Cell shape is variable, although most are round to oval, with abundant dull blue-gray cytoplasm. Granules are sometimes present, and they are usually fine, lightly staining, and numerous. Cytoplasmic vacuoles are often present. Monocyte nuclei are usually folded or kidney shaped. This variation in nuclear morphology is also seen in mice.

Platelets are derived in bone marrow from megakaryocytes. In mice, platelets are often clustered together but may also scattered individually. In both species, the diameter of platelets ranges

from 1 to 4 μm (Piper and Suzanne, 2012). Platelet edges are irregular, with pointed filaments and tentacle-like protrusions. The cytoplasm stains a light blue with small granules. No nucleus is present. Although platelets tend to adhere to one another, clumping can also be indicative of suboptimal anticoagulation at the time of blood collection. Platelets are crucial for thrombosis and hemostasis, and providing a catalytic surface for clotting factors.

1.3.2. Biochemical Compositions of Blood

The Biochemical compositions of blood such as blood plasma, urea, uric acid, creatinine, cholesterol, glucose, alkalin phosphatise (ALP) aspartate aminotransfrase (AST), alanine aminotransferase (ALT) and electrolytes are used to evaluate the toxic effects of medicinal plants. It was indicated that elevation of glucose, urea nitrogen, cholesterol, AST, ALT and albumin in serum blood shows that the administered herbal medicine has a toxic effect on animal model (Khlefat, 2001; Oyewole and Massaquoi, 2008). On the other hand, reduced level of glucose, urea, nitrogen and uric acid shows that the herbal medicine has limited toxic effect and does not interfere with renal tubular function. From these points of views, change in these biochemical compositions of blood as well as enzymatic activities in the serum blood is used to indicate toxic effect of medicinal plants. Such changes in biochemical compositions of blood are induced due to damage to hepatocytes and sever acute liver failure (Khlefat, 2001).

1.4. The Structure and Function of Liver

The liver is the central organ of metabolism. It serves as a filter against drugs and toxicants absorbed from the intestinal tract before passing to the systemic circulation (Michalopoulos and Defrances, 1997). Mouse liver weighs 1.3-1.5 gm forming approximately 5- 6% of the total body weight. It occupies the cranial third of abdominal cavity just caudal to the diaphragm and surrounded by a thin capsule (Glisson‟s capsule) (Piper and Suzanne, 2012). Similarly as for other mammals, mouse liver is multi-lobular organ. It consists of four lobes: right, left, median and caudate lobes. The left lobe is the largest liver lobe in mice located at the peripheral surface of the liver. The caudate lobe is small has two segments and located at the visceral surface of the liver. The right lobe is subdivided horizontally in to anterior and posterior portion. The median lobe has an incomplete fissure where falciform ligament attaches and divides the median lobe into two segments where the gall bladder is located in between (Thoolon *et al*., 2010).

The liver receives its blood supply via two sources, the portal vein and the hepatic artery. The portal vein serves as the drainage system of the capillary of the gastrointestinal tract, spleen, and pancreas. It contributes 75% of the total blood supply, while the hepatic artery serves the remaining 25 % of the blood supplies of the liver with arterial blood.

Both vessels enter the liver through the hilus where they branch to guide the blood to the periportal region of the liver lobules. From there, the blood flows through the hepatic sinusoids and drain in to the central vein at the center of the lobule. The central veins collect into hepatic veins which leaves the liver and drain in to inferior vena cava (Vollmer and Menger, 2009; Hoehme *et al*., 2010).

The metabolic zonation of the liver is the characteristics phenomenon for the site specific function of the liver cells. Hepatocytes display different metabolic functions according to their position along the porto-central axis of the liver lobule. There are two well known metabolic zones in the liver lobule, the periportal and pericentral zone. The periportal zone (zone1) involves hepatocytes close to the hepatic blood flow around the portal triad. The pericentral (perivenous zone, zone3) involves hepatocytes close to the central veins, in addition, there is the less defined mid lobular population of hepatocytes (zone2) (Jungermann and Kietzmann, 1996; Braeuning *et al.,* 2006).

Hepatocytes are polygonal epithelial cells. They are arranged in plate like cords separated by adjacent sinusoids. They are the predominant cells types of the liver, accounting for about 60-65% of the total cell number and 80 % of the liver mass (Jungermann and Kietzmann, 1996; Braeuning *et al.,* 2006).

The average diameter is 25-30 µm. About 25 % of the hepatocytes are binucleated (Weilbet *et al*., 1969; Mac Sween and Scothorne, 1994; Desmet, 2001). Hepatocytes are polarized epithelial cells having basal, apical and lateral surface. The basal surfaces face the sinusoidal epithelium and have microvilli to increase the surface area for exchange of materials between hepatocytes and the blood. The apical surface faces the adjacent hepatocytes and forms the bile canaliculi. It has microvilli to increase the surface area for bile secretion (Cardell, 1997).

Hepatic sinusoids are the microvascular structures. They are formed of fenestrated endothelial cells, Macrophages (Kupffer cells), lymphocytes and stellate cells. The sinusoidal cells account for about 30-40 % of the total cell number and approximately 6.3 % of the liver mass (Bioulac-Sage *et al*., 1990).

Endothelial cells account for 15-20 % of the total liver cells and 2.8 % of the liver volume (Kuntz, 2006).They are fenestrated cells lacking the basement membrane. They function as a filter that controls the exchange of materials between the blood and hepatocytes (Schuppan *et al*., 1998). The fenestrae of the endothelial cells exhibit heterogeneity along the porto-central axis of the liver lobules. The fenestrae at the periportal area are longer in size, fewer in number and less porous in comparison to the peri-central area of the live lobule (Wisse *et al*., 1986 and Vidal-Vanaclocha *et al*., 1985).

Kupffer cells constitute about 8-12 % of the total liver cells and 2.1 % of the liver volume. Phagocytosis to be high in the peri-central area of the liver lobule, and the number of Kupffer cell is higher in the periportal area (Bouwens et al., 1986; Lough *et al*., 1987;Tekoppel and Thuman,1990) .

Ito cells are also known as fat storing cells, hepatic stellate cells or lipocytes. They account for 3- 8 % of the total liver cells and 1.4 % of the liver mass. They lie in the space of Disse with higher frequency in the periportal area of the liver lobule. They function as storage for vitamin A. They are considered as the source of the hepatocytes growth factor (HGF) and the transforming growth factor beta (TGF-β) (Wake, 1974; Burt, 1999; Stockert and Wolkoff, 2001).

Different types of liver associated lymphocytes have been described including granular lymphocytes (pit cells) .Pit cells are natural killer cells showing a lower frequency compared to Kupffer cells. They play a role in defence against tumours and viruses (Nakatani *et al*., 2004).

1.4.1. Histopathology of Liver

The liver is responsible for metabolism and detoxification of most of the components that enter the body (Nunez, 2004). The strategic function of the liver allows maintaining the metabolic homeostasis from the intestinal tract via the portal vein and delivers metabolized harmless products to other body organs via hepatic vein (Michalopoulos and De France, 1997; Michalopoulos, 2007). It is the organ primary subjected to toxicants along with the kidneys. Hepatotoxicity implies chemical-driven liver damage and the chemicals that cause liver injury are called hepatotoxins. Liver plays a vital role in bio-transformation and sometimes clearing (detoxification) of chemicals that are susceptible to the toxicity. Certain medicinal agents in overdoses and sometimes even at therapeutic ranges may injure the liver. Other chemical agents, such as those used in laboratories and industries, can also cause injury to liver cell (Singh *et al*., 2012). Numerous medical plants and their formulations are being used for liver

disorders in ethnomedical practices and in traditional system of medicine (Vipin Kumar *et al*, 2013). The injured liver tissues indicated that, necrosis, inflammation, fibrosis, cirrhosis and hepatocarcinoma (Junnila *et al*. 2000; Karakus *et al*. 2011). The number of infiltrated neutrophils, macrophages, Kupffer cells, lymphocytes and natural killer (pit) cells are significantly increased. The activated macrophages are released and contributed to liver fibrosis, inflammation and injury (Canbay *et al*. 2004; Saile and Ramadori, 2007). The Chronic liver injury often leads to fibrosis, scar formation, and distortion of normal tissue architecture (Bennett and Brown, 2003).

1.5. Structure and function of kidneys

The bean-shaped mouse kidneys lie in a retroperitoneal position on either side of the vertebrae in the superior lumbar region. Extending approximately from T12 to L3, the right kidney receives some protection from the lower part of the rib cage. The right kidney is relatively cranial, adjacent to the right lobe of the liver; the left is more caudal (Young *et al*., 2006). However, in human the right kidney is usually slightly lower (caudal) than the left due to position of liver. Male mice kidney is relatively larger than the female; Similar to human, male kidney in human is slightly larger than the female kidney (Young *et al*., 2006). The kidney has lateral convex and medial concave borders with a vertical cleft called the renal hilum that leads into an internal space within the kidney called the renal sinus. A frontal section through a kidney reveals two major distinct regions: cortex, and medulla. The most superficial region, the renal cortex, is light in color and has a granular appearance. Deep to the cortex is the darker, reddish-brown renal medulla. The mouse kidney is unilobar (unipyramidal) with only a single papilla that extends deep into the renal pelvis. The mouse cortex has cone-shaped cortical labyrinths and medullary rays that extend from the outer medulla, which is divided into an outer and inner stripe. The inner portion of the medulla is the renal papilla, which extends into the renal pelvis and ureter.

Nephrons are the structural and functional units of the kidneys. In mice each kidney contains over 14,000 nephrons, which carry out the processes that form urine (Piper and Suzanne, 2012). Each nephron consists of a dilated portion, the renal corpuscle; the proximal convoluted tubule; the thin and thick limbs of Henle's loop; and the distal convoluted tubule. Each renal corpuscle consists of a tuft of capillaries, (the glomerulus), surrounded by a double-walled epithelial capsule called glomerular (Bowman's) capsule. The internal layer (the visceral layer) of the capsule envelops the capillaries of the glomerulus. The external layer forms the outer limit of the

renal corpuscle and is called the parietal layer of Bowman's capsule. Between the two layers of Bowman's capsule is the urinary space, which receives the fluid filtered through the capillary wall and the visceral layer. Each renal corpuscle has a vascular pole, where the afferent arteriole enters and the efferent arteriole leaves and a urinary pole, where the proximal convoluted tubule begins similar to human.

Kidney has vital functional role in the body next to the liver; every day the kidneys filter the fluid from the blood-stream, allowing toxins, metabolic wastes, and excess ions to leave the body in the form of urine, while returning needed substances to the blood. They also act as essential regulators of the volume and chemical makeup of the blood, maintaining the proper balance between water and salts and between acids and bases. Gluconeogenesis, producing hormones (renin and erythropoietin) and metabolizing vitamin D to its active form are other functions of the kidneys. Due to their main functional role, the same as the liver, kidneys are also the primary target organs for toxicity.

1.5.1. Histopathology of kidneys

The kidneys are routinely exposed to high concentrations of medications or their metabolites because their intrinsic functions are to metabolize, concentrate, and excrete compounds. Many dietary supplements have been associated with nephrotoxicity, either as a direct toxic effect, or secondary to liver dysfunction, rhabdomyolysis, or nephrolithiasis (Thomson *et al.*, 2002). The renal effects of various herbs can be harmful. These effects include: polyuria causing dehydration, acute renal failure, chronic renal insufficiency, and stone formation (Thomson *et al.*, 2002). Unknown usage of herbal medicine may cause more damage to endanger renal functions. Research has shown that herbal remedy use may be associated with acute renal failure and, may be detrimental for the patient with compromised renal function (Murcia *et al.*, 2004). Patients with renal insufficiency or renal failure may be at risk for further kidney damage as well as complications related to interactions of herbal remedies with complex renal therapy regimens (Murcia *et al.*, 2004). Nephrotoxicity is characterized by tubular necrosis; basal membrane disruption; mesangial cell contraction; proliferation and apoptosis, decreases in glomerular filtration and alteration in intraglomerular dynamics (Martinez-Salgado *et al.*, 2007).

1.6. Significance of the Study

Traditional herbal medicine and their preparations have been widely used for thousands of years to treat various ailments in developing and developed countries owing to its natural origin, accessibility and lesser side effects. Herbal plants are being increasingly utilized to treat a wide variety of clinical diseases (Gupta *et al*., 2004). Now a days the growing interest in studying herbal medicine is toxicity risk assessment of the various indigenous preparations used in the treatment of diseases (Yakubu *et al*., 2005). Various herbs can have harmful or beneficial effects. Therefore, the purpose of this study is to evaluate if there is any toxicity of *Maytenus gracilipes celastraciae* on some blood parameters, liver and kidney in mice‟s. This is important to have some information regarding the toxicity potential and efficiency of such plants utilized ethnobotanically to treat various ailments.

2. Objectives of the study

2.1. General Objective

- Evaluation of acute and sub-chronic toxicity of aqueous leaves extract of *Maytenus gracilipes celastraciae* (kombolcha) on some blood parameters, and histopathology of liver and kidney in mice.

2.2. Specific objectives

- To determine the LD_{50} of aqueous leaves extract of *Maytenus gracilipes celastraciae.*
- To investigate signs of toxicity in experimental mice.
- To evaluate the effect of the extract on general body weight and weight of liver, kidneys of the mice.
- To evaluate the effects of the aqueous leaves extract of these plant on some haematological and biochemical parameters of blood in the mice.
- To investigate any gross and microscopic histopathological changes of liver, and kidneys of the mice following treatment with the extract.

3. Materials and methods

3.1. Study design: laboratory based experiment

3.2. Study setting: AAU, School of Medicine, Histology, Physiology, Pharmacology, and Pathology laboratories.

3.3. Study area:

The study was conducted at Addis Ababa University, College of Health Science, School of Medicine, Department of Anatomy (Histology Laboratory).

3.4. Plant collection and Extraction

3.4.1. Plant Collection

Leaves of *Maytenus gracilipes celastraciae* were collected from Ankober woreda (Atsie Minilik loge) about 42 km away from Debre Birhan, Northern Shawa, Amhara Region, 130 km North of Addis Ababa during the month of May 2014. Specimens of the plant were identified by a taxonomist and a few samples were deposited at the National Herbarium in the College of Natural and Computational Sciences Addis Ababa University (AAU) with a Voucher specimen number (119/AMA/PHARM). The leaves were further dried under shade at room temperature to ensure easy grinding of the leaves and also protect from ultra violate rays as certain chemical constitutes of the leaves may be sensitive to sun radiation. Any extraneous materials from the leaves were also removed and carefully selected and crushed to fine powder with grinding machine. A yieled of 76 gm extract was obtained from 600 gm of fine powdered leaves of *Maytenus gracilipes*. The powder was stored in plastic bag till extracted with distilled water to obtain aqueous extracts.

3.4.2. Preparation of Aqueous Extract

A 100 gm dried leaf fine powder of *Maytenus gracilipes celastraciae* was macerated with 500ml of distilled water in Erlymleyer flask and allowed to rotate at speed of 150 per minute for six hours on orbital shaker to ensure complete mixing of the powdered particle with the solvent (distilled water) and then allowed to settle for about 30-60 minutes to separate the supernatant from residue. The supernatant was decanted from the residue, filtered through guaze ($0.1mm^2$ meshes) on to crucible and, or petridish. The crucible and, or petridish containing filtrate were

put into refrigerator to deep freeze until the filtrates became solidified. The solidified filtrate was allowed to freeze dry with lyophilizer for 3-4 days until the crude extract was dried. The dried crude extract was collected, and such procedure continued till the total extract was completely obtained and was kept in a desecrator to remove any moisture, until used for the experiment.

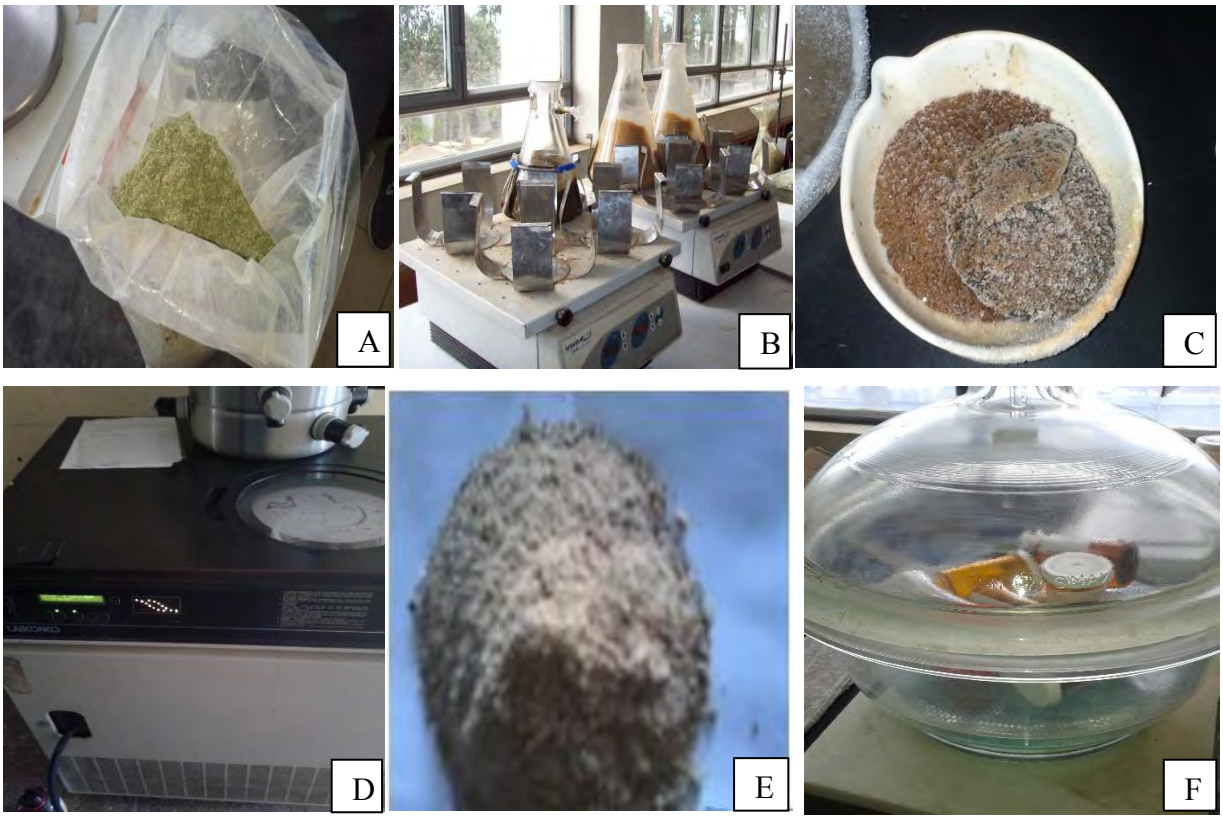


Figure 2.Photo graphs that show the process of Aqueous leaves extract of *M. gracilipes* celastraciea showing; (A) Dried leaf powder of *Maytenus gracilipes*, (B) Aqueous extraction process of *Maytenus gracilipes*,(C) crude leaf extract of *maytenus gracilipes*,(D) lyophilizer (E) extracted power of *Maytenus gracilipes*,(F) Extracted powder of *Maytenus gracilipes* within diseccator.

3.5. Experimental Animal Preparation

3.5.1. Acute Toxicity Study

3.5.1.1. Lethal dose determination of the aqueous leaves extract of *Maytenus gracilipes.*

Forty-five female adult Swiss albino mice, 2 – 3 month old weighing 25 – 30 gm, were obtained from animal breeding house of the Ethiopian Public Health Institute and randomly assigned into fifteen groups, including the control, each group containing 3 mice based on OECD protocol(OECD, 2001). The animals were acclimatized for a week to avoid any stress (Vipul *et*

al.,2007) and fasted for 3-4 hours before administration and 1-2 hours after administration (OECD, 2001). The weight of each animal within the group was recorded. Dose was calculated relative to their average body weight and administered orally to each mouse in a group as shown in table 1. The starting dose was selected from the fixed dose levels of 5, 50, 300 and 2000 mg/kg as a dose expected to produce evident toxicity. In this study the starting dose was 300 mg/kg, because of absence available evidences for toxicity of the plant (OECD, 2001). After oral administration of the desired dose, each animal was returned to the cage in its respective group. Animals were observed for signs of toxicity and lethality for 24 hours. Deaths occurred with 24 hours were recorded and LD_{50} was determined.

Table 1: Lethal dose determination of the aqueous leaves extracts of *Maytenus gracilipes celastraciea.*

Group	Dose (mg/kg)	Number of mice used
GI	Control	3
GII	300	3
GIII	2000	3
GIV	2500	3
GV	5000	3
G VI	5500	3
GVII	6000	3
GVIII	6500	3
GIX	7000	3
GX	7500	3
GXI	8000	3
GXII	8500	3
GXIII	9000	3
GXIV	9500	3
GXV	10000	3

3.6. Subchronic Treatment

Fifteen 8 weeks old adult Swiss albino mice weighing 25-30 gm for each male and female mice were used. The animals were obtained from animal breeding house of the Ethiopian Public Health Institute. The animals were kept in plastic cage under standard laboratory condition and were exposed to 12 hours light and dark cycle at standard temperature (21 ± 2^0C) and pressure (OECD, 2001). The animals were provided with standard commercial diet and were given tap water throughout the experimental periods.

Before the beginning of the experiment all animals were weighed and randomly assigned into 3 groups separately for each of the males and females (OECD, 2001).Containing 5 mice in each

group. Group I as control, and group II and III as experimental groups. The mice were acclimatized to laboratory conditions for about seven days to experimental protocol to avoid any stress (Vipul *et al*., 2007). Upon the commencement of the experiment, all animals were again weighed in their respective groups and their average body weights were recorded to calculate the dose accordingly. The weight recorded at the beginning of the experiment was the initial body weight, and animal weights were also recorded at the end of each week throughout the experimental period.

The control group was assigned as (group I) was administered distilled water. The experimental groups were given the aqueous extract treatment respectively as effective dose of 700 mg/kg/ body weight for group II and high dose of 2100 mg/kg/ body weight for group III for 90 days in 24 hours intervals. The administration rout for both extracts as well as the vehicle was through oral rout with oral gavage as per WHO guideline (WHO, 2000).

3.7. Blood collection for hematological and biochemical analyses

At the end of the experimental period, animals belonging to each group were weighed on a digital balance and each animal was anesthetized by diethyl ether and blood samples were withdrawn by cardiac puncture. Parts of the blood samples obtained from each mouse were collected in separate test tubes with an anti-coagulant, EDTA (ethylene diamine tetra-acetic acid) and the other in plain test tubes with no EDTA. Blood samples from EDTA containing test tubes were immediately processed for hematological parameters using Automated Hematological Analyzer, (SYSMEX RX21, Japan). White blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count (PLC),neutrophils count(NEUT), eosinophils count (EOSI),lymphocytes count(LMY) were determined. For biochemical analysis, the blood samples in the plain test tubes were allowed to stand for 3 hours for complete clotting and then centrifuged at 5000 rpm for 15 minutes using a bench top centrifuge (HUMAX-K, HUMAN-GmbH, Germany). The plasma was withdrawn and transferred into other clean vials. The sera were kept at -20°C until analysis for clinical biochemistry measurements. The concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, urea, and creatinine were automatically analyzed by (AUTO LAB18 clinical chemistry analyzer, Italy).

3.8. Animal Dissection and Tissue Collection

Following collection of blood samples, each animal was sacrified and the abdominal cavity was opened and the liver and kidney were carefully removed and cleared from any surrounding tissues by normal saline and put on clean paper and weighed quickly on digital balance.

Randomly the coronal section of some sample of the right and left kidney and some sample of transverse section of the left lobe, because it is a common site for histological sampling(Piper and Suzanne, 2012) of liver were preserved in 10% neutral buffered formalin fixative solution for 24 hours taken for histological processing.

3.9. Histological Processing

After overnight fixation, the liver and kidneys were washed with tap water to remove excess fixatives for several times and dehydrated with increased concentration of ethanol alcohol of 70% for two hours 80% for two hours, 90% for two hours, absolute alcohol (I, II and III) for one and half hour for each, and IV overnight. The dehydrated tissues were cleared in two changes of xylene (I and II) for one and half hours and two and half hours, respectively. The tissues were then infiltrated with three changes of paraffin wax (I, II and III) for one and half hours, two and half hours and overnight, respectively. Finally the tissues were embedded in paraffin wax in square metal plates forming tissue blocks, whereby each tissue block was labelled and stored at room temperature till sectioned.

Tissue blocks were sectioned in ribbons at a thickness of 5 µm with Leica rotary microtome (LEICA RM 2125RT, Germany). The ribbons of sections were taken at every 5^{th} sections and put onto the surface of a warm water bath at temperature of 40^{o}_{C}. The floating ribbons over the surface of warm water were mounted onto pre cleaned slides. The slides containing paraffin wax sections were arranged within the slide holder and placed in an oven with temperature of 40^{o}_{C} for overnight; to fix the tissue to the slides .The next day tissue sections were allowed to cool at room temperature for 30 minutes and stained progressively with routine Harris haematoxylin and eosin staining. For this two series of coupling jars were prepared. One for paraffin removal and hydration, and the other for dehydration and clearing. Sections were placed in xylene- I for 4 minutes and xylene II for 4 minutes to remove the paraffin from tissue and hydrated with decreasing concentrations of absolute alcohol I, II for four minutes each and 95% and , 80% of

alcohol for three minutes each. The tissue sections were washed with tap water for five minutes and stained progressively with Harris haematoxylin for 10 minutes, then washed under running tap water for five minutes. The slides were immersed in acidic alcohol for differentiation and controlling over stained haematoxylin for 1-3 second and then put in blueing solution (Sodium bicarbonate) until they became blue. After blueing, the slides were counter stained with eosin for one minute and then washed in tap water for five minutes. The sections were dehydrated with increasing alcohol concentration of 80%, 95%, absolute II and I for three minutes, each. The dehydrated sections were cleared with xylene II and I for three minutes each and permanently mounted on microscopic slides using DPX and cover slips and then observed by light microscope for investigations of any histological change, thereby the histology of the treated groups were compared with histology of the control group.

3.10. Light microscopy and photomicrography

Stained tissue sections of the liver and kidney were carefully examined under binocular compound light microscope (LEITZ WETZARE, Germany) and CX41RF, Philippines). Tissue sections from the treated groups were examined for any evidence of histopathological changes with respect to those of the controls. After examination, photomicrograph of selected samples of liver and kidney sections from both the treated and control mice were taken under a magnification of x20 objective lens by using (EVOS XL, USA) automated built-in digital photo camera.

3.11. Statistical Analysis

The result were analyzed statistically using one way analysis of variance (ANOVA) using the SPSS version 21 computer software to identify the possible difference between body weight , relative organ weight , haematological and biochemical values followed by student's t-test to compare the difference between controls and treated groups. All data were expressed as mean $\pm$ standard error of the mean (SEM). Differences at $p<0.05$ were considered to be significant.

4. Results

4.1. Signs of acute toxicity during LD_{50} Determinations

In this study for LD_{50} determination, the starting dose was 300 mg/kg, because of absence of available evidences of the toxic effect of the plant (OECD, 2001). Accordingly, in the aqueous leaves extracts treated mice, no signs and symptoms of toxicity on behaviours were observed from the starting dose up to dose level of 5000 mg/kg body weight and no mortality was observed up to the dose level of 9000 mg/kg/body weight. Depression, loss of appetite, piloerection and faster breathing were progressively increased as dose of the aqueous extract of the plant increased (Table 2). Mortality began at dose level of 9500 mg/kg/body weight of aqueous extract of the plant. More than half treated mice died in 24 hrs period following a single administration of the aqueous leaves extracts of the plant at dose level of 10,000 mg/kg/body weight. Therefore the LD_{50} of the aqueous leaves extracts of plant was about 10,000 mg/kg.

Table 2: Lethal dose determination of the aqueous leaves extracts of *Maytenus gracilipes celastraciea*

Group	Dose (mg/kg)	No of mice used	No of mice died	Signs and symptoms
GI	Control	3	0	None
GII	300	3	0	None
GIII	2000	3	0	None
GIV	2500	3	0	None
GV	5000	3	0	Depression, piloerection, and Fast breathing,
G VI	5500	3	0	Fast breathing, piloerection, depression.
GVII	6000	3	0	Depression, piloerection, fast breathing ,,
GVIII	6500	3	0	Depression, fast breathing
GIX	7000	3	0	Depression, fast breathing
GX	7500	3	0	Piloerection, fast breathing
GXI	8000	3	0	Depression, fast breathing
GXII	8500	3	0	Fast breathing, depression
GXIII	9000	3	0	Piloerection, depression
GXIV	9500	3	1	Fast breathing, piloerection
GXV	10000	3	2	Depression, fast breathing

4.2. General Observations during Subchronic Treatment of Aqueous Leaves extracts of *Maytenus gracilipes*

Among the animals administered with the repeated doses of the aqueous leaves extracts of *Maytenus gracilipes* at both 700 mg/kg and 2100 mg/kg body weight for 90 days, no death was observed throughout the experimental period. However, gentle signs of toxicity such as depression, piloerection, loss of appetite, and fast breathing were observed, among those mice treated with the aqueous leaves extract of *Maytenus gracilipes* at both doses as compared to the control group.

4.3. Effects of the Aqueous Leaves Extracts of *Maytenus gracilipes* on body weight

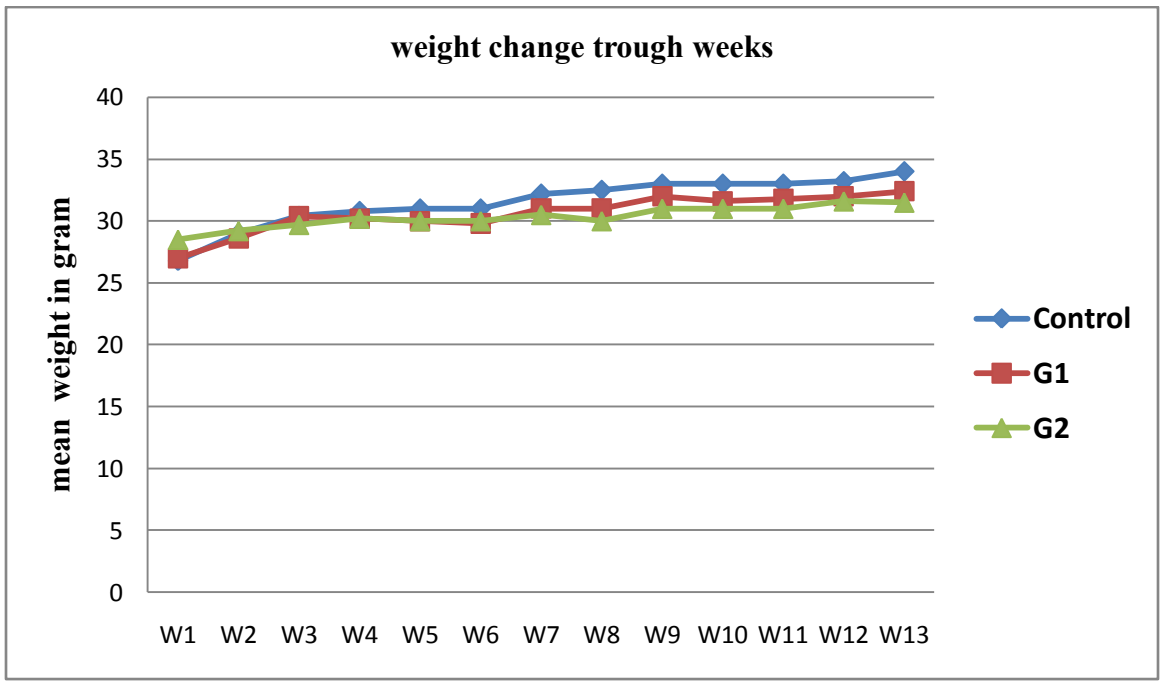
No statistically significant ($p>0.05$) body weight change was observed in the female mice treated with the repeated dose of 700 and 2100 mg/kg body weight/day of the aqueous extracts of the *Maytenus gracilipes* as compared to the controls. However, progressive body weight gains were recorded nearly in all groups of mice with time over the period of the experiment (table 3 & figure 3).

Table 3: The mean body weights of female mice treated with the aqueous extracts of *Maytenus gracilipes* at 700 and 2100 mg/kg doses as compared to the control group during 13 weeks of period.

Weeks	Groups		
	Control	700mg/kg	2100mg/kg
1	26.8±0.58	29.3±0.9 (0.77)	28.5±0.57(0.69)
2	29±0.145	29.6±0.74(0.31)	29.2±0.63(0.42)
3	30.4±0.81	30.4±0.67(0.49)	29.7±0. 48(0.6)
4	30.6±0.32	30.2±0.58(0.42)	30.2±0.58(0.41)
5	30.8±0.54	30±0.7(0.96)	30.5±052(0.39)
6	30.6±0.56	29.8±0.67(0.78)	31±0.71(0.96)
7	32.2±0.37	30.4±0.51(0.81)	31.4±0.7(0.97)
8	32.5±0.45	30.8±0.43(0.12)	31±0.67(0.9)
9	32±0.38	30.6±0.81(0.25)	30±0.71(0.8)
10	31.8±0.37	31.6±0.51(0.82)	30±0.55(0.11)
11	32.6±0.51	31.8±0.73(0.51)	31±0.51(0.15)
12	33.2±0.44	33±0.32(0.34)	31.6±0.45(0.81)
13	33.6±0.51	32.4±0.5(0.9)	32.4±0.5(0.79)

Values are expressed as Mean ± SEM, n=5. The figures in brackets indicate the calculated p values of the treatment groups as compared to the control.

Figure 3: Comparison of mean body weight change between female mice treated with 700 mg/kg (G1) and 2100 mg/kg (G2) doses of the extract with the control group



Similar to female mice, the male mice mean body weight change showed no statistically significant ($p>0.05$) change in the repeated dose of 700 and 2100 mg/kg body weight/day of the aqueous extracts treated mice as compared to the controls. However, Progressive body weight gains were recorded nearly in all groups of mice with time over the period of the experiment. However, at week 5, the weight of male mice treated at both 700 and 2100 mg/kg body weight doses slightly decreased from the former week by 0.47% and 0.2%, respectively and at week 12 the weight of male mice treated at 700 mg/kg and 2100 mg/kg body weight dose also non significantly decreased from the former week by 2% and 0.7 % respectively (table 4 & figure 4).

Table 4: Mean body weights of male mice treated with the aqueous leaves extracts of *Maytenus gracilipes*700 mg/kg and 2100 mg/kg doses as compared with the control group during the 13 weeks of period.

Weeks	Groups		
	Control	700mg/kg	2100mg/kg
1	27.4±0.92	26.5±087(0.17)	28±0.49(0.60)
2	28.6±0.11	27.2±0.86(0.22)	30.8±1.17(0.29)
3	31.2±0.39	32.6±0.50(0.81)	31.2±0.66(0.77)
4	33.7±1.57	34.5±0.59(0.67)	33.6±0.67(0.86)
5	37.8±1.49	34±1.48(0.15)	33.4±0.86(0.92)
6	38±1.14	36.2±0.66(0.77)	34.2±0.66(0.78)
7	38.8±0.86	36.6±0.81(0.83)	36.6±0.67(0.49)
8	38.6±0.58	35.8±1.06(0.79)	37.2±0.70(0.96)
9	39.2±0.90	36.6±0.92(0.77)	36.8±0.71(0.97)
10	39.4±0.69	37.8±1.24(0.65)	37.2±0.86(0.92)
11	39.6±0.51	38.8±0.66(0.78)	38.4±0.68(0.98)
12	40.4±0.94	39.4±1.15(0.96)	40.5±0.71(0.94)
13	40±0.70	38.5±1.25(0.39)	40.2±0.49(0.61)

Values are expressed as Mean ± SEM, n=5. The figures in brackets indicate the calculated p values of the treatment groups as compared to the control.

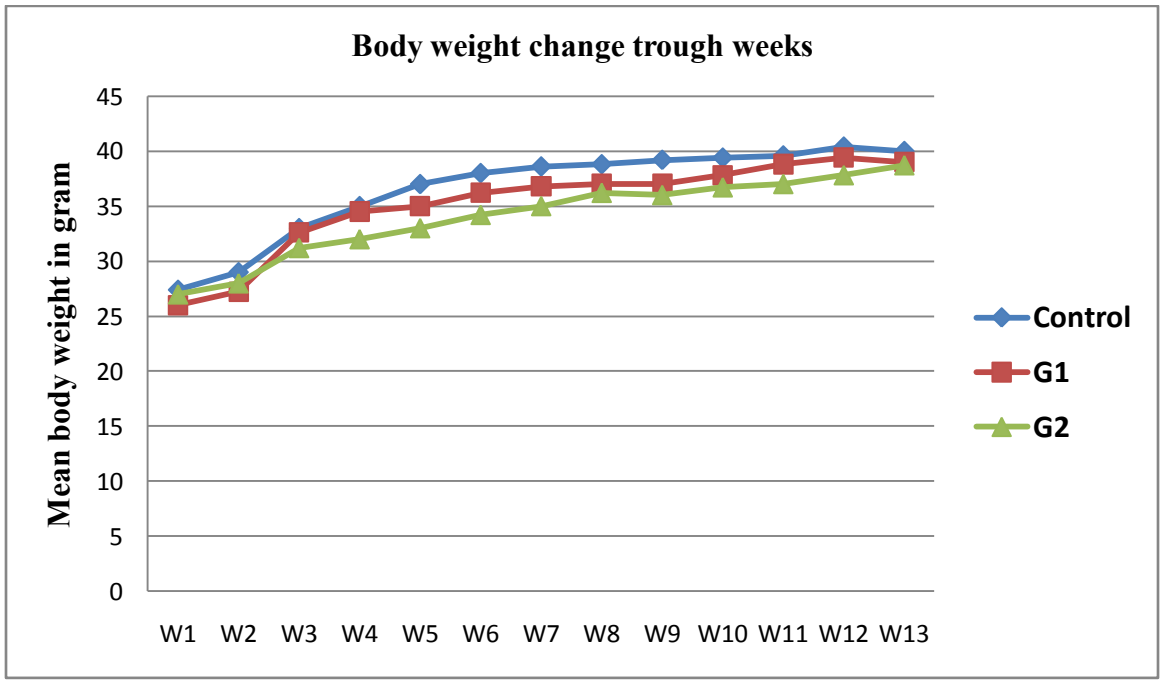


Figure 4: Comparison of mean body weight change between male mice treated with 700 mg/kg (G1) and 2100 mg/kg (G2) doses of the extract with the control group.

4.4. Effects of the aqueous leaves extract of *Maytenus gracilipes* on gross pathology and organ weight

Gross pathology of the liver and kidneys in female mice treated at 700 and 2100mg/kg body weight did not show abnormalities such as in spot, size, texture, color, necrosis and lesion as compared to the controls.

The mean organs weight treated female mice did not show statistically significant ($p>0.05$) difference as compared to the controls. Moreover, non significant increment in the mean weight of liver at higher dose (2100mg/kg) by 1.82% and the mean weight of kidneys increased non significantly by 1.78% at 700 mg/kg (table 5).

Table 5: Mean organ weights of female mice treated with 700 mg/kg and 2100 mg/kg body weight doses of the extract as compared to the control mice at the end of 90 days

Group	Organ weight	
	Liver(gm)	Kidney(gm)
Control	1.62 ± 0.36	0.73 ±0.07
700mg/kg	1.61±0.56(0.91)	0.76±0.69(0.28)
2100mg/kg	1.65±0.21(0.36)	0.72±0.68(0.92)

Values are expressed as Mean ± SEM, n=5. The figures in brackets indicate the calculated p values of the treatment groups as compared to the control.

Similar to the female mice observation of the target internal organs, liver and kidneys of treated male mice at 700 and 2100mg/kg/ day did not show noticeable abnormal changes such as in texture, shape, size, color and spot as compared to the controls. No sign of necrosis or lesion was also noted in these organs in all groups.

The mean weight of liver and kidneys in male treated mice at both doses showed no statistically significant ($p>0.05$) difference as compared to the controls. However, non significant decrement in the mean liver weight at both lower and higher doses by 3.4% and 4.7% respectively, and increased the mean weight of kidneys at the higher dose by 4.65% were observed (table 6).

Table 6: Mean organ weights of male mice treated with 700 mg/kg and 2100mg/kg body weight doses of the extract as compared to the control mice at the end of 90 days.

Group	Organ weight	
	Liver(gm)	Kidney(gm)
Control	1.78±0.51	0.82±0.06
700mg/kg	1.72±0.25(0.74)	0.8±0.46(0.9)
2100mg/kg	1.70±0.36(0.99)	0.86±0.64(0.6)

Values are expressed as Mean ± SEM, n=5. The figures in brackets indicate the calculated p values of the treatment groups as compared to the control

4.5. Effects of the aqueous leaves extract of *Maytenus gracilipes* on hematological parameters of blood.

No stastically significant ($p>0.05$) change was observed on the hematological parameters of female mice treated with the repeated doses of 700 and 2100 mg/kg body weight/day of the aqueous extract of *Maytenus gracilipes* as compared to the controls (table7). However, in female mice treated with the extract, the WBCs count showed nonsignificant decreased by 37% at dose of 700 mg/kg body weight, and increased by 12 % at 2100 mg/kg body weight. The RBCs count was decreased nonsignificantly by 1.1% at 700 mg/kg dose body weight/day and decreased by 3.4% at 2100mg/kg body weight /day as compared to the controls.

Other values of the remaining hematological parameters including HCB, HCT, MCV, MCH, and MCHC in the female mice treated with the extracts at doses of 700 and 2100 mg/kg body weight showed no statistically significant changes compared to the controls. Similarly, there is no significant change in the PLT, NEUT, BASO, and EOSI count between female mice treated with extract at both doses as compared to controls (table 7).

Table 7: Comparison of hematological parameters between female mice treated with 700mg/kg & 2100mg/kg doses of the extract with the controls.

Hematological Parameters	Control (G3)	700 mg/kg dose (G1)	2100 mg/kg dose (G2)
WBC ($x10^3/\mu L$)	6.2 ±1.3	4.5±1.2(0.4)	7.1±0.1(1.0)
RBC ($x10^6/\mu L$)	9.2±1.7	9.1±0.4(0.3)	8.9±1.0(0.5)
HGB (g/dL)	14.6±2.8	14.2±0.3(0.9)	13.5±1.1(0.8)
HCT (%)	44.3±9.5	41.5±2.2(0.3)	38.3±5.6(0.5)
MCV (fL)	44.5±11.4	41.3±1.8 (0.8)	42.4±0.1(0.2)
MCH (pg)	15.4±2.9	15.6±0.9(0.5)	14.5±0.7(0.2)
MCHC (g/dL)	27.8±0.8	28.1±0.5(0.1)	29±1.1(0.9)
PLT ($x10^3/\mu L$)	332±35	445±241(0.9)	547±9.6(0.4)
NEUT($x10^3/\mu L$)	21.4±1.1	23.06±1.1(0.9)	20.4±1.3(0.8)
BASO($x10^3/\mu L$)	0.31±0.5	0.37±0.3(0.27)	0.4±0.5(0.6)
EOSI($x10^3/\mu L$)	1.81±0.11	1.88±0.12(0.8)	1.97±0.8(0.9)
LYM($x10^3/\mu L$)	57.12±1.4	55.15±0.49(0.9)	52.24±0.6(0.7)

Values are expressed as Mean ± SEM, n=5. The figures in brackets indicate the calculated p values of the treatment groups as compared to the control

The hematological parameters of male mice treated with repeated dose of 700 and 2100 mg/kg body weight/day of the aqueous leaves extracts of *Maytenus gracilipes* did not show statistically significant ($p>0.05$) change as compared to the controls. However, WBCs count non significantly decreased by 10% at 700 mg/kg dose of the aqueous extracts of *Maytenus gracilipes* and increased by 31% at 2100 mg/kg body weight/day. RBCs count also non significantly increased by 0.67% at 700mg/kg dose and decreased by 17.5% at 2100mg/kg dose of body weight/day. On the other hand indices of red blood cells such as HGB, HCT, MCV, MCH, and MCHC in male mice treated with the aqueous leaves extracts of *Maytenus gracilipes* at both lower and higher doses did not show statistically significant changes as compared with the controls.

Other values of the remaining hematological parameters such as, NEUT, BASO, EOSI, LYM, and PLT in the male mice treated with the extract at both doses showed no statistically significant changes compared to the controls. (table 8).

Table 8: Comparison of hematological parameters between male mice treated with 700mg/kg & 2100mg/kg doses of the extract with the controls.

Hematological Parameters	Control (G3)	700 mg/kg dose (G1)	2100 mg/kg dose (G2)
WBC ($x10^3/\mu L$)	4.3±0.99	3.9±4.0 (0.17)	6.2±1.5(0.95)
RBC ($x10^6/\mu L$)	8.93±0.096	8.99±0.48(0.84)	7.6±0.7(0.32)
HGB (g/dL)	14.8±0.29	14.3±0.34(0.81)	13.9±0.12(0.46)
HCT (%)	51.1±0.85	48.2±1.0(0.32)	45±1.67(0.88)
MCV (fL)	57.2±1.50	55.6±0.65(0.08)	46.7±7.9(0.21)
MCH (pg)	16.5±0.41	15.3±0.44(0.49)	15.16±1.11(0.45)
MCHC (g/dL)	29.1±0.17	28±0.37(0.74)	28.2± 0.78(0.71)
PLT ($x10^3/\mu L$)	1105.3±1.3	743±43(0.86)	876±1.45(0.78)
NEUT($x10^3/\mu L$)	20.6±1.3	23.5±1.4(0.81)	24±1.2(0.72)
BASO($x10^3/\mu L$)	0.62±0.2	0.81±0.1(0.61)	0.83±0.5(0.51)
EOSI($x10^3/\mu L$)	2.1±1.0	2.3±0.2(0.63)	2.47±0.1(0.72)
LYM($x10^3/\mu L$)	63±2.4	52.2±1.2(0.82)	55±2.7(0.73)

Values are expressed as Mean ± SEM, n=5. The figures in brackets indicate the calculated p values of the treatment groups as compared to the control.

4.6. Effects of the aqueous leaves extract of *Maytenus gracilipes* on biochemical parameters of the blood.

The aqueous leaves extracts of *Maytenus gracilipes* did not show any statistically significant ($p>0.05$) changes in the female mice treated with 700 and 2100 mg/kg body weight/day on the selected serum biochemical parameters as compared to the controls. However, the serum levels of ALT nonsignificantly increased female treated mice at both lower and higher doses by 12% and 25 % respectively. Similarly serum levels of AST nonsignificantly increased by 26% at 700 mg/kg body weight/ day and by 23 % at 21000mg/kg. In addition, total bilirubin nonsignificantly increased at both lower and higher doses by 38% and 5% respectively. The kidneys functional test, urea nonsignificantly decreased by 6.8% at 700 mg/kg and increased by 11.6% at 2100mg/kg. Whereas creatinine nonsignificantly increased at 700mg/kg dose body weight by 0.1% and at 2100 mg/kg body weight by 13.7 %(table 9).

Table 9: Comparison of biochemical parameters between female mice treated with 700 & 2100 mg/kg body weight doses of the extract with the control group.

Biochemical Parameters	Control (G3)	700 mg/kg dose (G1)	2100 mg/kg dose (G2)
ALT (IU/L)	101.6±48	116±33(0.92)	136±12.7(0.12)
AST(IU/L)	113.3±57	153±23.5(0.84)	148±13.8(0.26)
Total Bilirubin	1.14±0.36	1.84±0.03(0.75)	2.24±15(0.65)
Urea (mg/dL)	44.0±8.6	41.18±13(0.68)	49.8±4.5(0.92)
Creatinine (mg/dL)	0.80±0.2	0.88±0.14(0.61)	1.02±1.12(0.78)

Values are expressed as Mean ± SEM, n=5. The figures in brackets indicate the calculated p values of the treatment groups as compared to the control

Similar to female mice the values of (AST, ALT, and Total bilirubin) in liver and kidneys urea and creatinine in the extract treated male mice with repeated dose at 700 and 2100mg/kg did not show stastically significant ($p>0.05$) changes as compared to controls.

The ALT values at both lower and higher doses, nonsignificantly increased by 2.7% and 5% respectively, AST also non significantly increased by 20.4% at 700 mg/kg dose and 14% at 2100 mg/kg dose), total bilirubin also non significantly increased by 9.9 % at 700 mg/kg dose and 19.5% at 2100mg/kg dose. Similarly creatinine increased non significantly by 36% at 700 mg/kg dose and increased by13% at 2100 mg/kg dose, and urea increased by 10.3 % at 700 mg/kg dose and 14 % at 2100 mg/kg dose).

The extract did not show statistically significant ($p>0.05$) changes in any of the selected serum biochemical parameters as compared to the controls (Table 10).

Table 10: Comparison of biochemical parameters between male mice treated with 700mg/kg & 2100 mg/kg body weight doses of the extract with the control group.

Biochemical Parameters	Control (G3)	700 mg/kg dose (G1)	2100 mg/kg dose (G2)
ALT (IU/L	96±6.12	98.7±3.41(0.73)	101±5.63(0.81)
AST(IU/L)	121±7.5	152±10.1(0.11)	141±13.7(0.92)
Total Bilirubin	1.81±0.65	2.01±0.98(0.61)	2.25±0.98(0.14)
Urea (mg/dL)	48.9±3.1	54.5±0.67(0.84)	57±1.60(0.51)
Creatinine (mg/dL)	0.53±0.21	0.55±0.35(0.76)	0.61±0.33(0.72)

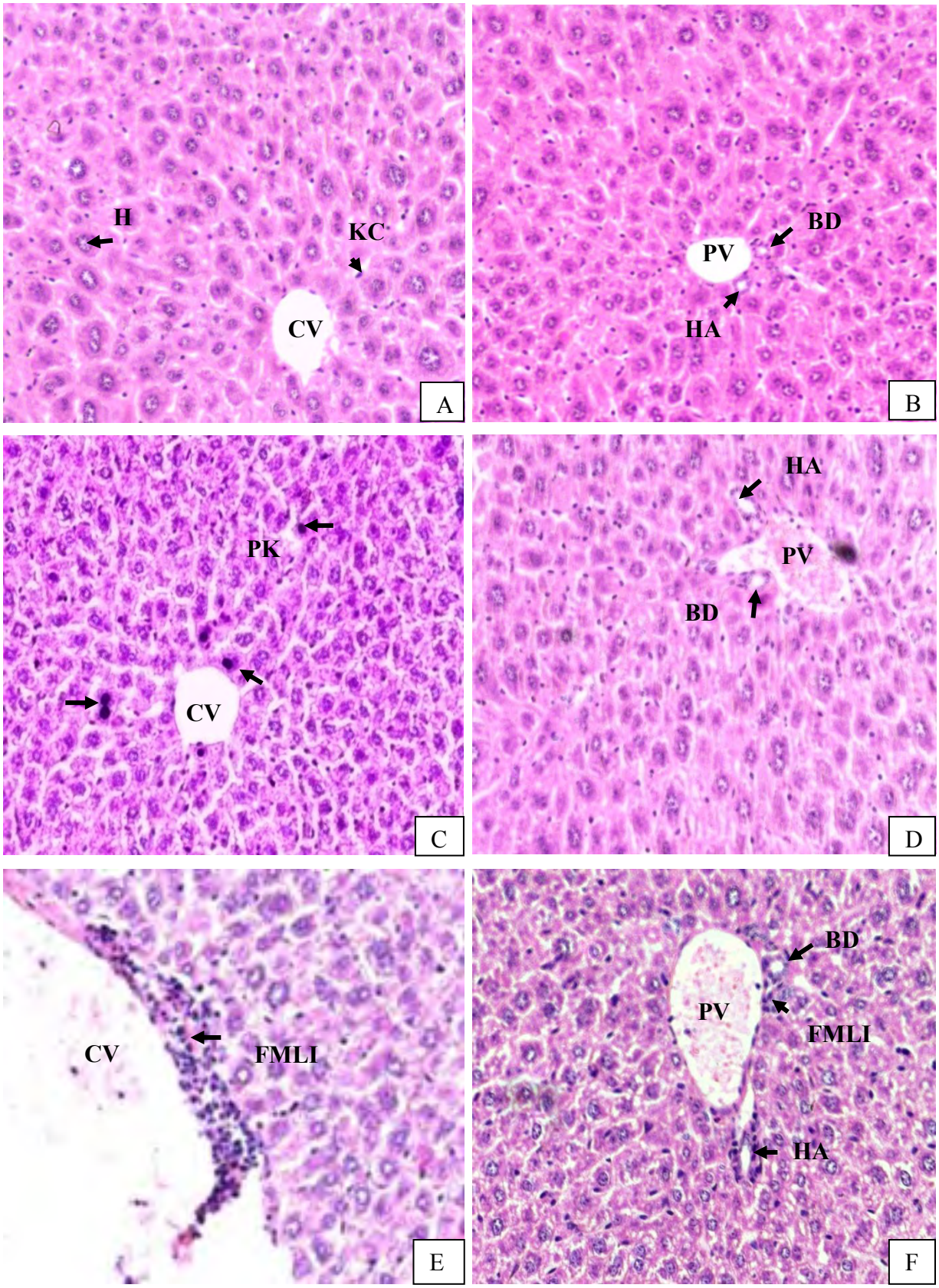
Values are expressed as Mean ± SEM, n=5. The figures in brackets indicate the calculated p values of the treatment groups as compared to the control.

4.7. Effects of the aqueous leaves extracts of *Maytenus gracilipes* on histology of the liver

Microscopic examination of liver sections from control mice showed the normal architecture of structural units of the hepatic lobules, formed by cords of hepatocytes separated by hepatic sinusoids (Figure 5A & B). The central vein and portal area containing branches of hepatic artery, bile duct and portal veins were maintained with their normal appearance. In comparison to the control, the general microscopic architecture of sections of liver tissue from the mice treated with the extracts at 700mg/kg dose body weight/day appeared to be not significantly affected after the 90 days administration (Figure 5C & D). However, liver section of mice treated with 700mg/kg body weight/day dose showed pyknotic nucleus in hepatocytes (Figure 5 C). In some areas of the liver sections of mice treated at 2100mg/kg body weight/day dose showed perivascular leukocytic cellular infiltration in the central and portal area (Figure 5 E & F).

There is no histopathological difference between male and female mice of liver section related to treated dose.

Figure 5: Photomicrographs of sections of liver from female control mice (A & B) and, mice treated with 700 mg/kg body weight/day (C & D) and 2100 mg/kg body weight/day (E& F).Note: pyknotic nucleus (**Pk**) in hepatocytes in mice treated with 700mg/kg body weight/day (C) , Focal mononuclear lymphocytic cellular infiltration (**FMLI**) in mice treated with 2100 mg/kg body weight/day (E&F) in central vein and portal area. Hepatocytes(**H**);Bile duct(**BD**);Hepatic artery (**HA**);Central vein(**CV**);Portal vein(**PV**);Kupffer cell(**KC**). (Sections were stained with H&E, X300).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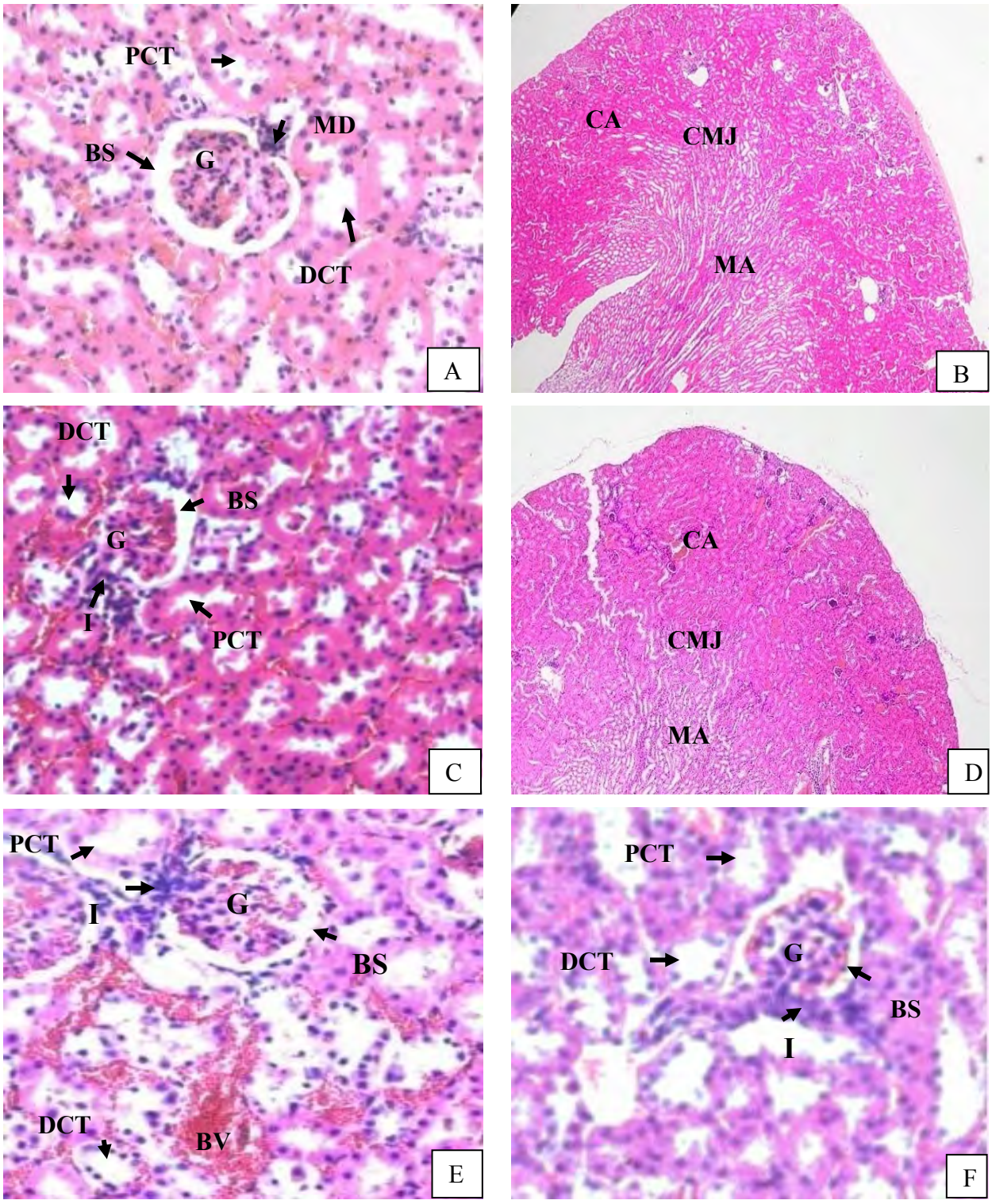
4.8. Effects of the aqueous leaves extracts of *Maytenus gracilipes* on histology of the kidneys

Histopathological examination of kidney sections of mice treated with the aqueous leaves extracts of *Maytenus gracilipes* at both 700mg/kg (Figure 6 C & D) and 2100mg/kg doses (Figure 6 E & F) indicated no structural disturbance as compared to the control mice (Figure 6A & B). The microscopic architecture of the kidneys in treated mice had similar appearance to that of the controls in which renal corpuscles maintaining their normal size of urinary space and normal tubular structures were observed with no sign of congestion. However, mononuclear lymphocytic infiltrations were observed in sections of kidneys of the mice treated with 700 mg/kg body weight (figure 6C) and 2100mg/kg dose body weight /day (Figure 6 E & F).

There is no histopathological difference between section of the kidneys of male and female mice related to treated dose.

Figure 6: Photomicrographs of sections of kidney of female control mice (A & B), mice treated with 700 mg/kg dose/day (C and D) and 2100 mg/kg dose body weight /day (E and F).Note: Mononuclear lymphocytic cellular infiltration in mice treated with 700 mg/kg dose body weight (C), and treated with 2100 mg/kg dose body weight (E&F). Distal convoluted tubule (**DCT**); Proximal convoluted tubule **(PCT)**; Macula densa**(MD)**; Bowman‟s space (**BS**); Glomerulus(**G)**; Blood Vessles(**BV)**; Cortical area(**CA)**; Cortico medullary junction(**CMJ);** Medullary area(**MA)**. (Sections were stained with H & E, X300 for A, C, E &F X60 for B&D).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5. Discussion

Herbal medicine is still used by about 75-80% of the world population, mainly in the developing countries for primary health care (Godkar,2003). Herbs are the basis for the development of modern drugs, and medicinal plants have been used for many years in daily life to treat diseases all over the world (Agbor *et al*., 2007). The wide usage of these herbal medicine for self-medication is a result of the fact that the general public believes them to be safe and do not have any compromising health effects (Obici *et al*., 2008). However, because there has been a lack of scientific studies of the toxicity and adverse effects of these remedies, so further investigations are vital. Because a number of cases of renal and hepatic toxicity have been reported following the use of phytotherapeutic products (Corns, 2003; Hilaly *et al*., 2004; Isnard *et al*., 2004; Saad *et al*., 2006). There are numerous examples of potential side effects associated with the most commonly used herbal and other types of complementary and alternative medicine (Kefale, 2005).

The increase in number of users and scarcity of scientific evidences on the safety of the medicinal plants have raised concern regarding toxicity and detrimental effects of these remedies. Clinical evidences of safety and efficacy in medicinal plant contribute to a better health care system and towards the potential use of herbal medicine. Medicinal plants commonly contain various bioactive chemical constitueonts which have the potential to cause beneficial and/or detrimental effects. Experimental screening method is therefore important in order to ascertain the safety and efficacy of traditional and herbal products and also to establish the active components of the herbal products (Mythilpriya *et al*.,2007). Toxicity screening models provide important preliminary information to help select natural remedies with potential health beneficial properties for future work (Pour *et al.,* 2011).

Determination of LD_{50} which is a single oral dose, at which 50 % of mortality occurs, is an initial screening step in the assessment and evaluation of the toxic characteristics of medicinal plants as it helps to identify the safety margin of the medicinal plant under investigation. In the present study, the oral $LD_{50,}$ of the aqueous leaves extracts of *Maytenus gracilipes* was about 10,000mg/kg body weight/day dose.

Acute toxicity study on the aqueous leaves extracts of *Maytenus gracilipes* at dose higher than 5000mg/kg revealed manifestations like depression, piloerection, fast breathing, and salivation.

No gross pathological changes such as in color, organ swelling, spot, texture and atrophy or hypertrophy were observed after single administration of the extracts as compared with the control group. This may suggests the safety nature of the plant.

Subchronic toxicity study was conducted with repeated administration of 700mg/kg body weight and 2100mg/kg body weight of the aqueous leaves extracts of the plant to investigate its effects on liver , kidney, and haematological and biochemical parameters.

As stated in the result daily treatment with both lower and higher doses of the extracts for a period of 90 days did not show any toxicity related mortality and changes in general health, and behaviour as compared to the controls.

One of the indices of toxicity assessment after exposure to toxic substances is body weight change (Vahalia *et al.,* 2011). Significant changes in body weight have been used as an indicator of adverse effects of drugs and chemicals (Raza *et al*., 2002),although Harizal *et al*. (2010) reported that the significant increment in the body weights of animals are more closely related to body fat accumulation rather than to the toxic effects of drugs or chemicals. In addition, Rhiouani *et al* (2008) suggested that reductions in the body weights of animals in toxicity studies may be associated with normal physiological adaptation responses to plant extracts or chemical constituents, which lead to low appetite and, hence, lower caloric intake by the animals. High doses of plant extracts or compounds might also induce stress in the animals, thereby reducing their food intake, which may lead to reductions in their body weights (Harris *et al*., 1998). In the present study, subchronic treatment with the extracts of *Maytenus gracilipes* did not produce statistically significant increment or decrement in body weight changes as compared to the controls in both sexes. The insignificant changes in the increment of body weights with the extract as compared to the control at the end of 13 weeks treatment indicate the plant is safe and even it may have nutritional value.

No significant difference in relative weight of liver and kidney of the extracts treated mice were observed when compared with the control group in both male and female mice. This may be due to the absence of toxicity of the extract on target organs (liver and kidney).

The hematopoietic system is one of the most sensitive targets for toxic chemicals and an important index of physiological and pathological status in human and animal (Li Xia orong *et al*.,2010). Because of this, hematological parameters were evaluated to obtain further toxicity related information not detected by direct examination of organs and body weight analysis. Studies on haematological parameters could show abnormalities in body metabolic processes, and the blood profile. In clinical pathology, the core haematology tests recommended include total WBC count, RBC count, platelet count, hemoglobin concentration, hematocrit (or packed cell volume), mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration (Weingand *et al.,* 1996).The primary reasons for assessing the RBC count is to check anemia and to evaluate normal erythropoiesis. Hemoglobin level indicates the amount of intracellular iron, while hematocrit, representing the volume of RBC helps to determine the degree of anemia or polycythaemia. The mean cell hemoglobin level is a significant index for folic acid or Vit B12 (Ganong, 1999).

Red blood cell count such as the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) are the most useful indicators in the diagnosis of anemia in most animals (Akpamu *et al.,* 2011).
In the present study the analysis of total RBC count, hematocrit, hemoglobin, and other indices (MCH, MCV, and MCHC) did not show significant changes following repeated administration of the aqueous leaves extracts of *Maytenus gracilipes* at both 700 and 2100mg/kg body weight/day doses as compared to the controls. These results indicate that there is no lysis of blood cells, bleeding, anemia and inhibition in blood cells synthesis or bone marrow suppression by any of the active constituents in the extracts of *Maytenus gracilipes*. In line with these results, histopathological evaluation of the organs (kidneys &liver) collected from treated animals showed normal architecture as compared to the control group, indicating that subchronic administration did not bring detrimental changes and morphological disturbances.

Leukocytes are first line defence for the body and increase when infections occur. In this study there is no significant change in the WBC count after 90 days of subchronic treatment with

aqueous leaves extracts of *Maytenus gracilipes.* These show the absence of toxic effects of the extract in treated mice. The non significant increment of WBC count treated within both lower and higher doses as compared with the control may suggest for normal response of the mice to foreign bodies or stress associated with the repeated treatment with the extract.

Coagulation studies are other great important parameters for considering the role of blood. Platelets are fragments of cells that participate in blood clotting. They initiate repair of walls of blood vessels and they also considered as an acute phase reactant to infection or inflammation; plateletcrits show the precise method of determining the degree of acute blood loss, while mean platelet volume (MPV) is used when investigating the ability of a drug/extract to enhance blood clotting (Ganong, 1999). In this study there was no significant increment of platelets (thrombocytosis) and decrease in platelets (thrombocytopenia) in the extract treated mice at both lower and higher doses. This suggests that the chemical constituents of the extracts of *Maytenus gracilipes* may not cause thrombosis.

Clinical chemistry parameters, in combination with haematology and urinalysis data, remain a valuable tool to obtain information on kidney and liver toxicity. Because of their functional roles in the body, the kidneys and liver are two major organs that play important roles in detoxification. A number of cases of renal and hepatic toxicity have been reported following the use of phytotherapeutic products (Corns, 2003; Hilaly *et al*., 2004; Isnard *et al.*, 2004; Saad *et al*., 2006). The injury of liver may affect the integrity of the hepatocytes leading to release of membrane bounded enzymes. A selected panel of biomarkers should be measured for the identification of hepatocellular or hepatobiliary injuries. The panel for hepatocellular injury should include: measurements of Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TB). AST and ALT are classical enzymes in liver function test and biomarkers to recognise diagnosis of drug-induced liver injury (Ozer *et al*., 2009). ALT is considered to be more specific and sensitive indicator of hepatocellular injury than AST in rats, mice, dogs, rabbit and non-human primates (Farah *et al.*; 2011). It is localized primarily in the cytosol of hepatocytes and is considered to be a sensitive marker of hepatocellular damage in these animal species when compared to the levels of AST.

Mostly the AST concentrations were consistently higher than ALT (Aniagu *et al*., 2004). Usually, about 80% of AST is found in the mitochondria whereas ALT is a purely cytosolic

enzyme. Therefore, AST appears in higher concentrations in tissues of liver and kidneys in comparison to ALT. Moreover, AST is not specific for the liver only but it is also found in other organs like the heart, brain, kidney and skeletal muscle (Aniagu *et al*., 2004).

The significant increment of these enzymes (ALT and AST) above the normal value in the blood serum indicates liver toxicity (Ozer et al., 2009). When the magnitude of ALT increase is usually greater than that of AST it shows liver injury, whereas when the magnitude of AST elevation is greater than of ALT it shows muscle damage (Nathwani *et al*., 2005; Ramaiah, 2007). In the present study concentrations of both ALT and AST in blood serum level did not significantly ($p>0.05$) changed in the treated groups at both lower and higher doses as compared to the controls. These indicate that the aqueous leaves extracts of *Maytenus gracilipes* have no adverse effect on the histology of the liver.

Measurements of urea and creatinine levels in the blood are usually performed to evaluate kidney function (Newman and Price, 1999). Urea is the major nitrogen containing metabolic end product of protein catabolism and Creatinine is a waste product of muscle energy metabolism. Creatinine and urea concentrations are used for the assessment of renal sufficiency (Smith *et al*., 2006). In cases of acute or chronic renal toxicity, these two parameters are usually significantly increased to four or five times higher than the normal values. The rise in serum level of these chemicals indicates a decline or failure in renal function to filter waste products from the blood and excrete them in the urine (Whelton *et al*., 1994). Creatinine, and urea are normally filtered from the plasma, and they are reabsorbed or secreted by the proximal tubules to a minor extent. Tubular secretion leads to overestimation of GFR and it is higher in laboratory animals than in man. Creatinine is a better marker of glomerular function than urea (Prause and Grauer 1998; Medaille *et al*. 2004). Elevated creatinine is a reliable indicator of impaired glomerular filtration or alterations in renal blood flow and severe tubular dysfunction along with urea. In this study renal function test did not significantly ($p>0.05$) change in both treated groups as compared to the control. This indicated that the extract of *Maytenus gracilipes* has no toxic effects on the kidneys.

Histopathological examinations of the selected organs (liver and kidney) provide information to strengthen the findings on biochemical and haematological parameters. Elevated liver enzymes always shows concurrent changes in the liver microscopically. For instance, centrolobular

degenerative changes, necrosis and fatty liver always associated with elevated liver enzymes (Ishak and Zimmerman, 1995). These changes were not observed in the livers of the mice in the present study. However, histological examination of the liver sections of extract treated mice showed pyknosis at 700 mg/kg body weight dose and focal mononuclear leukocytic cellular infiltration around the central vein and portal area at 2100 mg/kg body weight dose. Condensation of the nuclear chromatin (pyknosis) occurs when a cell receives a signal to initiate apoptosis (Ebaid *et al.,* 2007). When the cell death that is toxic or immunologically mediated occurs via apoptosis**,** in which isolated hepatocytes become shrunken, intensely eosinophilic, clear space (vacuolation), disturbed sinusoidal space and focal necrosis (Kumar *et al*., 2002). This in line with the evidence of liver damage is usually indicated by the fatty change which is further indicated by the form of cytoplasmic vacuoles in the liver cell (hepatocytes) (Ebaid *et al.,* 2007).

However, in the present study there is no disturbed sinusoidal space, focal necrosis, clear space (vacuolation), this may suggests that the plant extracts was relatively safe. More over mononuclear leukocytic infiltration showed may be due to the chemical constituents of the extracts.

Microscopic examination of sections of kidneys revealed numerous protein casts in the renal tubules, which is one of the hallmarks of acute renal toxicity (Hazilawati *et al*., 2009b). Lesions that is typical in chronic renal toxicity including interstitial fibrosis, chronic inflammation and dilatation of the renal tubules (Farah *et al*., 2011). In the present study, the microscopic examination of kidney sections of treated mice has no significant histopathological presentations at both lower and higher doses of the treatment groups as compared to the controls. The microscopic architecture of sections of kidney in treated groups had similar appearance to the controls; have no dilated urinary space, fibrosis or chronic inflammation. However, focal perivascular lymphocytic infiltration were observed in small areas of kidney sections of mice treated with 2100mg/kg body weight dose, this may be associated with the chemical constituents of the plant. Hence there is no significant histopathological difference as compared to the controls in both organs. The aqueous extracts of *Maytenus gracilipes* is therefore assumed to be safe for both target organs as found in this study.

6. Conclusion

From the present result it can be conclude that the aqueous leaves extracts of *Maytenus gracilipes* is safe as demonstrated from its high LD_{50}.

In the subchronic study treatments of the extracts at both 700mg/kg and 2100mg/kg body weight dose did not show significant change on the hematological and biochemical parameters as well the gross structures of liver and kidneys. In addition, the general behaviour, organ and body weights of treated mice were not significantly affected. However, Pyknotic nucleus were observed over kidney at 700 mg/kg and mononuclear lymphocytic infiltrations were observed over kidney and liver sections of mice treated at higher (2100mg/kg) doses; this may associated with the chemical constituents of the extracts. Therefore we can conclude that the plant is relatively safe when administered orally for 90 days at both lower and higher doses.

7. Recommendations

From this study it could be recommended that

- Investigation on the histopathological effects of *M.gracilipes* on other organs such as brain, heart and GIT should be carried out.
- Further acute and sub-chronic toxicity study in other rodent species (rat) should be carried out.
- Further studies are needed to find out the reason and chemical constituents responsible for the leukocytic mononuclear cellular infiltrations.
- Further studies are needed to find out the chemicals constituents which are found in the plant.

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8.1. Appendix-I

Preparation of working chemicals/solution

1. **10% Neutral Buffered Formalin**

40% formaldehyde	100 ml
Distilled water	900 ml
Sodium dihydrogen phosphate monohydrate	4 gm
Disodium hydrogen phosphate anhydrous	6.5 gm

2. **Harris's Hematoxylin (H)**

Hematoxylin crystals	2.5 gm
Absolute alcohol	25 ml
Potassium alum	50 gm
Distilled water	500 ml
Sodium iodate	0.5 gm
Glacial acetic acid	20 ml

3. **1% Alcoholic Eosin (E)**

Eosin Y, water soluble (CI 45380)	1 gm
95% Ethanol	100 ml
Glacial acetic acid	0.5 ml

4. **1% Acidic alcohol**

70% alcohol	500 ml
Hydochloric acid, concentrated	5 ml

5. **Preparation of sodium bicarbonate/bluing solution**

Sodium bicarbonate	2.5 gm
Distilled water	1000 ml

8.2. Appendix-II

Tissue processing procedures (Bankrapht, 2013)

1. Fixation	
10% Neutral Buffered Formalin	24 hrs
2. Washing	
Tap water	several changes
3. Dehydration	
70% Ethanol	2 hrs
80% Ethanol	2 hrs
90% Ethanol	2 hrs
Absolute alcohol I	$1^1/_2$ hrs
Absolute alcohol II	$1^1/_2$ hrs
Absolute alcohol III	$1^1/_2$ hrs
Absolute alcohol IV	overnight
4. Clearing	
Xylene I	$1^1/_2$ hrs
Xylene II	$2^1/_2$ hrs
5. Infiltration	
Paraffin wax I	$1^1/_2$ hrs
Paraffin wax II	$2^1/_2$ hrs
Paraffin wax III	overnight

8.3. Appendix-III

Heamatoxylin and Eosin (H & E) Tissue Staining Protocol

1. **Deparaffinization**
   - Xylene I — 4 min
   - Xylene II — 4 min
2. **Rehydration**
   - Absolute alcohol I — 4 min
   - Absolute alcohol II — 4 min
   - 95% Ethanol — 3min
   - 80% Ethanol — 3 min

Rinse in distilled water — 5 min

Stain in Hematoxylin — 10 min

Rinse in running tap water — 5 min

Decolorize in acid alcohol — 1-3 sec

Rinse in running tap water — 5 min

Immerse in Sodium bicarbonate solution — 1 min

Rinse in running tap water — 5 min

Counter stain in Eosin — 1 min

3. **Dehydration**
   - 80% Ethanol — 3 min
   - 95% Ethanol — 3 min
   - Absolute alcohol II — 3 min
   - Absolute alcohol I — 3 min
4. **Clearing**
   - Xylene II — 3 min
   - Xylene I — 3 min