

ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCE
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**THE EFFECT OF CHRONIC TREATMENT OF THE ETHANOLIC
FRUIT EXTRACT OF *Embelia schimperi* ON BLOOD
PARAMETERS, AND LIVER AND KIDNEY OF RATS**

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September, 2016
Addis Ababa, Ethiopia

**THE EFFECT OF CHRONIC TREATMENT OF THE ETHANOLIC
FRUIT EXTRACT OF *Embelia schimperi* ON BLOOD
PARAMETERS, AND LIVER AND KIDNEY OF RATS**

**A Thesis submitted to the School of Graduate Studies of Addis Ababa
University in partial fulfillment of the requirements for the degree of
Master of Science in Anatomy**

By: Mohamed Zewdu

September, 2016

Addis Ababa, Ethiopia

DECLARATION

This is to certify that the thesis prepared by Mohammed Zewdu, entitled: The effect of chronic treatment of the ethanolic fruit extract of *Embelia schimperi* on blood, liver and kidney of rats 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 **Very good** remark.

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LIST OF ABBREVIATIONS

AAU	-----	Addis Ababa University
ALT	-----	Alanine Amino Transferase
AST	-----	Asparate aminotransferase
ANOVA	-----	Analysis of Variance
bwt	-----	body weight
Cl ⁻	-----	Chloride ion
DDR	-----	Department of Drug Research
DMSO	-----	Dimethyl sulphoxide
DPX	-----	Dibutyl Phthalate in Xylene
dL	-----	deciliter
EDTA	-----	Ethylene Diamine Tetraacetic Acid
EPHI	-----	Ethiopian Public Health Institute
fL	-----	femtolit
FWT	-----	Final Body Weight
GGT	-----	Gamma-Glutamyl Transferase
H ⁺	-----	Hydrogen Ion
(HCO ₃ ⁻)	-----	Hydrogen Carbonate Ion
H & E	-----	Hematoxylin and Eosin
HCT	-----	Hematocrit
Hgb	-----	Hemoglobin
IWT	-----	Initial Weight
K ⁺	-----	Potassium ion
L	-----	Lymphocyte

LD ₅₀	-----	Lethal Dose that kills half of the animals
MCH	-----	Mean Corpuscular Hemoglobin
MCHC	-----	Mean Corpuscular Hemoglobin Concentration
MCV	-----	Mean Corpuscular Volume
Na ⁺	-----	Sodium Ion
OECD	-----	Organization for Economic Co-operation and Development
PLT	-----	Platelet
pg	-----	pictogram
RBC	-----	Red Blood Cell
Rpm	-----	Revolution per minute
SDh	-----	Sorbital Dehydrogenase
SEM	-----	Standard Error of the Mean
SPSS	-----	Statistical Package for Social Science
μL	-----	microlitre
WBC	-----	White Blood Cell
WHO	-----	World Health Organization

ABSTRACT

Background: *Embelia schimperi* is a plant traditionally used for its medicinal value in many parts of the world including Ethiopia. There are several reports about the pharmacological properties of *Embelia schimperi* such as ethno-anthelmintics, and antibacterials. However, there are limited published reports about the possible toxicological effects of this plant especially long-term (chronic) toxicity.

Objective: The aim of this study was to evaluate the effect of chronic treatment of ethanolic extract of dried fruit of *Embelia schimperi* in rat models.

Methods: The fruits of the plant were collected from Bahir Dar area; Ethiopia, dried, crushed into powder and soaked in 80% ethanol. The filtrate from each soaking was defatted and concentrated by a vacuum rotary evaporator to remove the solvents. The sample was set on a water bath of temperature 40°C to remove the remaining solvent (water). The final dried mass was weighed, packed in plastic cup and kept at -4°C until used. The extract was then orally administered to rats at doses of 400 and 1600mg/kg bwt/day for 24 weeks. Initial and final body weights and absolute weight of liver and kidney were recorded. Blood samples were collected for the analysis of hematological and biochemical parameters. The animals were then sacrificed and dissected carefully to collect the liver and kidney for histopathological studies.

Results: Ethanolic fruit extract of *Embelia schimperi* at doses of 400 & 1600 mg/kg bwt/day was not significantly associated with body weight change and relative organ weights of liver and kidney. From hematological and biochemical parameters, only platelets and AST concentration were significantly associated with fruit extract. The histopathological findings showed liver and kidney tissue inflammations among study groups. However, considerable inflammatory cells were observed in kidney sections of rats treated with 1600 mg/kg bwt/day as compared to the controls.

Conclusion: Chronic treatment with ethanolic fruit extracts of *Embelia schimperi* in rats has not shown toxicity though there is significant change in platelets count and AST concentration. However, plant extract might have mild toxicity as indicated by inflammatory cells observed in kidney sections of rats.

Key words: *Embelia schimperi*, Fruit, Ethanolic extract, Chronic Toxicity, Rats.

1. INTRODUCTION

1.1 Background of the study

Plant derived products or natural products have been used for medicinal purposes for centuries. The use of natural products with healing properties is as old as human civilization and plant products were the main sources of drugs (Rates, 2001). The practice of traditional medicine has expanded globally and gained popularity. It has not only continued to be used for primary health care of the poor in developing countries, but also in countries where conventional medicine is predominant in the national health care system (WHO, 2000).

Medicinal plants are used primarily for the treatment of numerous diseases like cardiovascular diseases (Ouedraogo et al., 2004), hepatic illness (Phillipson & Wright, 1991) and for various types of parasitic infections (Endale et al., 1998).

Parasitic helminthes are of major public health and economic importance to both man and livestock. Most diseases caused by helminthes are of chronic, debilitating nature; they probably cause more morbidity and greater economic and social deprivation among humans and animals than any single group of parasites (Suleiman et al., 2005). Even though there are several synthetic anthelmintics available against these parasites, the fact remains that a large proportion of the world's population still do not have an access to, or cannot afford to pay for the high cost of modern medicines, particularly in remote rural areas in poor countries (WHO, 2002), limiting the effective control of parasitic helminthes. Besides, the continued usage of modern anthelmintic drugs is also posing a major problem of drug resistance by several parasitic species (Sangster, 2008; Alfredo et al., 2010), stimulating the desire to search for additional chemotherapeutic agents that might allow more efficient control of helminth parasites (Hammond et al., 1997). There is thus an urgent need for newer and inexpensive drugs that are able to act for longer periods before resistance sets in. In this context, traditional medicines, based largely on medicinal plants, offer a major and accessible source of health care, especially to people living in developing countries.

Human intestinal parasitic worms live in the intestines by sucking blood or eating intestinal wall. They are documented as a serious public health problem as they cause iron deficiency anemia, growth retardation in children and other physical and mental health problems (WHO, 1996).

Many drugs are available against these worms with the most striking problem being adaptation or resistance of the drugs by parasites. Multidrug resistance against anthelmintics in human and animal pathogenic helminthes has been increasing in prevalence and severity. This has become a global phenomenon in gastro intestinal nematodes of farm animals (Ronald et al, 2008).

The long standing tradition of eating raw meat in Ethiopia has established a craving for raw beef. This custom is so rampant and the tapeworm infestation is so extensive that over 80% of the adult population have to take a tapeworm expellant (taenicidal medication) every 3 months (Rollo, 1970). Tapeworm infection (Taeniasis) is one of the most common parasitic diseases in Ethiopia. Even though the disease may be caused by different platyhelminthes, *Taenia saginata* (beef-tapeworm) is more prevalent in the country because of the habit of eating raw or not properly cooked beef (Pankhurst, 1965). Finding crawling proglottids on one's person body is taboo and associated with a great shame (Desta, 1995). Therefore, there is a recurrent necessity of removing the worm from the body and this has brought to the use of a large variety of taenicidal herbs in different parts of the country. However, the regular intake of different taenicidal herbs has been associated with a variety of side effects, including liver disease (Tsega, 1977), gastro intestinal ailments (Chernishov et al., 1978) and eye complications (Rokos, 1969).

Recently, the development of anthelmintic resistance and the high cost of conventional anthelmintic resistance led to the evaluation of medicinal plants as an alternative source of anthelmintics (Dereje et al., 2009). The most frequent problems that are associated with medicinal plants are the lack of clinical, toxicological and pharmacological studies (Ronald et al., 2008).

Therefore the growing interest in herbal medicine demands information on the effects of the various plant preparations used in the management of diseases (Atawodi, 2005; Toma

et al., 2009). The incidence of adverse effects and sometimes life-threatening conditions allegedly emanating from herbal medicines has been reported among various ethnic groups (Elvin-Lewis, 2001). Consequently, it has become imperative to ascertain the toxicity profile of these medicinal herbs.

Embelia schimperi (“Enkoko” in Amharic) is the most commonly used traditional herb against the widely prevalent tapeworm parasite (Keay & hepper, 1972). *Embelia schimperi*, native to regions of Africa, is also one of the most commonly used traditional taenicial herbs in different regions of our country, Ethiopia (Hedberg et al., 2003). In Ethiopia *Embelia schimperi* fruits are, after kosso (*Hagenia*), the most popular worm medicine. Dry fruits are chewed and the juice is swallowed (one teaspoon of fruits, three times a day), or the dry fruits are hulled and coarsely ground and one teaspoon of powder swallowed with water three times a day. If this is not effective enough, the fruits are mixed with kosso. The seeds of *Embelia schimperi* are dried, powered and eaten to eliminate hookworms (Heidi & Girma , 2013)

However, there are no adequate studies on toxicity of chronic treatment by this medicinal plant. Thus, the aim of the present study was to evaluate toxic effects of the crude ethanolic fruit extract of *Embelia Schimperi* on blood profiles, body weight, as well as weight and histopathology of liver and kidney.

1.2 Literature Review

1.2.1 Traditional medicine

The World Health Organization (WHO) defines traditional medicine as health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral-based medicines, and spiritual therapies, applied singularly or in combination to treat, diagnose and prevent illnesses and maintain well-being (WHO, 2001).

It has been estimated that 80% of the world population rely on herbal traditional medicine for their primary health care (Abasar et al, 2008, cited in (Qureshi & Kumar, 2010). Traditional medicines, including plant derived medicines, have been, and continue to be, used in every country around the world in some capacity. In much of the developing world, 70–95% of the population relies on traditional medicines for primary health care

(WHO, 2011) which is well known in rural areas (Sandhu and Heinrich, 2005). Moreover, WHO has officially launched an international program in promoting traditional medicine that included promotion and development of basic and applied research in traditional medicine (WHO, 1978). Hence, traditional medicine has become a topic of increasing global importance to play a pivotal role in the health care system of large proportion of world's population especially in developing countries where traditional medicine has a long history and is part of indigenous culture (Aniagu et al., 2005; Fekadu, 2002).

Ethiopia has diverse flora and fauna. The Ethiopian flora is estimated to contain between 6000 and 7000 species of which about 10% are endemic (Hareya, 2005), and most of the flora are used as an important herbal medicine of the country (Teferi and Heinz, 2003). Reports indicated that medicinal plants occur throughout the country as important sources of traditional medicine and indigenous health care system of the country (Aberra et al., 2005; Teferi and Heinz, 2003). It has also been reported that most of the Ethiopian population uses traditional medicine due to the cultural acceptability of healers and the relatively low cost of traditional medicine as well as difficult access to modern health facilities (Kebede et al., 2006), without adequate knowledge of their adverse effects.

Traditional medicinal plants could be useful in an integrated health care delivery system with curative effects. However, such plants must not be toxic (WHO, 1978), as many medicinal plants have been found to induce fatal hepatic effects and severe acute liver failure with marked hematological and biochemical alterations (Oluwole, 2001; Adedapo et al., 2007; Okpuzor, 2009). The reason for these problems as reported by world health organization is that, Traditional medicine practices have been developed within different cultures in different regions without parallel development of national or international standards and methods for evaluating them (WHO, 2000).

For example, exposure of the kidneys to circulating toxins leads to pathological changes resulting in disruption of glomerular functions. Consequently, the renal tubular function will be affected. Thus, toxic effects of undermined traditional medicinal plants could cause tissue or organ damage (Dapar et al., 2007).

Therefore, as medicinal plants continue to receive attention and more of their medicinal values discovered day by day, there is a need to investigate the effects of their consumption on liver and kidney functions using animal model as these organs are commonly exposed to toxicity.

Embelia schimperi, whose toxicity profile has not adequately been investigated, is one of the most commonly used traditional medicinal plants in different regions of Ethiopia (Hedberg et al., 2003).

1.2.2 Embelia Schimperi Vatke

The plant *Embelia schimperi* Vatke, known by its local name “*Enkoko*”, belongs to the family Myrsinaceae. This is a large family consists of nearly 1000 species of trees and shrubs spread over 33 genera (Januario et al., 1992) including four genera namely Myrsine, Embelia, Rapanea and Maesa which are widely used for traditional medicines (kokwaro, 1976). Embelia is a genus of climbing shrubs in the family Myrsinaceae.

Embelia schimperi Vatke is one of the most commonly used plants against the widely prevalent tapeworm parasite in Ethiopia. It is native to east Africa (Kenya, Tanzania, Uganda); west-central tropical Africa (Burundi, Cameroon, Rwanda, Zaire); west tropical Africa (Nigeria); south tropical Africa (Angola, Malawi, Mozambique, Zambia, Zimbabwe); southern Africa (Namibia) (Keay & hepper, 1972) and north east tropical Africa (Ethiopia). In Ethiopia, it is found in Gondar, Gojam, Wello, Arsi, Shewa, Welega, Ilubabor, Kefa, Sidamo and Bale (Hedberg et al., 2003).

Embelia schimperi is climbing shrub that reaches the height of 2-13 meters. It has branches with prominent lenticles. The leaves are usually alternate or oblanceolate with slightly revolute margin, rounded base and acuminate apex. Its flowers are pedicellate with rust colored hairs. The fruit, 5-8mm in diameter, is orange yellow, reddish-green to black red in color when ripen. Each fruit often has one seed that has a diameter of 5-7mm. The seed is brown in color with irregular orange markings when ripen (Hedberg et al., 2003). In Ethiopia, the plant can be found growing along creeks on mountain slopes, in dense moist montane forests, in montane thicket-borders and along rivers around grassy fields, at altitudes of 1700-2800m.



Figure 1: Photograph of *Embelia schimperi* (taken during data collection by the researcher, Bahir Dar- *Ethiopia*, 2012).



Figure 2: Photograph of *embelia schimperi* (Modified from Mergo L et al., 2013)

The plant produces flowers and fruits mostly from January to September. However, the fruits are collected from the wild plants for sale on the market the whole year (Hedberg et al., 2003). The fruits and roots of *Embelia schimperi* are used as anthelmintics in Africa. It is used among the traditional Masai people of Tanzania and Kenya to eliminate adult *Taenia saginata*, the beef tapeworm. Report by Bogh et al., (1996), indicated that the crushed seeds of *Embelia schimperi* taken orally by the Masai people have an anthelmintic effect against human intestinal tapeworms. In India, the closely related

Embelia ribes has had similar usage (Gupta et al., 1976). This plant is also widely used as taenicide in Ethiopia. The traditional uses of the different species of Myrsinaceae are given in table 1.

Table 1: Traditional uses of different species of Myrsinaceae (Kokwaro, 1976)

Plant	Traditional use
<i>Myrsine africana</i>	2-3 Handfuls of fruits are chewed for roundworm and tapeworm treatment and remedy for chest pains and stiff joints. No harm is done when too much is taken.
<i>Embelia schimperi</i>	Fruits chewed as both vermifuge and purgative. Dried fruits and roots are boiled or soaked in water and infusion drunk for intestinal worms.
<i>Rapanea melanphloes</i>	Fruits used as anthelmintic when chewed and eaten in porridge to expel intestinal worms.
<i>Maesa lanceolata</i>	Fruits used as purgative to remove worms and as remedy for sore throats or eaten to cure tapeworm

Literatures reported the efficacy of the extracts of Myrsinaceae species as anthelmintics (Bogh et al., 1995) and other activities (Githui et al., 1991; Midiwo et al., 1995). The LD₅₀ value of the crude methanolic fruit extract of *embelia schimperi* was reported to be more than ten thousand mg/kg (Dawit et al., 2012) in mice. The LD₅₀ value is a dose that, when administered in an acute toxicity test, is expected to cause death in 50% of the treated animals in a given period.

The study by Belachew Desta (1995) showed that the treatments with aqueous and hydro-alcoholic extracts of *Embelia schimperi* were found to be safe in mice. In another eight weeks sub-chronic toxicity study, it has been reported that oral administration of the crude methanolic extract of the plant did not cause significant alterations in the tested parameters (body weight change, hematological and biochemical parameters as well as histology of liver and kidney in mice (Dawit et al., 2012). A mild inflammation was observed around the portal areas of the liver tissues of the mice treated with 1200mg/kg bwt/day of the crude extract. The plant has shown no signs of toxicity at 400 mg/kg bwt of the mice (Dawit et al., 2012). However, the effects of long term administration of the plant extract needs to be evaluated.

1.2.3 The blood and its components

Since blood plays a key role in the delivery of nutrients, hormones, metabolic excretion and immunological processes as well as homeostatic responses (Clark & Wallis, 2003), its parameters are still most highly accurate, sensitive and reliable that investigators use and depend on for the purpose of disease diagnosis, prevention and treatment (Irshaid & Mansi, 2009).

Blood is a specialized connective tissue composed of formed elements including erythrocytes (red blood cells), leukocytes (white blood cells), and platelets (thrombocytes) which are suspended in a fluid extracellular matrix component known as plasma (Junqueira & Carneiro, 2005). The total volume of blood in the normal adult rat is about 5.6 to 7.1ml/100g of body weight (Sharp & Villano, 2012), which is about 6 liters in adult human (Ross & Pawlina, 2011). The red and white blood cells constitute the major non-fluid component of the blood.

Erythrocytes (red blood cells) are terminally differentiated anucleated cells. The number of circulating erythrocytes in the blood of normal adult rat is about $5-10 \times 10^6/\mu\text{l}$ making up the greatest portion of formed elements of the blood (Sharp & Villano, 2012).

Leukocytes (white blood cells) are defensive cells that migrate from the blood stream into tissues to perform their functions by generally defending the body against foreign substances. The number of leukocytes is much smaller than that of RBCs. In a healthy adult rat, there are about $3-17 \times 10^3/\mu\text{l}$ white blood cells (Sharp & Villano, 2012). According to the type of granules in their cytoplasm and the shape of their nuclei, leukocytes are classified into two general groups: granulocytes (neutrophils, eosinophils, and basophils) and agranulocytes (lymphocytes and monocytes). Granulocytes contain specific granules and agranulocytes lack these granules. Neutrophils are the most numerous WBCs as well as the most common granulocytes and constitute 13-26 % of circulating leukocytes. Eosinophils constitute 0-4% of a typical blood sample. Basophils are the least numerous of the WBCs that accounts for 0-1% of the total leukocytes (Sharp & Villano, 2012).

Lymphocytes are the main functional cells of the immune system and they are the most common agranulocytes that accounts for about 65-83% of the total blood leukocytes in adult rat. Monocytes constitute 0-4% in a total leukocytes and differentiate into the various phagocytes (CRL, 1998 and Sharp & Villano, 2012).

Platelets are small anuclear cell fragments derived from the cytoplasm of bone marrow megakaryocytes and play an important function in the control of excess bleeding from an injured vessel (Kaplan & Jackson, 2011). Their shape and small size enables to be pushed to the edge of vessels, placing them in the optimum location required to constantly survey the integrity of the vasculature (Harrison, 2005). There are about 200 - 1,500 x 10³/μL platelets in healthily adult rat blood (CRL, 1998 and Sharp & Villano, 2012).

The hematological parameters such as Hgb, RBC, HCT, MCV, MCH, MCHC, WBC, L, and PLT are useful indices to assess the toxic potentials of plant extracts in living systems (Iniaghe et al., 2013). For example, damage and destruction of the blood cells are hostile to normal functioning of the body.

Plasma, the fluid component of blood, transports nutrients as well as hormones from their site of synthesis or absorption distributing them to different cells of the organism and collects metabolic residues from all cells removing from the blood by the excretory organs (Junqueira & Carneiro, 2005). Biochemical profiles of toxicity are usually studied from blood serum. The blood serum is a yellowish fluid obtained after fibrinogen and clotting factors are removed from plasma (Rose & Pawlina, 2011).

In the serum, liver associated enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), as well as others gamma-glutamyl transferase (GGT), sorbitaldehydrogenase (SDH) are commonly used as markers of hepatic injuries (Gaskill et al., 2005; Haldar et al., 2007 and James et al., 2000). An increase in the serum levels of these enzymes is a reflection of liver dysfunction due to liver injuries (Adebayo et al., 2005; Gaskill et al., 2005; Seifert et al., 2008 and Shahraki et al., 2007). The increase in serum ALT (cytoplasmic enzyme) activity is typically associated with hepatocellular membrane damage and leakage of the enzyme from hepatocytes (Gaskill et al., 2005 and James et al., 2007). Normally kidneys filter serum

urea and other substances present in the blood. The presence of high serum urea and creatinine concentration in the blood indicates more deteriorative effect on the kidneys, which can adversely affect the functioning of kidneys (Adebayo et al., 2003).

1.2.4. The structure, function and histopathology of liver

The liver is the largest reddish-brown internal organ constituting about 2.5% of the body weight in adult human. It lies mainly in the right upper quadrant of the abdomen and has 2 major lobes (the left and the right) and two small (caudate and quadrate) lobes. It is surrounded by a dense, irregular collagenous connective tissue known as Glisson capsule, which gives rise to septae that subdivide the liver into lobes and lobules ((Rose & Pawlina, 2011). The rat's liver occupies the cranial third of the abdominal cavity. The liver mass represents approximately 5% of the total body weight in rats and 2.5% in adult humans (Kogure et al., 1999 and Martins & Neuhaus, 2007). In rats weighing between 250 and 300 g, the liver mean weight was 13.6 g and the liver transverse diameter measured from 7.5 to 8.0 cm. The superior–inferior diameter measured from 3.8 to 4.2 cm, while the anterior–posterior diameter ranged from 2.2 to 2.5 cm. (Martins & Neuhaus, 2007). A thin connective tissue capsule that is externally lined by peritoneal mesothelial cells covers the parietal and visceral surfaces of the rat's liver (Thoolen et al.2010).

The rat's liver is made up of 4 lobes, like the human liver, and they are named after the portal branches that supply them. These lobes are middle or median lobe (ML), right lobe (RL), left lateral lobe (LLL) and caudate lobe (CL) (Martins & Neuhaus, 2007). In a study by Kongure et al. (1999), comparing the rat and human liver, it was shown that the hepatic lobes of the rat are equivalent to the human liver.

The median lobe (ML) is the largest, accounting for approximately 38% of the total liver weight. It is in continuity with the left lateral lobe (LLL) and is subdivided by main fissure (MF) into a large right medial lobe (RML) and a smaller left medial lobe (LML). The right lobe (RL) is located on the right side of the inferior vena cava and comprises about 22% of the total liver weight. It is divided by a horizontal fissure into the superior right lobe (SRL) and inferior right lobe (IRL). The left lateral lobe (LLL) is situated over

the anterior aspect of the stomach and represents about 30 % of the total liver weight. Its medial portion is covered by the left part of the medial lobe. It has no fissures. The caudate lobe (CL) is situated behind the LLL and comprises 8–10% of the total liver weight. It is divided into two portions: the caudate process (CP) that encircles the inferior vena cava and bridges into the right lobe, and the Spiegel lobe, which has an anterior portion or anterior caudate (AC) and a posterior portion or posterior caudate (PC) in the form of discs, each representing 4% of the liver mass. The anterior part of the CL is located anterior to the esophagus and stomach, while the posterior part is located behind these structures and its pedicle lies inferior (Martins & Neuhaus, 2007). In a study by Kongure et al. (1999), comparing the rat and human liver, it was shown that the hepatic lobes of the rat are equivalent to the human liver. Different from human, dog, pig and mouse, rats do not have a gallbladder (Thoolen et al., 2010).

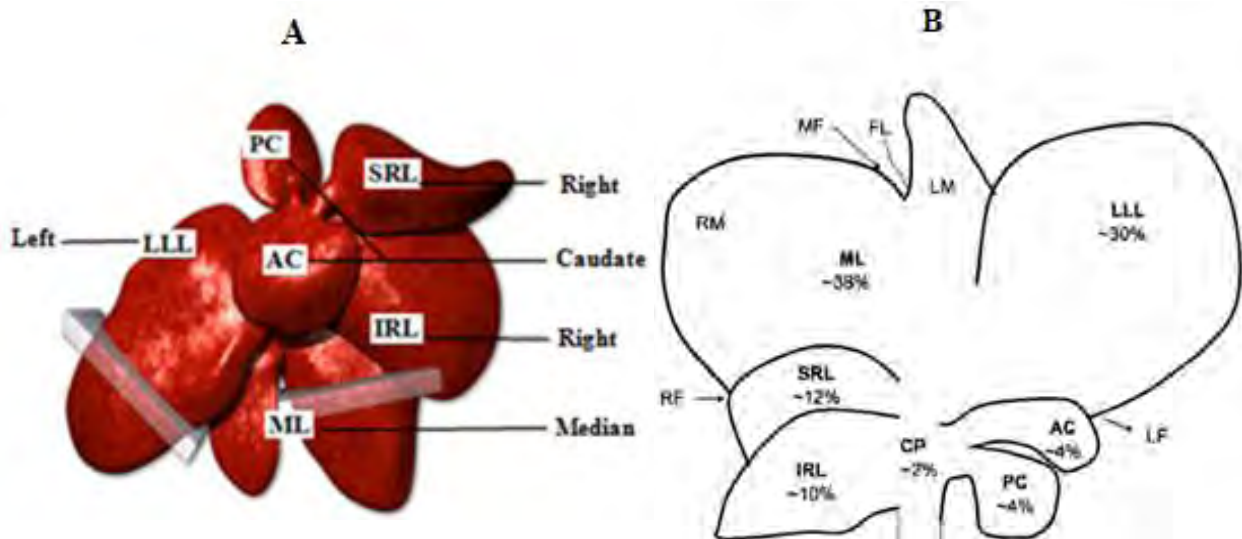


Figure 3: Rat liver lobes, (A), visceral aspect. (B) Visceral surface of the rat liver showing mean relative weight of each lobe in approximate percentages. CP, caudate process; AC, anterior caudate lobe; PC, posterior caudate lobe; SRL, superior right lateral lobe; IRL, inferior right lateral lobe; ML, median lobe; RML, right portion of the medial lobe; LML, left portion of the medial lobe; LLL, left lateral lobe; MF, median fissure; LF, left fissure; RF, right fissure and FL, falciform ligament (Ruehl-Fehlert et al., 2003 and Martins & Neuhaus,2007).

The liver has a dual blood supply: the hepatic portal vein and the hepatic artery. Most of the blood (approximately 75%) is delivered to the liver via the hepatic portal vein that drains the spleen, stomach, intestines, and pancreas whereas the hepatic artery supplies

approximately 25 % of the blood (Thoolen et al., 2010). The lobes of the rat appear to have similar fundamental portal and hepatic vascular systems, and thus segments, comparable to that of human liver (Kogure et al., 1999). However, the portal vein bifurcates in human liver, whereas it trifurcates in the rat's liver giving three main portal branches that determine the following fissures: left portal fissure (LF), right portal fissure (RF) and main portal fissure (MF) (Martins & Neuhaus, 2007). These fissures form separations between the lobes of the rat's liver as shown in (Fig.3). Branches of the hepatic artery and portal vein along with bile ducts are seen in the portal triads (Junqueira & Carneiro, 2005). The bile ducts join to form the hepatic duct leading to the small intestine in rats and to the gallbladder in mice. Blood flows from the portal areas to the central vein in the center of each lobule while bile flows from the center of the hepatic lobule to the portal areas and on to the hepatic duct (Thoolen et al., 2010).

As an important interface for processing blood from the digestive system, the liver is uniquely situated with respect to the venous blood flow. Blood from the portal vein passes through the substance of the liver before entering the systemic circulation making it the first organ to receive metabolic substrates and nutrients as well as toxic substances (Rose & Pawlina, 2011). Branches of the portal vein called portal venules empty into hepatic sinusoids that drain into central veins. Both the portal and arterial blood percolate through the liver sinusoids and exit by way of the hepatic vein (Junqueira & Carneiro, 2005), eventually draining into inferior vena cava.

Despite gross differences, the microscopic features of the rat's and human livers are more or less similar. The liver is structurally organized into polygon-shaped lobules as seen in light-microscope sections. The normal rat liver is divided into the classic hepatic lobules. Each lobule is formed of central vein located at the center and cords of hepatic cells radiating peripherally from the central vein. The cell cords are separated by narrow blood sinusoids. Each lobule is bound by scanty connective tissue. At the apices of lobules, are portal islands of connective tissue each containing a branch of hepatic artery, a branch of hepatic portal vein and bile ductile forming portal triads (Demetris, 2008).The parenchymal cells of rat liver, hepatocytes, are polyhedral cells with acidophilic cytoplasm and each cell had a rounded pale stained nucleus. The sinusoidal surface of

hepatocytes has numerous microvilli that increase the surface area available for exchange and absorption of substances from portal blood (Malarkey et al., 2005 & Demetris, 2008).

Hepatic sinusoids are capillaries that are larger and more irregular in shape than ordinary capillaries. These sinusoids conduct nutrients/hormone-rich portal venous and high-oxygenated arterial blood slowly past the hepatocytes. Cells lining the hepatic sinusoids include Kupffer cells, endothelial cells, and Ito cells) (Laskin, 1996; Demetris, 2008 and Thoolen et al., 2010). Endothelial cells, are long slender cells with extended processes, and constitute the major cellular element of the hepatic sinusoidal lining with a fenestrated endothelium. The fenestrae refer to large holes in the endothelial cells that allow easy passage of nutrients and lipids from the portal blood to the hepatocyte surface. A narrow perisinusoidal space (the space of Disse) separates endothelial cells from underlying hepatocytes. Kupffer cells are macrophages of the liver derived from monocytes. These cells are found between endothelial cells and on the luminal surface within the sinusoids. Their main functions are to break down aged erythrocytes, remove bacteria or debris that may enter the portal blood from the gut, clear foreign materials, in particular endotoxin, from the portal circulation. Ito cells, located in space of Disse, are fat storing stellate cells with small lipid droplets containing vitamin A (Laskin, 1996 and Junqueira & Carneiro, 2005).

The liver is a vital organ that has the major functions including secretory, storage, metabolic, and excretory. Its secretory function involves the production and release of 600 to 1200 mL of bile per day. It manufactures most of plasma proteins, blood clotting-factors (Effendy et al., 2006). Liver cells store metabolites such as large amounts of glucose in the form of glycogen and amino acids in the form of triglycerides, vitamins, from diet (Guyton & Hall, 2006). It detoxifies and eliminates drugs such as pesticides, herbicides and toxic substances (Effendy et al., 2006) remove damaged red blood cells from the blood in co-ordination with spleen (Bigoniya et al., 2009).

Because of its central role in xenobiotic metabolism and portal location within the circulation (Jones, 1996 & Thoolen et al., 2010) as well as accumulation of toxins in the body faster than the capacity of liver to process and remove them results in hepatic damage (Effendy et al., 2006). Early evidence of liver damage is usually manifested by

the fatty change that is indicated by the form of cytoplasmic vacuoles in hepatocytes. These vacuoles will displace the nucleus to one side. The hepatocytes will enlarge and their nuclei appear darkly stained (Ebaid et al. 2007). Moreover, Zhang and Wang (1984) suggested that the cytoplasmic vacuolation is mainly a consequence of considerable disturbance in lipid inclusions and fat metabolism occurring during pathological changes. When there is more severe metabolic disruptions, the hepatocytes will undergo hydropic degeneration and become swollen (Effendy et al., 2006). These affected cells will gradually undergo necrosis, which is an indication of severe hepatic damage.

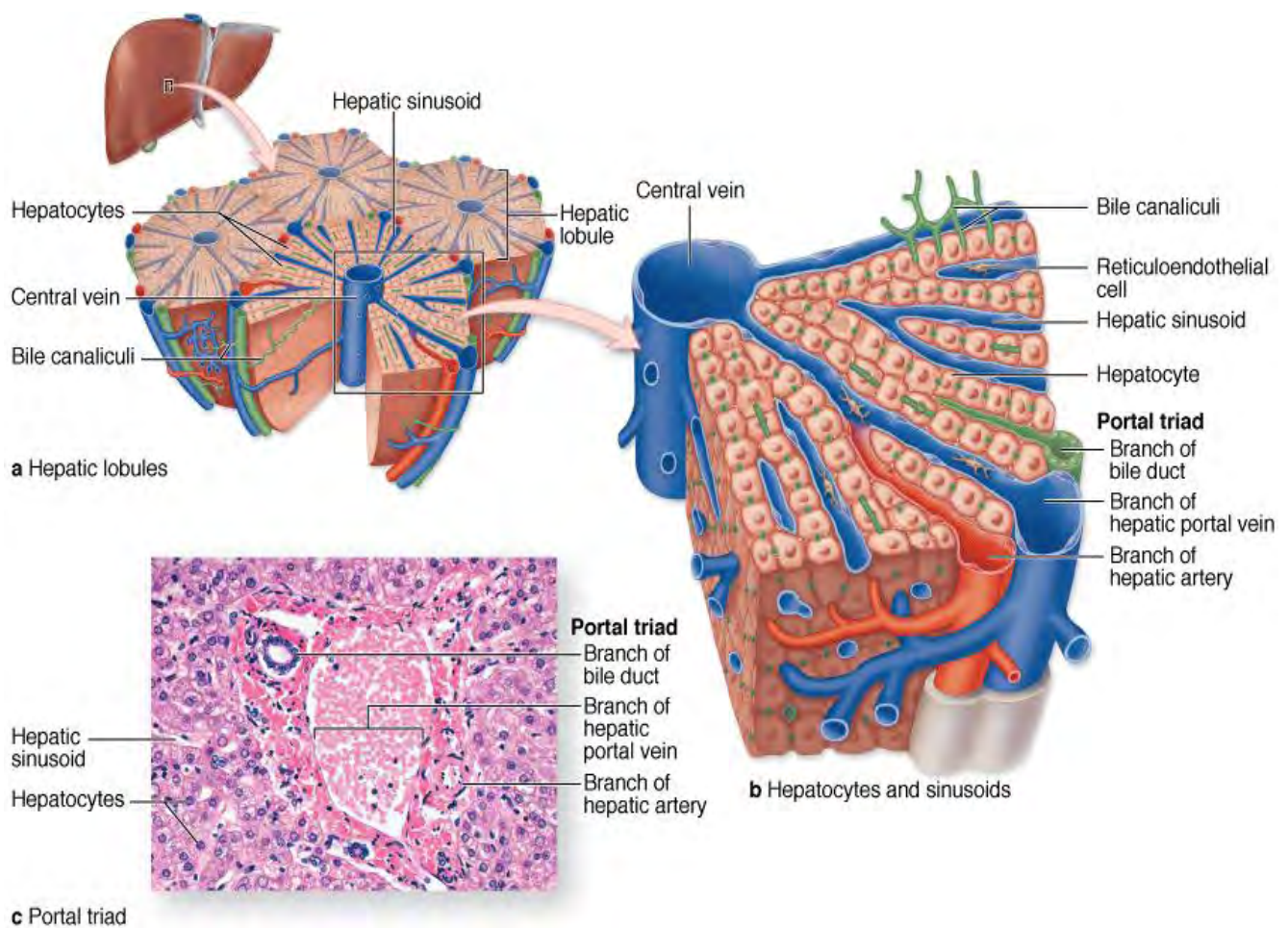


Figure 4: Diagram of human liver (a) central vein and portal triad. (b): Sinusoids, which run between plates of hepatocytes and drain into the central vein. (c): Micrograph showing components of the portal triad. X220. H&E. (Junqueira & Carneiro, 2012)

1.2.5. The structure, function and histopathology of the kidney

The kidneys of the rat are bean-shaped, smooth, reddish-brown in color, and covered by a thin connective tissue capsule adherent to sub capsular connective tissue. The rat kidneys lay alongside vertebral column in the abdominal cavity, and the right kidney situated cranially than the left. Each kidney has dorsal and ventral surfaces, medial and lateral borders, and an upper and lower poles. The lateral border is convex while, the medial border is concave with indentation, called the hilus, where major renal vessels enter and leave, and the ureters originate (Onyeanusil et al., 2009 and Al-Samawy, 2012).

On the cut surface of bisected kidney, 2 distinct regions can be identified: a pale outer region, the cortex, and a dark granular inner region, the medulla. In humans, the medulla is divided into 8 to 18 renal pyramids whose base is positioned at corticomedullary boundary, and apex extends towards renal pelvis to form papilla. On the tip of each papilla are small openings of distal ends of collecting ducts (of Bellini). Cortical parenchyma extends into spaces between adjacent pyramids; forming columns of Bertin. A medullary pyramid with surrounding cortical parenchyma including columns of Bertin and subcapsular cortex constitutes a renal lobe (Fogo et al., 2006; Tortora & Derrickson, 2012). Extending outward from upper dilated end of renal pelvis, are 2 or 3 outpouchings, major calyces. From each major calyx, several minor calyces extend toward the papillae of pyramids and drain urine formed by each pyramidal unit. In contrast to human kidney, the kidney of rat has single renal pyramid, termed “unipapillate,”and its papilla directly surrounded by renal pelvis. Otherwise, these kidneys resemble human kidney in their gross and microscopic structures (Webster et al., 1947; Fogo et al., 2006 & Al-Samawy, 2012).

The functional unit of the kidney is the nephron. Each human kidney contains about $1-1.4 \times 10^6$ nephrons (Junqueira & Carneiro, 2012), which contrasts with approximately 30,000 nephrons in each adult rat kidney (Rytand, 1938 & Webster et al., 1947). The components of each nephron include the renal corpuscle, the proximal convoluted tubule, the thin and thick limbs of Henle’s loop, the distal convoluted tubule and the collecting tubules and ducts (Al-Samawy, 2012, & Junqueira & Carneiro, 2012).

The renal corpuscles are composed of glomerulus and Bowman's capsule. The glomeruli are spherical collections of interconnected arterial capillaries within Bowman's/urinary space. The Bowman's space is continuous with proximal convoluted tubule at urinary pole. The glomerular hilus, where afferent and efferent arterioles enter and leave, is vascular pole. The Bowman's capsule is made of 2 thin cellular layers: the outer parietal layer lined by simple squamous epithelium and inner visceral layer formed by epithelial cells, podocytes (Verland, 1998 & Rose & Pawlina, 2011).

The visceral epithelial cells (podocytes) cover the outer aspect of the glomerular capillaries. Each podocyte has a large body containing the nucleus and cytoplasmic extensions, forming small finger-like processes called foot processes (pedicles), that interdigitate with similar structures from adjacent cells and cover the capillaries. The space between adjacent foot processes form filtration slit. Adjacent foot processes are joined together by a thin membrane called the slit-pore diaphragm (Fogo et al., 2006). The filtrate that leaks out of the glomerulus enters the Bowman's space through a complex filtration barrier. This barrier is formed of 3 main layers: visceral epithelial cells on the filtrate side, a basement membrane and fenestrated endothelium of capillaries on the blood side. The capillary tufts are supported by the mesangium that represents the intraglomerular continuations of the arteriolar walls (Verland, 1998 & Fogo et al., 2006).

The renal tubules form the remaining portion of the nephron, which include proximal convoluted tubules (PCT), the loop of Henle, with descending and ascending limbs, and the distal convoluted tubule (DCT). PCT is a very tortuous tubule longer than the DCT. The PCT cells have abundant long microvilli on luminal surfaces forming a prominent brush border for reabsorption (Junqueira & Carneiro, 2012). The loop of Henle, interposed between the proximal and distal convoluted tubules, is a U shaped structure with descending and ascending limbs that are composed of simple epithelia (cuboidal near the cortex and squamous deeper in the medulla) (Junqueira & Carneiro, 2005).

DCT is made up of simple cuboidal cells that differ from cells of PCT in being flatter, smaller and having no brush border. The initial, straight part of distal tubule contact with the vascular pole of the renal corpuscle of its parent nephron form part of a specialized structure, the juxtaglomerular apparatus. At the point of contact with the arterioles, the

cells of the distal tubule become columnar, closely packed and form a thickened spot, macula densa. Adjacent to the macula densa, smooth muscle cells of the tunica media of afferent arteriole is modified in to juxtaglomerular cells. Also at the vascular pole are extraglomerular mesangial cells, lacis cells, with supportive functions. The collecting tubules and ducts lined by cuboidal epithelial cells become columnar as they penetrate deep in to medulla. Renal interstitium is the space between renal tubules, and blood and lymph vessels (Fogo et al., 2006 and Junqueira & Carneiro, 2005).

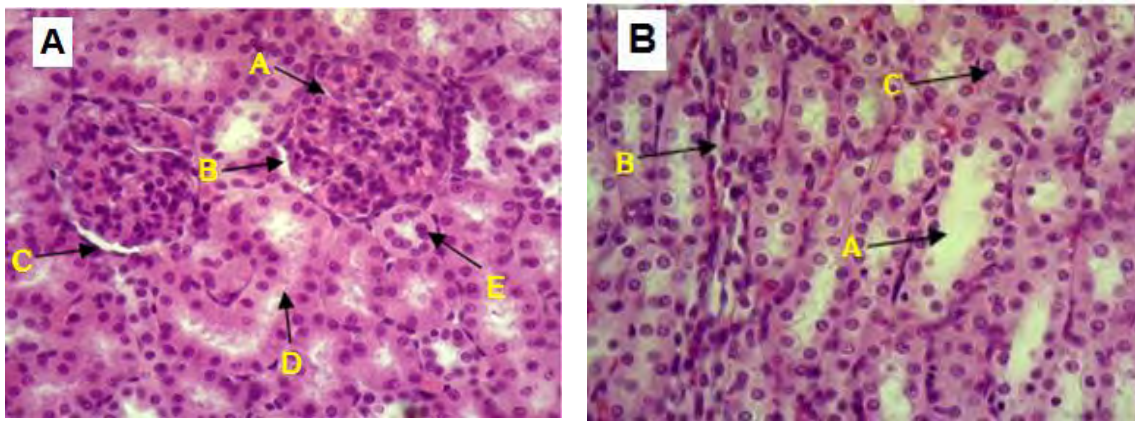


Figure 5: (A); A-Glomerulus. B-Visceral layer. C-Parietal layer. D-Distal tubule. E-Proximal tubule. (B); A-Collecting tubule... B-Thin limb... C-thick descending limb. (400 × H & E), (Al-Samawy, 2012).

The kidneys are the major excretory and osmoregulatory organs that regulate fluid osmolality and volume, fluid and electrolyte balance, eliminate foreign chemicals, and help maintain acid base balance of the body (Verland, 1998; Rose & Pawlina, 2011). Nephrons filter water and soluble components from the blood and selectively reabsorb some components such as ions (e.g., Na^+ , K^+ , Cl^- , HCO_3^- , and phosphate), water, glucose and amino acids (Rose & Pawlina, 2011). They also selectively secrete inorganic ions (Na^+ , K^+ , and H^+) to maintain stable concentrations in the extracellular fluid. Water and other substances that are not reabsorbed from renal tubule constitute urine. The kidneys are therefore the vital organs to maintain total body homeostasis (Klaassen, 2008) where their functional impairment is worthy.

A toxic insult to the kidney by a xenobiotic may disrupt any or all of renal functions and could have profound effects on the total body metabolism (Klaassen, 2008). In response to damaging stimuli, there may be proliferation of endothelial cells lining glomerular capillaries and visceral epithelial cells investing the outer surface of glomerular capillary, and congestion of glomerulus (Robbins & Cotran, 2005). Many traditional medicinal plants have found to cause renal damage most commonly in the outer cortex (Dapar et al., 2007). Marked increase in serum urea and creatinine are also indications of renal functional impairment due to pathological changes on the kidney tubular structure caused by plant products (Atawodi et al., 2013). In the tubules of nephrons for example, there were tubular necrosis and chronic inflammatory cellular infiltrations following oral administration of *Phyllanthus Amarus* leaf extract (Eweka & Enogieru, 2011). Moreover, chronic use of opioids showed renal tubular vacuolization, focal necrosis and haemorrhage (Atici et al., 2005).

1.3 Significance of the study

Recently, the development of anthelmintic resistance and the high cost of conventional anthelmintic drugs led to the evaluation of medicinal plants as an alternative source of anthelmintics (Dereje et al., 2009). On top of this some medicinal plants contain hazardous compounds that might be harmful to the host organism (Effendy et al., 2006) especially in the long term administration of herbal remedies. Therefore, it is necessary to investigate the toxicity of local medicinal plants usually employed by herbalists in the treatment of diseases (Oyewole & Massaquoi, 2008).

Embelia schimperi is one of the most commonly used traditional taenicidal plants in different regions of our country. However, there is paucity of information regarding the toxic effect of chronic treatment by the plant extract in spite of its use in folk medicine practice. The objective of the present study is therefore to evaluate the toxic effects of chronic treatment by crude fruit extract of *embelia schimperi* in rats. The information obtained from this study can be used as one of the evidences in the attempt to develop anthelmenthic drugs from *Embelia schimperi* for treatment and control of intestinal parasitic diseases. It can also be used by researchers as baseline information for further study on the area.

2. OBJECTIVES OF THE STUDY

2.1 General objective

To investigate the toxic effects of chronic treatment by crude ethanolic fruit extract of *Embelia schimperi* on body weight, blood profiles, liver and kidney tissues in rats.

2.2 Specific objectives

- To evaluate the effect of fruit extract on the general body weight
- To investigate the effect of fruit extract on the relative weight of the liver and kidney
- To assess the effect of fruit extract on blood profiles
- To evaluate the effect of fruit extract on histology of liver and kidney

3. METHODS AND MATERIALS

3.1 Plant material collection and extraction

The fruits of *Embelia schimperi* were collected from Bahir Dar area about 487 kms North West of Addis Ababa, Ethiopia. The plant specimen was identified and deposited at Herbarium of Department of Drug Research (DDR), Ethiopian Public Health Institute (EPHI). The fruits of the plant were cleansed from extraneous materials, dried at room temperature, ground to powder and 6500 grams of the powdered material was obtained. The ground plant material was weighed, moistened and packed into a percolator of two liter-capacity and soaked with 80% ethanol. Each soaking was collected after 48 hours of saturation and the percolate was filtered with Whatman filter paper (18.5cm in diameter). The filtrate was defatted by petroleum ether and concentrated by a vacuum rotary evaporator (BUCHI Rotavapour, R-205, and Switzerland) at 40°C with a flask-rotation of 60 Rpm in order to remove the ethanol. Reduced pressure was applied to speed up the concentration. The ethanol was recovered in a flask for use in another soaking. In the drying process or after Rotavapour concentration, the remaining solvent, that is the 20%-water, was subsequently removed by setting the sample on a water bath of temperature 40°C (Debella, 2002). A total of 630 gram of crude ethanolic extract of the fruit was obtained over four series/batches. The percentage yield of extract was 9.7%. The dried extract collected was weighed, labeled, packed in plastic cup, and kept at -4°C until used.

3.2 Experimental Animals preparation

The animals employed in this study were adult male and female rats of 8-12 weeks old weighing 180-210gm. 59 rats obtained from animal breeding house of EPHI in Addis Ababa, Ethiopia, were used for both acute and chronic toxicity studies. For LD_{50s} determination (acute toxicity study), thirty-five nulliparous and non-pregnant female rats were equally divided into 6 treatment groups (group I-VI) and one control group (groupVII). Each group contained five rats. The reason for selection of female rats was based on recommendation of OECD 420. The preferred rodent species is the rat, although other rodent species may be used. Normally females are used. This is because literature surveys of conventional LD₅₀ tests show that usually there is little difference in

sensitivity between the sexes, but in those cases where differences are observed, females are generally slightly more sensitive. However, if knowledge of the toxicological or toxicokinetic properties of structurally related chemicals indicates that males are likely to be more sensitive then this sex should be used. When the test is conducted in males, adequate justification should be provided (OECD, 2001). For the chronic toxicity study, 24 rats were randomly distributed into three groups (group I, II & III) each containing eight rats. Each group contained four male and four female rats and were kept in separate cages to prevent sexual mixing. The three groups of rats were randomly assigned as Groups I, II and III. Group I and II animals received ethanolic fruit extract of *Embelia schimperi* at a dose of 400 mg/kg/bwt/day and 1600 mg/kg/bwt/day, respectively. Group III animals served as control and received a vehicle (2% DMSO solution). The animals were acclimated to a laboratory condition for a week before the commencement of the experiment. The animals had unrestricted access to standard commercial diet and water and maintained at standard temperature of (22±3 °C) and relative humidity of (50-60%) with 12 hr light/dark cycles until the end of the experiment.

3.3 Administration of the plant extract

Each group of animal was given different doses of ethanolic fruit extract of *Embelia schimperi* orally using intragastric catheter or stomach tube. The method of administration for acute toxicity study and LD₅₀ determination of the crude ethanolic fruit extract of *Embelia schimperi* was following OECD guideline 420 (OECD, 2001). The extracts were given once after the animals were fasted overnight with a free access for water. After the period of fasting, the animals were weighed and the dose was calculated according to their body weight, then the test substance was administered accordingly (OECD, 2001). For the chronic toxicity study, the rats in the experimental groups received the crude ethanolic fruit extract of *Embelia schimperi*, while the rats in the control group were given 2% Dimethylsulfoxide (DMSO) every 24 hours for consecutive 24 weeks. Route of administration was intra-gastric using a ball-tipped stainless steel feeding needle fitted to a 5ml syringe. All equipments used were clean and placed in an oven after each administration to prevent any contamination.

3.4 Acute toxicity study and LD50 determination

The acute toxicity and LD₅₀s of the crude ethanolic fruit extract of *Embelia schimperi* was determined following OECD guide line 420 (OECD, 2001). The crude ethanolic fruit extract of *Embelia schimperi* was administered orally at the doses of 400, 1,000, 2,000, 3,000, 4,000, and 5,000 mg/kg body weight of crude ethanolic fruit extract of *Embelia schimperi* for treatment groups I, II, III, IV, V and VI respectively. The control group (group VII) was administered orally 2% DMSO solution.

Once-daily, cage side observations for behavioral changes were made. These included salivation, piloerection (erection of the hair), urinary incontinence, diarrhea, and locomotion. Mortality, if any, was determined over a period of two weeks.

3.5 Chronic toxicity study

The rats in Group I and II were treated with the extract at doses of 400 and 1600 mg/kg bwt/day respectively, while rats in Group III (control) were treated with 2% Dimethylsulfoxide (DMSO) every 24 hours for consecutive 24 weeks. Route of administration was intra-gastric using a ball-tipped stainless steel feeding needle fitted to a 5ml syringe. Preparations of the doses and duration of administration for chronic toxicity study were based on the WHO guideline (WHO 2000) and the recommendation of guideline document OECD 452 (OECD, 2009).

3.6 Data collection

Data were collected from the experimental animals before and after they were sacrificed.

3.6.1 Body and organ weight measurement

The animal's body weight was recorded at the start of treatment and once a week thereafter. The weight recorded shortly before administration of the test substance was considered to be initial body weight. Final body weight was recorded on the last day after 12 hours fasting following administration of test substance (WHO, 2000) and OECD 452 (OECD, 2009). Absolute organ weight of liver and kidney was measured immediately after rats were sacrificed. Liver and kidney were carefully removed from each rat and weighed using electronic balance with 0.001 precision.

The relative organ weight was calculated from body weight and absolute organ weight of liver or kidney using the following formula (Aniagu et al., 2005 and wonder et al., 2011).

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)}}{\text{Rat's body weight on sacrifice day (g)}} \times 100$$

3.6.2 Blood collection for hematological and biochemical analyses

Blood sample was collected before rats were sacrificed. The rats were anaesthetized using cotton wool soaked in diethyl ether vapour (inhalation) in desiccators Jar. When they became unconscious, they were quickly brought out of the jar. Blood samples were then obtained through cardiac puncture using sterile needle fitted to 5ml syringe and directly introduced in to two groups of test tubes (Adedapo et al., 2008). The test tubes with anticoagulant, ethylene-diaminetetraacetic acid (EDTA) were used to collect blood samples for analysis of haematological parameters while test tubes without anticoagulant were used to collect blood for biochemical estimation.

Hematological parameter such as hematocrit (HCT), hemoglobin (Hgb), total counts of RBC and WBC, mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), lymphocytes (L), and platelets (PLT) were measured using Automated Hematology Analyzer (Symex-XT, 1800i-Japan). The blood samples in test tubes were allowed to clot, and then centrifuged at 5000 Rpm for about five minutes to obtain the serum. The serum was employed to analyze the biochemical parameters that are commonly used for liver and kidney function tests including urea, creatinin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using Automated Clinical Chemistry Analyzer (Huma Star 80-Germany).

3.6.3 Animal dissection, tissue collection and histological processing

The abdominal cavity of the animals was opened by a vertical midline incision with scissors cut from the neck to pubis. The liver and kidney were gently isolated, all extraneous tissues (e.g., fat) removed, and immediately weighed (Sellers et al., 2007) on electronic balance. Then, 3-5mm thick, strips of tissue samples were randomly taken (Ruehl-Fehlert et al., 2003) from the liver and coronal section of right kidneys lengthwise with a scalpel through the renal pelvis. Tissue samples were transferred by a blunt

forceps into a labeled test tubes containing 10% neutral buffered formalin (Adedapo et al., 2008) that completely immerses the tissues for the purpose of fixation. After overnight fixation, the tissues were washed for 8 hours in running tap water (Singh, 2006). This removes excess fixatives and prevent over fixation of tissues. After washing, the tissues were dehydrated with graded series of alcohol: one hour each in 70% alcohol, 80% alcohol, 95% alcohol, absolute alcohol-I and two hours in absolute alcohol II. The tissues were cleared with two changes of xylene for one and half an hour each (Singh, 2006; Mohan, 2007). After clearing, they were infiltrated in two changes of melted paraffin wax for one and half an hour that has a melting point of 56°C (52-64°C) (Mohan, 2007). The paraffin infiltrated tissues were then placed carefully into squared metallic plate block moulds into which liquid paraffin wax was poured with the help of Electro-thermal Wax Dispenser to form tissue blocks. The paraffin was allowed to harden around each tissue section to a solid mass of block confirming to the shape of a mold. The blocks were labeled before harden. The resulting solid paraffin blocks containing the tissue were then removed from the mold and sectioned in ribbons at a thickness of 5µm using a rotary microtome (Leica RM 2125, Leica Microsystems Nussloch GmbH, Germany).

The paraffin ribbons containing the tissues were allowed to float onto the surface of a warm water bath at 45°C to spread and remove folds in the sections. The slides were arranged in slide racks and were placed in an oven with a temperature of 60°C for 10-15 minutes. The tissue sections were allowed to cool and dry at room temperature and stained with routine Hematoxylin and Eosin staining method (H and E).

Staining solutions were prepared using the formula given by Clopton (2006). The slides to which the tissue sections attached were placed in xylene I for 5 minutes and xylene II for 2 minutes so as to dissolve the paraffin. The sections were then immersed in a series of descending alcohol concentrations: absolute alcohol I, II and 95% alcohol for 2 minutes each, 70% alcohol for 3 minutes and 50 % alcohol for 2 minutes to remove xylene after which distilled water (for 2 minutes) was used to hydrate the tissue.

The hydrated sections were immersed in Harris Hematoxylin containing jar for 6 minutes and washed under running tap water for 3 minutes. The slides were immersed in 1% acid alcohol for differentiation and controlling overstrained hematoxylin for 20 seconds and

then immersed into running tap water and bluing solution (Sodium bicarbonate solution) for 1 minute each. After bluing, the slides were immersed in running tap water for 2 minutes then counterstained with eosin for 1 minute. Finally, tissue sections were dehydrated with increasing alcohol concentrations of 50%, 70%, 95%, absolute alcohol I and II for 2 minutes each and cleared with two changes of xylene for 3 minutes each (Kiernan 2008). The slides were mounted by adding a drop of Dibutyl phthalate in xylene (DPX) mounting medium on the section to cover the microscopic glass with cover slip. This was done with care to prevent bubble formation between the tissue and the glass cover (Singh, 2006). Finally, the slides were labeled with pertinent identification information and placed in a slide box (Bancroft et al., 1990)

3.6.4 Light Microscopy

For histopathological investigations, stained tissue slides of liver and kidney were examined at different magnifications (x10, x40 and x100 objectives) using light microscope: MC 80 DX Microscope Camera (Carl Zeiss, Germany). This investigation was carried out in the histology laboratory of Anatomy Department, School of Medicine, AAU. After examination of histological slides of all groups, photomicrographs of selected samples of liver and kidney from both extract treated and vehicle treated rats were taken using digital camera installed microscope. The photomicrographs were directly saved on to portable USB flash drive inserted to the microscope. A magnification of x40 and x10 were used for photomicrography for both liver and kidney tissue sections. Based on observations, comparisons were made between treatment and control groups.

3.6.5 Statistical analysis

Data were analyzed using the statistical software package SPSS version 16 for windows program. All the values in the test are presented as mean and standard error of the mean (mean \pm SEM). Statistical differences between the means of different groups were evaluated by one-way analysis of variance (ANOVA). P-values <0.05 were considered significant.

4. RESULTS

4.1 Acute toxicity study and LD₅₀ determination

An experiment was conducted to study the acute toxicity and LD₅₀ of the crude ethanolic fruit extract of *Embelia schimperi* in female rats. After treatment with the plant extract, cage side observations were made for any behavioral changes such as salivation, piloerection (erection of the hair), urinary incontinence, diarrhea, and locomotion. Animals did not show any of these behavioral changes except for those rats treated at doses of 4,000 and 5,000mg/kg. The rats treated with *Embelia schimperi* at 4,000 and 5,000mg/kg showed weak motor activity (hypoactivity) and erection of hair. However, these changes were not apparent after the first 24 hours of the follow-up. All the extract treated animals were as active as the control animals. During this short-term acute toxicity study, no prominent signs of toxicity were recorded among experimental animals. As indicated in table 2, administration of the crude ethanolic fruit extract of *Embelia schimperi* at doses of 400, 1,000, 2,000, 3,000, 4,000 and 5,000mg/kg did not produce any death in female animals during the fourteen days period of experiment for acute toxicity. This result indicated that the oral median lethal dose (LD₅₀) is higher than 5,000mg/kg. Since no death was recorded at the maximum administered dose, the LD₅₀ of the plant extract was found to be higher than 5,000 mg/kg.

Table 2: Acute toxicity of ethanolic fruit extract of *Embelia schimperi* in rats. (N=5).

Group	Dose (mg/kg/)	Number of died rats	Signs of toxicity observed
I	400	0	No toxic changes observed
II	1000	0	No toxic changes observed
III	2000	0	No toxic changes observed
IV	3000	0	No toxic changes observed
V	4000	0	Hypo activity and piloerection was observed in animals in the first 24 hours of administration, but after this period they became normal.
VI	5000	0	Hypo activity and piloerection was observed in the animals in the first 24 hours of extract administration but after this period they became normal
VII	0	0	-----

4.2 The effect of the plant extract on the body weight

As shown in figure 3, the growth of rats treated with the plant extract was retarded throughout the study period, especially between the 5th to 7th weeks compared to the control group.

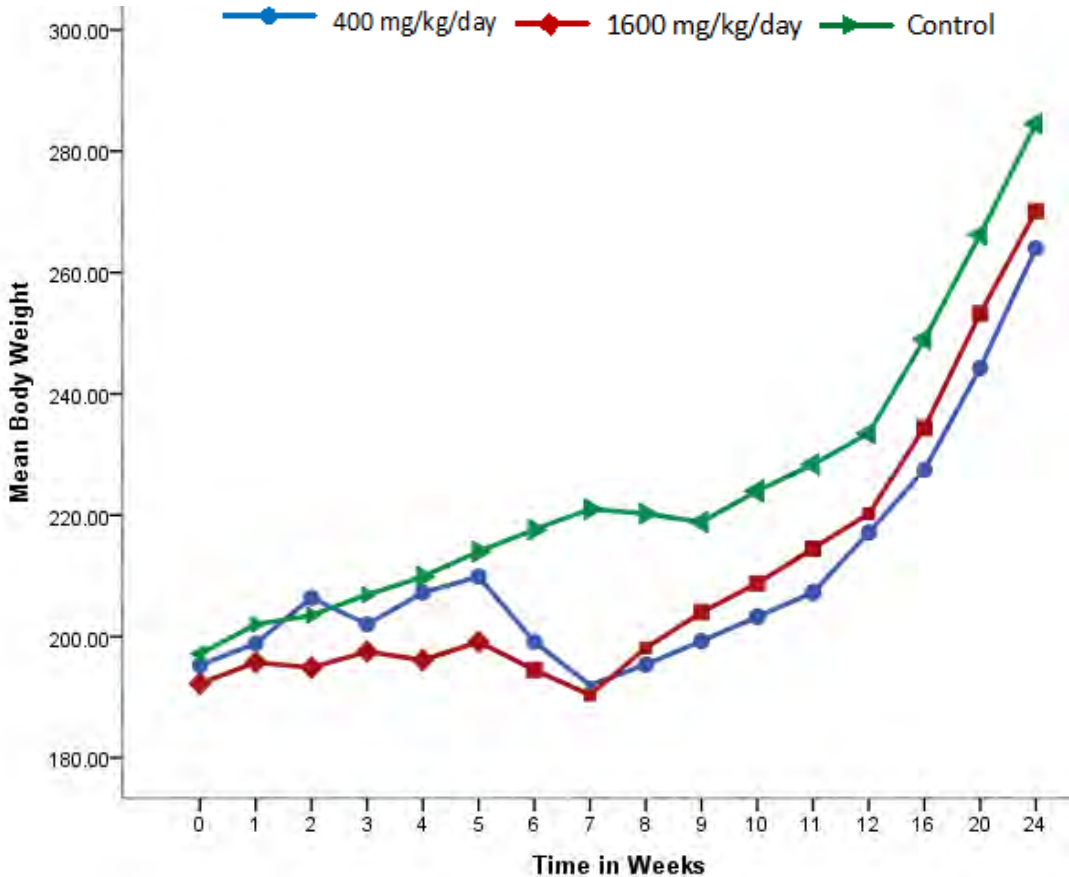


Figure 6. Line graph of mean body weight changes of groups of rats (control = 0 mg/kg/day; G1= 400 mg/kg/day; G2 = 1600 mg/kg/day; $N = 8$) given daily doses of Ethanolic fruit extract of *Embelia schimperi* during chronic toxicity study.

As indicated in table 3, the mean body weight change in rats treated with the crude ethanolic fruit extract of the plant was lower (35.75 and 79.13 gm) as compared to the controls (87.63 gm). However, the differences were not statistically significant ($P < 0.05$) when treatment groups were compared with the control.

Table 3: Comparison of mean body weight change among ethanolic fruit extract of *Embelia schimperi* treated groups and the controls.

Group	Initial weight	Final weight	Weight difference	% of body
	IWT (in g)	FWT (in g)	(FWT-IWT) (in g)	weight change
Control	197.12±4.16	284.75±11.99	87.63±9.64	44.4
400 mg/kg/day	195.25±4.14	264.00±11.45	68.75±9.44	35.38
1600mg/kg/day	192.25±3.43	270.12±6.30	79.13±5.31	41.16

Values are expressed as mean ± SEM, N = 8/group

4.3 The effect of the plant extract on relative weights of liver and kidney

As shown in table 3, there was no statistically significant change in the relative organ weights of liver and kidney of the treated rats as compared to that of the control.

Table 4: Comparison of relative organ weight of liver & kidney among ethanolic fruit extract of *Embelia schimperi* treated groups and the controls.

Group	Relative organ weight (g/100g of bwt)	
	Liver	kidney
Control	3.77±0.17	0.3375±0.02
400mg/kg/day	3.73±0.08	0.3412±0.01
1600mg/kg/day	3.77±0.10	0.3375±0.01

Values are expressed as mean ± SEM, N=8/group

4.4 Effect of the plant extract on hematological parameters

The effect of chronic treatment of ethanolic fruit extract of *Embelia schimperi* on haematological parameters of blood is illustrated in table 4. Different values of haematological parameters were obtained between the controls and treatment groups. Such differences were, however, found to be statistically not significant ($p>0.05$) for most of haematological parameters. Although statistically not significant, slight reductions in Hgb, MCH and MCHC at doses (1600 mg/kg bwt/day and 400 mg/kg bwt/day) as well as, RBC, HCT and MCV at 400 mg/kg bwt/day were observed in extract treated groups as compared to controls.

Though statistically not significant ($p>0.05$) the values of WBC and L recorded at both doses (1600 mg/kg bwt/day and 400 mg/kg bwt/day) were found to increase as compared to the counter controls. However, significant increases in the values of PLT ($p<0.05$) were observed at both doses (1600 mg/kg bwt/day and 400 mg/kg bwt/day).

Table 5: Comparison of Hematological parameters among rats treated with ethanolic fruit extract of *Embelia schimperi* and controls.

Haematological Parameters	Control	400mg/kg/ bwt/day	% of mean difference	1600mg/kg/ bwt/day	% of mean difference
Hgb (g/dl)	21.81±.26	21.49±.21	-1.47	21.71±.27	-0.46
RBC (M/UL)	8.47±.22	8.43±.22	-0.47	8.58±.28	1.29
HCT (%)	46.03±.67	45.84±.69	-0.41	46.75±.81	1.56
MCV (fl)	54.11±.794	51.38±1.32	-5.05	55.54±.60	2.64
MCH (pg)	25.89±.44	25.26±.27	-2.43	25.25±.37	-2.47
MCHC (g/dL)	47.81±.51	46.79±.42	-2.13	46.53±.28	-2.68
WBC (K/UL)	8.30±1.26	9.20±.26	10.84	8.59±.78	3.49
L (K/UL)	6.42±.99	7.23±.16	12.62	6.69±.47	4.21
PLT (K/UL)	692.25±38.95	862.75±47.91*	24.63	845.88±13.89*	22.19

Values are expressed as mean ± SEM. * $P<0.05$, N=8/group

4.5 Effect of the plant extract on biochemical parameters

Some biochemical parameters in the blood serum of rats treated with ethanolic fruit extract of *Embelia schimperi* were analyzed and compared to the controls. The result showed that biochemical parameters AST and ALT were increased in the rats treated with the plant extract at both doses as compared to those of the control rats. The increases in ALT were not statistically significant at both doses. However, serum concentrations of AST was significantly increased ($p<0.05$) in rats treated with 1600 mg/kg bwt/day. There were decreases in creatinine levels in rats treated at 400 mg/kg bwt/day and increases in rats treated at 1600 mg/kg bwt/day as compared to controls. These creatinine changes were not statistically significant. Also, the decrease in the levels of urea was not significant.

Table 6: Comparison of Biochemical parameters among rats treated with ethanolic fruit extract of *Embelia schimperi* and controls.

Biochemical Parameters	Control	400mg/kg/ bwt	% means difference	1600mg/kg/ bwt	% mean difference
ALT (IU/L)	102.00±7.27	109.50±5.30	7.35	103.38±6.66	1.35
AST (IU/L)	81.38±8.17	101.38±3.99	24.58	110.38±7.69*	35.64
Creatinine (mg/dL)	0.72±.04	0.71±.03	-1.4	0.81±.01	12.5
Urea	40.50±.96	36.25±.98	-10.49	39.75±1.99	-1.85

Values are expressed as mean ± SEM. *P<0.05, N=8/group.

4.6 Effect of the plant extract on histology of the liver

In addition to body weight and organ weight changes, biochemical and hematological parameters, histopathology of the plant extract on liver and kidney were investigated in this study. As shown in figures: 7B and 7A and 7C and 7A, Histopathological examination of liver sections obtained from rats treated with the ethanolic fruit extract of *Embelia schimperi* at both doses (400mg/kg bwt/day and 1600 mg/kg bwt/day) show periportal focal lymphocytic inflammations as compared to the control.

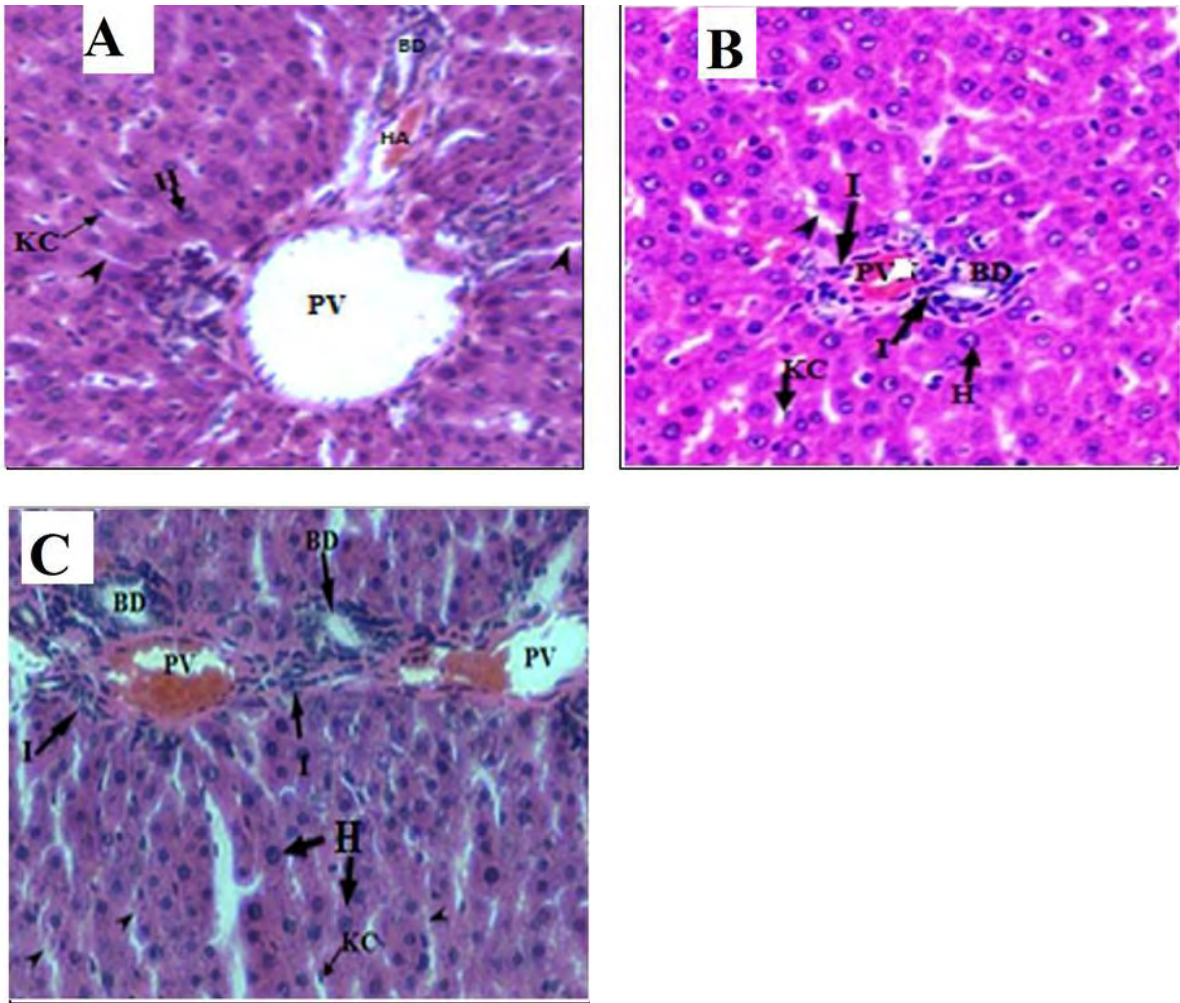


Figure 7: Photomicrographs of liver sections (H & E stain, X400): (A): photomicrograph of liver sections of control rats. (B): photomicrograph of liver sections of rats treated with 400mg/kg bwt/day. (C): photomicrograph of liver sections of rats treated with 1600mg/kg bwt/day. There were periportal focal lymphocytic inflammations in the liver sections of treated rats at both doses (400mg/kg bwt/day and 1600 mg/kg bwt/day) as compared to control. H = Hepatocytes, KC = Kupffer Cells, PV = Portal Vein, BD = Bile Duct, I = Inflammation, PV = Portal Vein.

4.7 Effect of the plant extract on histology of the kidney

The histopathological effect of the ethanolic fruit extract of *Embelia schimperi* was done on the kidney section that was stained with hematoxylin and eosin. Microscopic observation indicated that there was difference between the kidney sections of the control (Figure 8. A), and rats treated with doses of 400 mg/kg bwt/day and 1600mg/kg ethanolic fruit extract of the plant (Figure 8. B, C & D). Renal histology of rats treated at 400 mg/kg bwt/day show narrowed bowman's space and glomerular cellularity (Figure 8. B) as well as periglomerular lymphocytic infiltration Figure 8. C) as compared to controls. The kidney sections of rats treated at 1600mg/kg bwt/day showed more periglomerular lymphocytic infiltration around the parietal layer of bowman's space (Figure 8. D). The lumens of some tubules were also narrowed as compared to controls.

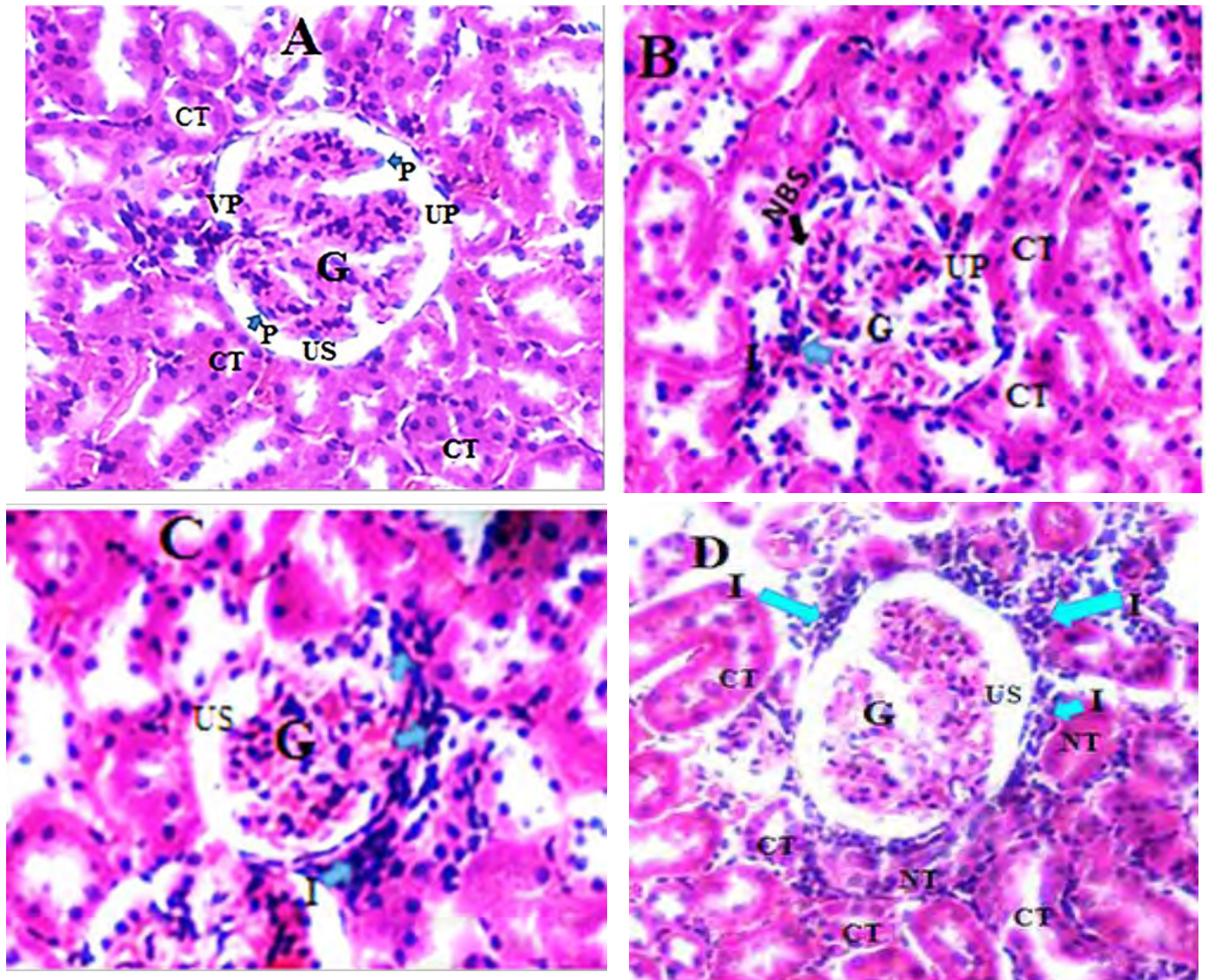


Figure 8: Photomicrographs of kidney sections (H & E stain, X400): (A): photomicrograph of kidney sections of control rats. (B): photomicrograph of kidney sections of rats treated with 400mg/kg bwt/day. (C): photomicrograph of kidney sections of rats treated with 400mg/kg bwt/day. (D): photomicrograph of kidney sections of rats treated with 1600mg/kg bwt/day. In the above photomicrographs, notice the following changes: NBS in (B) narrowed bowmans space; I or arrows in (C and D) periglomerular inflammation; NT in (D) narrowed lumen of convoluted tubule. G = Glomerulus, UP =Urinary pole, CT = Convoluted tubule, VP =Vascular pole, US =urinary space.

5. DISCUSSION

In a global context, various medicinal plants and botanical preparations have been widely adapted as primary therapeutic agents or supplements for treating various human ailments. This has maintained greater popularity all over developing world and the use is rapidly on the increase. Based on the findings that herbal medicines are abused, there is a great need to look into their acute and chronic toxicity effects (Afolabi et al., 2012).

Acute toxicity/LD₅₀ studies are usually initial screening experiments performed in the assessment of toxic manifestations of compounds. The data from these studies give a clue on the range of doses that could be used in subsequent toxicity testing (e.g. sub-chronic or chronic) and may reveal the possible clinical signs elicited by the substance under investigation (Akhila et al., 2007; Ping Y et al., 2013). In the present acute toxicity study, single dose administration of the crude ethanolic fruit extract of *Embelia schimperi* up to a dose of 5,000 mg/kg to female rats did not show behavioral changes like, salivation, urinary incontinence, diarrhea, piloerection (erection of the hair), and hypoactivity. The following day after treatment (24 hours post treatment), all rats were as active as the controls and no death was observed. In the present study, LD₅₀ of the crude ethanolic fruit extract of *Embelia schimper* was found to be greater than 5,000 mg/kg, which may be accepted as safe (OECD 2001). In agreement with this finding, the LD₅₀ study conducted on the same plant extract by Debebe et al. (2015) found the LD₅₀ being greater than 5,000 mg/kg. The study of Dawit et al. (2012) also indicated that the LD₅₀ of *Embelia schimperi* is greater than 10,000 mg/kg. In the present study conducted in female rats, a sensitive sex to acute toxicity (OECD, 2001), there was no death among rats in all the dose groups throughout the two weeks of the experimental follow up. Thus, the crude ethanolic fruit extract of *Embelia schimperi* can be considered as a substance with low toxicity.

In the present study, the effects of long-term treatment (24 weeks) with crude ethanolic fruit extract of *Embelia schimperi* on body weight, relative organ weight, some hematological and biochemical parameters of the blood, and histopathology of liver and kidney were investigated in rats. The doses of test substance evaluated were 400 mg/kg

bwt/day and 1600 mg/kg bwt/day. These doses were selected based on the results of LD₅₀/acute toxicity study (Akhila et al., 2007).

In the current work, a general body weight gain was observed in both rats treated with crude ethanolic extract of fruits of *Embelia schimperi* and the controls. The body weight gain in treated animals was less than those of the controls. There was a decrease in body weight during the 5th to 7th weeks of this study in treated animals. However, after 7th week, there was body weight gain that continued to increase until the end of the study. This regain in body weight might be due to tolerance of the effect of plant extract as reported by others (Ogbonnia et al, 2010). Moreover, the observed general body weight change was not significantly different in treated and control groups as well as dose-independent. Similarly, significant difference was not observed between relative organ weights of liver and kidney of the treated and control rats. This finding is in line with reports of studies on sub-chronic toxicity of methanolic fruit extract of *Embelia schimperi* in mice (Dawit et al.,2012) and chronic treatment of aqueous extract of *Artemisia afra* in rats (Mukinda and Syce, 2007). The pattern of body weight change observed in the present study was not in a dose dependant manner. These depicted that the plant extract might have caused no significant changes in their food intake and utilization of food indicating normal metabolism in the animals, which in turn suggests as reported by Mukinda and Syce (2007) that chronic treatment of aqueous extract of *Artemisia afra* in rats did not retard the growth of rats. Thus, the absence of significant differences in the body weight and relative weights of the liver and kidney provides support for the safety of the plant extract under investigation.

In this study, hematological parameters (such as Hgb, RBC, HCT, MCV, MCH, MCHC, WBC, L, and PLT) were investigated in the treated and control groups. The results of the investigation revealed no statistically significant differences in hematological parameters between treated and control groups. Damage and destruction of the blood cells like red blood cells are hostile to normal functioning of the body.

Red blood cell count and RBC indices (Hgb, MCV, MCH and MCHC) observed in the present study at both doses (400 and 1600mg/kg body weight) were not significantly different from controls. This result suggests that long-term use of crude ethanolic extract

of fruits of *Embelia schimperi* has no differential effect on matured RBCs and change in the rate of production of RBCs (erythropoiesis).

The absence of significant difference in the values of RBC and Hgb between treated and control groups imply that there might be no abnormal change in the oxygen-carrying capacity of the blood and the amount of oxygen delivered to the tissues following the extract administration. This finding was in agreement with previous reports of Dawit et al. (2012) on the effect of sub-chronic administration of methanolic extract of *embelia schimperi* in mice and Ping Y et al. (2013) on acute and sub-chronic toxicity of *euphorbia hirta methanol extract* in rats where the plant extracts did not significantly alter total RBC count, Hgb, MCV, MCH and MCHC in treatment group as compared to the controls. However, there were slight changes of these parameters in both directions that may have resulted from normal variation among animal groups as reported by Panunto et al. (2011). These findings imply that the crude ethanolic extract of fruits of *Embelia schimperi* does not possess the potential to induce anemia. This was further corroborated by other worker (Adeyemi et al., 2010) who reported that sub-chronic treatment of the ethanol extract of the leaves of *Sphenocentrum jollyanum* did not produce anemic effect which indicated the absence of the potential to induce anemia. Though statistically not significant, rats treated with the ethanolic fruit extract of *Embelia schimperi* at both doses (1600 mg/kg bwt/day and 400 mg/kg bwt/day) were found to have an increased white blood cell (WBC) and lymphocyte counts as compared to the counter controls. An increase in the number of WBC in the circulatory system of the animals is a reaction of animals to foreign substances (Adebayo et al., 2010) which may indicate a stimulation of the immune system to protect the animals against infection that might have been caused by compounds from plant extracts. This finding was similar with the previous report by Dawit et al. (2012) on the effect of sub-chronic administration of methanolic extract of *embelia schimperi* in mice that increase in WBC and lymphocyte count was indicated.

Significantly elevated platelet count was observed in the treated animals as compared to their counter control groups. This finding was in contrary to the study by Dawit et al. (2012) who reported sub-chronic administration of methanolic extract of *embelia schimperi* has slightly decreased platelet count in mice. The increase in platelet count in

treated animals may be due to difference in the duration of extract administration that is chronic in the present study that might have prolonged stimulatory effect on thrombopoietin (Yakubu et al., 2007; Amadi et al., 2012).

Ordinarily, liver cell damage by drug or any other hepatotoxin is characterized (Aniagu et al., 2005) and assessed by the level of plasma enzymes (AST, ALT, etc) (Ramaiah, 2011). In this study, serum concentrations of AST and ALT were increased in treated rats. The increment of AST concentration was significantly higher in rats treated with a dose of 1600 mg/kg bwt/day as compared to controls. This was different from the finding of sub-chronic toxicity study of same plant by Dawit et al. (2012) who reported that the concentrations of AST in the treated mice were not statistically significant as compared to the controls. This could be due to either severe liver tissue injuries (Martins, 2006) or due to the fact that the release of AST is in higher concentration from different tissues such as liver, kidneys, heart and pancreas (Aniagu et al., 2005). However, ALT which is more specific marker enzyme and indicative of hepato-cellular damage (Ozer et al., 2008) is not significantly different between treated and control groups. Therefore, the elevated AST concentration observed in treated rats in this study might not ascertain that the extract has insulted hepato-cellular toxicity. The serum concentrations of creatinine and urea in rats treated with ethanolic fruit extract of *Embelia schimperi* were not significantly changed as compared to the controls. There was a decrease in creatinine of rats treated with 400mg/kg bwt/day of the extract. The serum concentration of creatinine was increased in rats treated with 1600mg/kg bwt/day of the extract. The non-significant changes in creatinine and urea might indicate the non-toxic effect of the extract in the kidney function. These were in agreement with the previous study by Dawit et al., (2012) who reported that sub-chronic oral treatment of methanolic extract the same plant did not have nephrotoxic actions.

Histological examinations of the liver and kidney tissues of rats treated with the ethanolic fruit extract of *Embelia schimperi* showed changes in treated animals as compared to their concurrent controls. The histological changes observed were focal lymphocytic inflammations in the liver sections near the portal areas (periportal inflammations) in treated animals. More focal periportal inflammations were observed in the animals

treated with 1600mg/kg bwt/day. This was in agreement with sub-chronic toxicity study by Dawit et al. (2012) who reported that the presence of inflammation around the portal areas of the liver sections in the 1200mg/kg bwt/day treated mice might be due to the effect of the bioactive compounds in the plant extract. Moreover, in the present study, the observed inflammations in liver sections of rats treated with 400mg/kg bwt/day that is low dose might be due to prolonged treatment of the plant extract. This finding could indicate presence of ingredients in the ethanolic extract of the fruits of *Embelia schimperi* that may damage liver cells of rats when administered for longer duration especially at higher doses. This was corroborated by Effendy et al. (2006) who suggested that Kacip fatimah extract contains bioactive ingredients that are harmful to the liver in higher doses. The bioactive compounds in plant extracts may contain free radical scavenging molecules that can cause substantial biological damage of the tissues (Rodriguez et al., 2008; Effendy et al., 2006) by a complex reaction involving vascular responses like migration and activation of leukocytes (Tedong et al., 2007; Salawu et al., 2009). The observed inflammatory changes in the liver around the portal areas of treated rats could mean that the extract contains ingredients that might have toxic effects on the liver.

The histological examinations of the kidney sections of the rats treated at both doses revealed histological changes. The changes observed in the kidney sections of rats treated with 400mg/kg bwt/day of the extract showed inflammatory cells near the bowman's space (periglomerular infiltration), few cells inside the glomerulus (glomerular cellularity) and narrowed bowman's space. The rats treated with 1600 mg/kg bwt/day of the extract indicated increased peri-glomerular cellular infiltration around the parietal layer of the bowman's space and narrowed lumen in some of convoluted tubules. These results differ from the finding by Dawit et al. (2012) who reported that sub-chronic (eight weeks) treatment of mice with crude methanolic fruit extract of *Embelia schimperi* at doses of 400 mg/kg bwt/day and 1200 mg/kg bwt/day did not cause any histological changes in the kidneys. This variation might have occurred probably due to the longer duration of treatment period; animal models used and dose difference. According to studies by others, some histopathological changes were observed following treatment with herbal preparations. For instance, oral administration of *Ocimum gratissimum* leaf extract insulted renal tubular degeneration in rabbits (Effraim et al., 2001); *Securida*

calongepedunculata has been found to elicit tubular necrosis with glomerular haemorrhage (Dapar et al., 2007); oral administration of *Phyllanthus amarus* caused tubular necrosis and chronic inflammatory cell infiltrates (Eweka and Enogieru, 2011). In the present study, histological changes observed were periglomerular inflammatory cells that appeared more in the rats treated with 1600mg/kg bwt/day. Moreover, few narrowed bowman's space and tubular lumen were observed. This difference from previous study by Dawit et al. (2012) might be due prolonged treatment (Ezekwesili. et al., 2010) by the plant extract. However, the finding of the present study is in agreement with Ezekwesili et al. (2010) who reported presence of inflammatory cells point to the fact that inflammatory reaction played a major role in the genesis of the observed pathological lesions. Therefore, even though severe type of pathological changes like tissue necrosis are not observed in this study, the inflammatory cellular infiltrations indicated that the plant extract should be used with caution if intended for prolonged use.

6. CONCLUSION

This study showed that the chronic oral administration of crude ethanolic fruit extract of *Embelia schimperi* at doses of 400 & 1600 mg/kg bwt/day did not cause significant changes in body weight and organ weight.

Chronic oral administration of crude ethanolic fruit extract of *Embelia schimperi* at the tested doses did not cause destruction of blood cells or alterations in hematopoiesis.

The long-term oral administration of crude ethanolic fruit extract of *Embelia schimperi* did not result significant changes in the levels of ALT, creatinine and urea.

Chronic oral administration of crude ethanolic fruit extract of *Embelia schimperi* caused considerable kidney tissue inflammation at higher dose of 1600 mg/kg bwt/day.

7. RECOMMENDATION

Based on the findings of this study, the following recommendations are forwarded:

The ethanolic fruit extract of *Embelia schimperi* should be used with caution if intended for prolonged use.

Further studies are recommended to be carried out on the remaining organs of the body such as the brain, the heart, stomach/GIT structures, spleen and testis/ovaries) before allowing administration/or use of the plant in humans.

Further investigations on different species of animals such as pigs, rabbits, dogs and goats should be performed.

Toxicological studies on fractionate other than the crude extracts of the plant are recommended.

Limitations of the present study included: small sample size, study investigated only some parameters and tissues (example blood, liver and kidneys), only low dose and high dose of the test substance were used, a medium dose should also have been included. Therefore, extrapolation of results may be limited to only the study conditions.

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9. APPENDIX I

CHEMICAL PREPARATION

10% Neutral Buffered Formalin

10% Formalin (90% distilled H ₂ O and 37% Formalin)	1000ml
Sodium dihydrogen phosphate monohydrate (NaH ₂ PO ₄ .H ₂ O)	4g
Sodium monohydrogen phosphate anhydrous (Na ₂ HPO ₄)	6.5g

Harris' Hematoxylin

Hematoxylin Crystal	2.5g
Ethanol, 100%	25ml
Ammonium or Potassium Alum	50g
Distilled water	500ml
Sodium Iodate	1g

Eosin

Eosin Y (Yellow) CI 45380	0.5g
Ethanol, 95%	100ml
Glacial acetic acid	0.5ml

1% Acid Alcohol

Ethanol, 70%	500ml
HCl, concentrated	5ml

Bluing Solution

Sodium bicarbonate	2.5g
Ethanol	1000ml
Distilled water	500ml

10. APPENDIX II

TISSUE PROCESSING SCHEDULES TO FORM PARAFFIN BLOCK FOR MANUAL TECHNIQUE

Solution	Duration
Fixation	
Buffered formalin, 10%	over night
Washing	
Running tap water	8 hrs
Dehydration	
Alcohol, 70%	1 hr
Alcohol, 80%	1 hr
Alcohol, 95%	1 hr
Absolute alcohol	1 hr
Absolute alcohol II	2 hrs
Clearing	
Xylene I	1½ hr
Xylene II	1 ½ hr
Infiltration (in paraffin oven)	
Paraffin wax I 56°c (52-64°c)	1½ hr
Paraffin wax II 56°c (52-64°c)	1½ hr

11. APPENDIX III

HEMATOXYLIN AND EOSIN (H & E) STAINING SCHEDULES FOR PARAFFIN SECTIONS

Chemicals, solutions	Duration
Xylene I	5min
Xylene II	2min
Absolute alcohol I	2min
Absolute alcohol II	2min
Alcohol, 95%	2min
Alcohol, 70%	3min
Alcohol, 50%	2min
Distilled water	2min
Hematoxylin	6min
Tape water	3min
Acid alcohol	20sec
Tape water	1min
Bluing solution	1min
Tape water	2min
Eosin	1min
Xylene I	3min
Xylene II	3min