ADDIS ABABA UNIVERSITY COLLEGE OF HEALTH SCIENCE

SCHOOL OF GRADUATE STUDIES

Evaluation of Acute and Subacute Toxicity of Methanol Extract of *Syzygium guineense* Leaves on the Histology of the Liver and Kidney and Biochemical Compositions of Blood in Rats.

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Anatomy

Thesis

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Declaration

This is to certify that the thesis prepared by Million Loha entitled: Evaluation of acute and subacute toxicity of methanol extract of *Syzygium guineense* leaves on liver and kidney and blood parameters of Rats. And submitted in partial fulfillment of the requirements for degree of Master of Science (MSc) 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 dully acknowledged.

The thesis has passed with **Very good** remark.

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List of abbreviation

AAU	Addis Ababa University
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ALT	Alanine transaminase
AST	Aspartate transaminase
BW	Body weight
^{O}C	Degree Celsius
DPX	Dibutyl phthalate in xylene
EDTA	Ethylene Diamine Tetra-acetic acid
EPHI	Ethiopia Public Health Institute
H & E	Heamatoxylin and Eosin
HCT	Hematocrit
HGB	Hemoglobin
LD50	Median lethaldose
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
Mg	Milligram
Kg	Kilogram
g	Gram
OECD	Organization of Economic Co-operation and Development
PLT	Platelet count
RBC	Red blood cell
SPSS	Statistical Package for Social Science
WBC	White Blood Cell
WHO	World Health Organization

Abstract

Plant medicine, is the oldest form of health care known to mankind. Syzygium guineense *is one of the many species of Ethiopian medicinal plants which have a long history of use as remedies for various ailments such as dysentery, diarrhea and hypertension. In most countries, herbal medicines and related products are introduced into the market without any mandatory safety or toxicological evaluation. Therefore this study was conducted to investigate the histopathological effect of hydromethanol extract of S.* guineense *on liver and kidney; and blood parameters of rats. For acute toxicity study the rats were randomly divided into three groups (n=4). The control group received distilled water, while the experimental groups, received 2000 mg/kg and 5000 mg/kg 80% methanol extract of* S.guineense *leaves orally. For subacute toxicity study the rats were randomly divided into three groups (n=6). The control group received distilled water, while the experimental groups, received 500 and 1500mg/kg 80% methanol extract of S.guineense leaves orally for 28 days. At the end of the experiment, blood samples and liver and kidneys were collected for gross pathology, hematology, clinical chemistry and histopathology evaluation. In the acute toxicity study rats treated with 2000 and 5000mg/kg showed no toxicological signs observed on behavior, gross pathology, and body weight of rats. In the subacute toxicity study rats have showed no significant changes on behavior, gross pathology, body weight, hematological and biochemical parameters except glucose ($p < 0.05$) as compared with the control group. There were no significant differences in the gross and histopathology of the liver and kidneys of experimental animals in extract exposed groups and their counterpart control. These results show that hydromethanol extract of S.* guineense *did not produce adverse effects in treated rats after acute and subacute treatment. Reduction in serum blood glucose level may indicate its therapeutic values, but further detailed studies should be carried out to recommend its therapeutic use.*

Keywords*:* Syzygium guineense*, medicinal plant, toxicological evaluation*

1. Introduction

1.1 Background of the study

Plant medicine, is the oldest form of health care known to mankind. Herbal medicine/plant medicine flourishes today as the primary form of medicine for perhaps as much as 80% of the world's population (WHO, 2002). Usually, a specific part of the plant (root, leaves, fruit, flowers, and seeds) is formulated into a suitable preparation, for example, compressed as tablets or made into pills, used to make infusions (teas), extracts, tinctures, ointments, or creams. Many medicines commonly used today are of herbal origin. Indeed, about 25% of prescription drugs contain at least one active ingredient derived from plant material (Bashar and Omar, 2011**)**.

Plants have been selected and used empirically as drugs for centuries, initially as traditional preparations then as pure active principles, with this knowledge and accumulated practice passing from generation to generation (Taylor *et al,* 2001).

The identification of plants useful to human beings from natural stands commenced in prehistoric times. Experiments and trials were the two main ways through which humans have learnt the various uses of plants. The use of plant resources for medicinal and other purposes is one of a number of practices developed by ancient people. Plant derived medicines are used in all civilizations and cultures and, hence, plants have always played a key role in health care systems worldwide. In most developing countries, the indigenous modes of herbal treatment are a parts of the culture and the dominant method of healing therapy. These remedies, with a considerable extent of effectiveness, are socially accepted, economically viable and, mostly, are the only available source (Thadani, 2006).

Traditional medicine is Africa's culture, future and heritage because the region has a rich bio-resource base: about 6,377 plant species are used in tropical Africa, more than 4,000 of these as medicinal plants. It is estimated that 90% of traditional medicine in Africa is plant-based (WHO, 2003).

The various literature available show the significant role of medicinal plant in primary health care delivery in Ethiopia where 70% of human and 90% of livestock population depend on traditional medicine like many developing countries particularly that of Sub-Saharan African

countries. Endemic medicinal species restricted to Ethiopia are of primary concern to Ethiopia and to the world as well and thus need serious attention (Endashaw, 2007).

The Ethiopian Flora is estimated to consist of between 6000 – 7000 species distributed in about 245 plant families. Although the exact number is still unknown, a large number of the species, i.e., about one-third of the families, have been employed in traditional medicinal practices (Tadesse and Demissew, 1992).

Many species of Ethiopian medicinal plants have a long history of use as remedies.The Ethiopian traditional medical system is mainly a subcategory of the African traditional medical system with some influence from Egypt and Greece and has its own characteristic features. Ethiopian traditional life is painted with the hallmark of widespread use of traditional medicinal plants with various levels of sophistication within the indigenous medicinal lore. It is blended with religious thinking and various beliefs and need further investigation (Endashaw, 2007).

1.2 Toxicological studies

Toxicology can be defined as branch of science that deals with poisons, and a poison can be defined as any substance that causes a harmful effect when administered, either by accident or design, to a living organism. Broader definitions of toxicology, such as "the study of the detection, occurrence, properties, effects, and regulation of toxic substances," although more descriptive, do not resolve the difficulties. Toxicity itself can rarely, if ever, be defined as a single molecular event but is, rather, a cascade of events starting with exposure, proceeding through distribution and metabolism, and ending with interaction with cellular macromolecules (usually DNA or protein) and the expression of a toxic end point. This sequence may be mitigated by excretion and repair. Poison is a quantitative concept, almost any substance being harmful at some doses but, at the same time, being without harmful effect at some lower dose. Between these two limits there is a range of possible effects, from subtle long-term chronic toxicity to immediate lethality (Ernest, 2004).

The large array of toxic chemicals produced by plants (phytotoxins), usually referred to as secondary plant compounds, are often held to have evolved as defense mechanisms against herbivorous animals, particularly insects and mammals. These compounds may be repellent but not particularly toxic, or they may be acutely toxic to a wide range

of organisms. Many chemicals that have been shown to be toxic are constituents of plants that form part of the human diet (Ernest, 2004). Therfore the aims of this study is to assess the toxic effects of leaves of *S. Guineense* on liver and kidney of the rats.

1.3 *Syzygium guineense*

*Syzygium guineense (*Willd) D.C (*Myrtaceae*), Vernecular name 'Dokima" (Amharic) and in English as "water berry" is a medium sized or tall ever green tree 15-30m high with edible fruits. It is widely distributed in sub Saharan Africa, from Senegal to Eritrea, Ethiopia, Somalia Zaire, Rwanda, Zambia, Malawi, Zimbabwe, South Africa and others (Teketay *et al*., 2002, Abou *et al.,* 2005). *S. guineense* (*Myrtaceae*) is an odorous species native of the wooded savannahs and tropical forests of Africa. It is one of the ten most recorded tree species in the Bonga and Boginda forest in Ethiopia (Sisay, 2008).

S. guineense is included among the African plant species that are active against malaria. In southern Uganda it is widely used for malaria (Ssegawa and Kasenene, 2007). Traditionally, many morphological parts of this plant have been utilized in the management of various ailments in many Ethiopian communities. For example, oral administration of its fruits and bark are used for the treatment of dysentery and diarrhea and infusion prepared from its leaves, fruits or bark is used for treatment of hypertension (Abebe *et al.,* 2003).

Its main uses are as charcoal, timber, tool handles, food, medicine, fodder, bee forage and tannin/dye (Guinand and Lemessa, 2000). Published reports in Tanzania indicate that the methanol extract of the bark has a hypotensive effect (Malele *et al.,* 1997). Moreover, the plant is endowed with antibacterial effects against many species of bacteria. Its bark is used in traditional medicine to treat gasto-intestinal upsets and diarrhea (Tsakala *et al.,* 1996, Hamil *et al.,* 2000, Oluwolé *et al.,* 2002). In Cameroon, the wood of *S. guineense* is used as fuel for the household, for construction and for carpentry (Matig *et al.,* 2006). Oral administration of twigs and leaves are used against hookworm and leaves against amenorrhea and madness. *S. guineense* sap yields a black dye used to color textiles. The antibacterial properties of the watery extract of fruits and bark of *S. guineense* have been demonstrated on different strains of bacteria responsible for diarrhea (Mukherjee *et al.,* 1998, Ashebir and Ashenafi, 1999). Ethanol (EtOH) extracts of the stem bark of *S. guineense* showed molluscicidal activities and cardiovascular properties, mainly the reduction of blood pressure (Oketch *et al.,* 1998). Other biological properties such as anti-

inflammatory, analgesic and immunological activities of different part of *S. guineense* have been reported (Djoukeng *et al.,* 2005, Parakashtha *et al.,* 2010).

The chemical composition of *S. guineense* leaves includes pectic polysaccharides and two immunologically active polysaccharide fractions such as arabinogalactan type II polysaccharide, called Sg50A1, other polysaccharide fraction is a mixture of oligosaccharides of the pectic type, called Sg50A2 (Ghildyal *et al.,*2010). Its wild, oval fruits are edible with high concentrations of Ca, Mg, Fe, K and P (Saka and Msonthi, 1994, Ambé*.,* 2001). Antibacterial activity of triterpenes isolated from fruits and bark of *S. guineense* has been demonstrated (Djoukeng *et al.,* 2005). Phytochemical screening of the plant revealed that *S. guineense* leaf extract contain flavonoids, tannins, saponins and carbohydrate. Alkaloids and cardiac glycosides are also present. Furthermore; another finding on phytochemical composition of *S. guineense* extracts are proteins, lipids, polyphenols, steroids ; except for the Ethanol/ water extract of the bark, which contains more coumarins. The result indicates that the bark has the highest level of polyphenols. These phytochemical constituents are physiologically active compounds possessing great potential for therapeutic and prophylactic uses (Ior *et al.,* 2012, Moukette *et al.,* 2014).

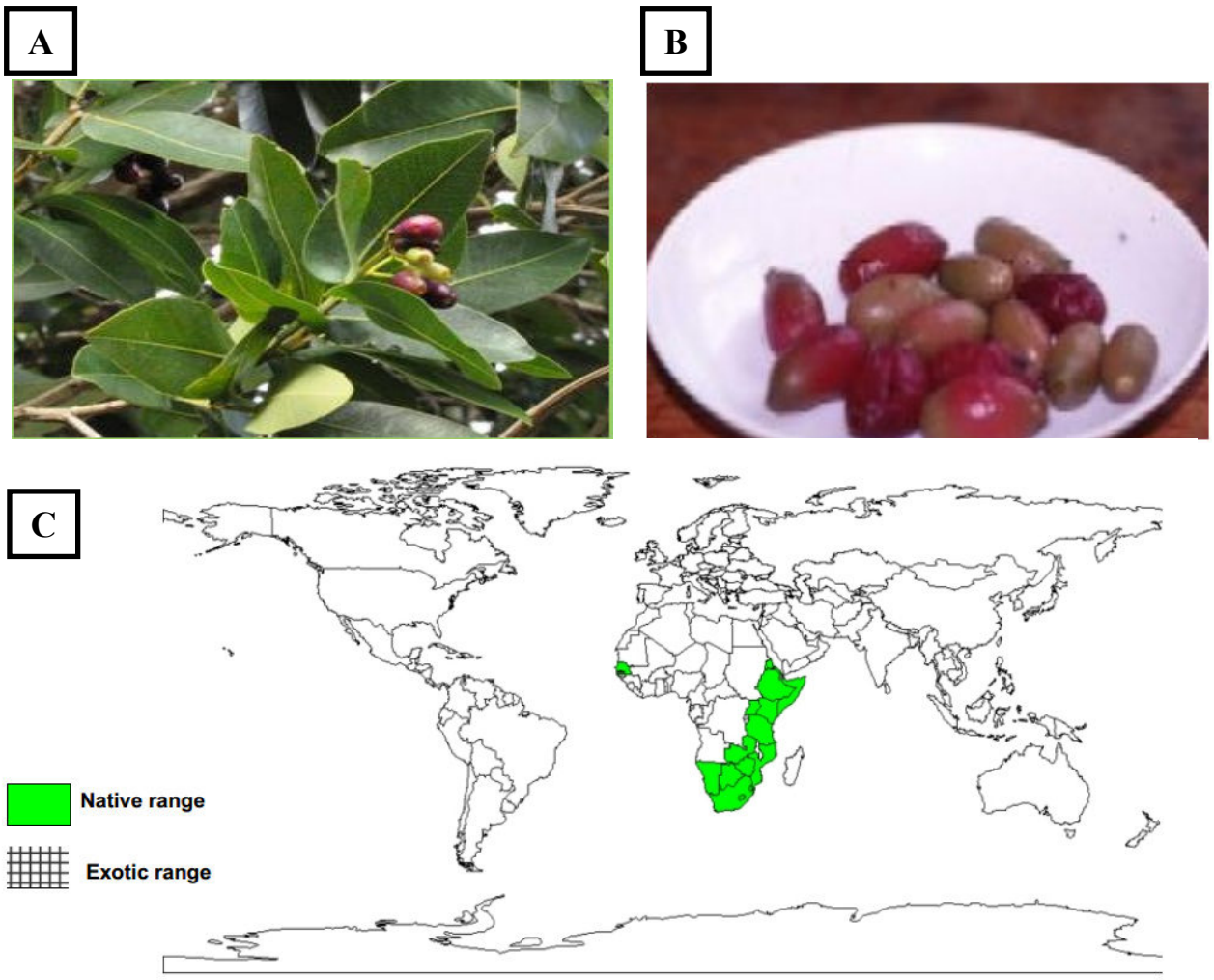


Figure 1: A and B, Photograph showing leaves and seeds of *S. guineense* respectively and C, map showing *Syzygium guineense* distribution around the world (Orwa et al. 2009, http://www.plantzafrica.com/plantqrs/syzygiumguin.htm)

1.4 Literature review

The study conducted in Nigeria on anti-venom studies of *Olax viridis* and *Syzygium guineense* extracts studied against *Naja katiensis* venom rats reveal that plant extracts were administered orally at the dose of 400 mg kg-1 b.w of rats and 1 h later, the venom (0.08 mk kg-1) and has no significant change in blood glucose level in those group taking only extracts of *O. viridis* and *S. guineense* without envenomation (there was no significant increase in their blood glucose before and after envenomation). The lipid profiles (triglyceride and cholesterol) were increased by the plant extracts. Creatine Kinase activity showed significant reduction of activities of the enzyme administered with S. Guineense administration (Omale *et al.,* 2013).

Another study conducted in Cameroon on *Syzyguim guineense* extracts show antioxidant activities and beneficial activities on oxidative stress induced by ferric chloride in the liver homogenate show that the extract of the leaves has the best protective activity against lipid peroxidation compared to the other samples and vitamin C used as a positive control. The inhibition of lipid peroxidation by the ethanol leaf extract is significantly higher than that of methanol extract of the same part of the plant, 40 fold higher than the negative control (Moukette *et al.,*2014). The study on anti-Inflammatory and analgesic activities of the ethanolic extract of the leaf of *S. Guineense* in rats and mice in Nigeria showed that the intraperitoneal LD_{50} of the ethanolic extract of *S. guineense* was found to be 3.807 g/kg. Intraperitoneal administration of concentrations of 500 mg/kg and 1000 mg/kg the extract was found to possess significant ($P<0.05$) analgesic effects on the hot plate model, but only the concentration of 1000 mg/kg possessed significant ($P<0.05$) anti-inflammatory and analgesic effects on the writhing test. Lower concentration of 200 mg/kg possessed insignificant ($P>0.05$) anti-inflammatory and analgesic effects. (Ior *et al.,* 2012).

The study conducted on antimalarial activity of 80 % methanol extract of the stem bark of *Syzygium guineense* (Willd.) DC. (Myrtaceae) in mice infected with *Plasmodium berghei* indicated that the acute toxicity study caused no mortality, at 2000mg/kg dose within the first 24h as well as on the following 14 days. Physical and behavioral observations of the experimental mice also revealed no visible signs of overt toxicity like lacrimation, loss of appetite, tremors, hair erection, salivation and diarrhea (Zeleke, 2015). Another study by Nigatu,

(2004) showed that the LD_{50} of Leaf tips aqueous extracts, twigs 80% methanolic extracts, stem bark aqueous extracts, stem bark 80% methanolic extracts, and fruit 80% methanolic extracts were 14.10, 2.91, 5.12, 8.77 and >10.0g/kg respectively.

Study done on oral treatment of the aqueous leaf extract of *S. guineense* at dose of 200, 400 and 600mg/kg bw daily for six weeks with investigations in the liver and kidney tissue sections of the mice showed morphological changes at the highest doses of the extract. The liver revealed hemmorhegic centrilobular necrosis and cytoplasmic vacoulation of the hepatocytes: and in the kidney tubulointerstitial inflammation, shrinkage and congestion of the glomerulus were observed at higher doses. Hematological investigations showed lowered Hgb concentrations that might indicate the development of mild anemia. Decreased body weight gain and increased organ weights of the liver and kidney were observed in contrast to the control (Amare, 2009)

The study conducted in investigation of antihyperglycemic and hypoglycemic activity of Ajuga remota and *Syzygium guineense* on mice in Ethiopian Public health institute showed significant reduction in blood glucose level by aqueous extract on alloxan induced diabetic mice. Results of the present study on the hydro alcoholic extract of *S.guineense* did not show any significant reduction in blood glucose level of both normoglycemic and alloxan induced hyperglycemic mice. However, the aqueous extract reduced the blood glucose level of both normoglycemic and diabetic mice at all doses tested i.e. 50,100 and 200mg/kg in time dependent manner starting from half an hour after treatment. Acute toxicity study revealed the non-toxic nature of the crude aqueous extract of *S. guineense*. No mortality or toxic reactions were found up to 6000mg/kg body weight (Worku, 2009).

The study conducted in Brazil to evaluate toxicity of *Syzygium cumini* leaves in rodents show that no sign of acute toxicity to rats and mice, at doses up to 2 and 6 g/kg respectively, or death up to the 14^{th}day of observation, even at doses above 5 g/kg when administered orally. Mortality were observed after the intraperitoneal administration of the extract in mice, causing death in 70% of the animals at a dose of 0.5 g/kg, and 100% when using a dose of 1 g/kg (LD_{50} 0.489 g/kg). When administered intraperitoneal at the highest dose (2 g/kg), the extract caused 67% death in rats. The intraperitoneal route, although not used in humans, was selected to determine

the inherent toxicity of extract, since the effects of an oral dose are subject to systemic bioavailability and hepatic extraction. It was found that the administration at doses of 0.05, 0.1 and 0.25 g/kg did not interfere with weight gain in the rats nor in the feed consumption of the animals for 90 days. No significant difference in body weight gain was noted between the control and the treated groups with 0.05 and 0.1 g/kg at 180 days of treatment. However, the male rats group dealt with the highest dose (0.25 g/kg) of *S. cumini* did not have gain of body weight from 15thweek until the end of the treatment (Silva *et al.,* 2012).

The serum levels of urea from the animals treated at doses of 0.05, 0.1 and 0.25 g/kg were reduced by 19.4, 13.8 and 18.4%, respectively, after 30 days, while creatinine showed a reduction of 15.4% with a dose of 0.25 g/kg, when compared to the control group. Regarding the investigation of hepatic function, in this work the only change observed was an increase in the serum levels of alkaline phosphatase, at the higher dose in the 30-day treatment, which occurred of isolated form and was not followed by any alteration in the value of the transaminase in any of the groups treated. There was no change in the serum concentration of glucose in animals treated for 30, 90 and 180 days with the *Syzygium cumini*. In rats treated for 180 days, no differences in the serum levels of ALP, ALT, AST, BUN, total protein, albumin, creatinine, glucose and Ca^{2+} was found between extract-treated groups and the control groups. It was found that the levels of HDL, triacylglyceride and cholesterol of extract-treated groups were significantly lower than the control group. With reference to the hematologic parameters evaluated, the group treated for 180 days did not show any alteration treatment-related effects when compared to the control group. Microscopic examination of the selected organs (liver, lungs, kidneys, stomach, intestine, heart and pancreas) did not reveal any treatment-related effects (Silva *et al.,* 2012).

Another study conducted on islet regenerative potential of purified fraction of *Syzygium cumini* seeds *on* streptozotosin (STZ) induced diabetic mice showed that lethal concentration (LC_{50}) analysis SC2 (active constituent of *S. cumini*) of fraction was 12.33mg/ml. The total dosage of 2.1 mg of SC2 fraction was administered to mice within 21 days which is very minimal to show any toxicity. SC2- treated mice showed sustained reversal of in experimental diabetes as evidenced by restoration of of normoglycemia, increase in glycolytic enzyme glucose – 6 – phosphate- dehydrogenase (G6PD) and hepatic and muscle glycogen along with increase in plasma insulin and C- peptide levels. Glycogen contents of diabetic mice were significantly lower than those of the normal control mice ($p<0.01$) (Menakshi and Bimba, 2011).

1.5. The blood

Blood is a specialized connective tissue in which cells are suspended in fluid extracellular material called plasma. Propelled mainly by rhythmic contractions of the heart, about 5 L (in human) of blood in an average adult moves unidirectionally within the closed circulatory system. The so-called formed elements circulating in the plasma are erythrocytes (red blood cells), leukocytes (white blood cells), and platelets (Junqueira and Carneiro, 2012). Total blood volume of an average normal adult rat is about 5.6 - 7.1 ml/100g of body weight. The RBCs are the smallest and most numerous cells of blood, the number of circulating erythrocytes in the blood of normal adult rat is about 7-10 x 10^6/µl. The reference value of hemoglobin (HGB) in adult rat measures about 11-19.2g/dL and that of hematocrit value (HCT) is about 35 - 64% (Pass and Freeth, 1993).

In normal adult rat the reference values for RBC indices such as mean corpuscular HGB (MCH) and mean corpuscular HGB concentration (MCHC) are 14.3 – 19.5 pg and 26.2 – 40 g/dL respectively (Pass and Freeth, 1993). The number of leukocytes is much smaller than that of RBCs; in fact, in a healthy adult rat there are only 6 to 18 x 10^3 white blood cells per µL of blood (Pass and Freeth, 1993). Unlike erythrocytes, leukocytes do not function within the bloodstream but use it as a means of traveling from one region of the body to another. They have diversified functions, and are one of the body's chief defenses against infection. According to the type granules in their cytoplasm and the shape of their nuclei, leukocytes are divided as granulocytes (include the neutrophils, eosinophils, and basophiles) and agranulocytes (include lymphocytes and monocytes). The normal reference values of neutrophils, eosinophils, basophiles, lymphocytes and monocytes are 1.95 – 3.38x10^3/mm^3, 0.03 - 0.08x10^3/mm^3, 0.01 - 0.03x10^3/mm^3, 6.03 – 9.12x10^3/mm^3 and 0.01 – 0.04 x 10^3/mm^3, respectively (Pass and Freeth, 1993; CRL, 1998 and Delaney, 2008).

Platelets are small, disk–shaped, non-nucleated cell fragments derived from megakaryocytes in the bone marrow (Gartner and Hiatt, 2007). Platelets promotes blood clotting and helps repair gaps in the walls of blood vessels, preventing loss of blood (Junqueira and Carneiro, 2012). There are about 500 - 1,300 x 10^3/µL platelets in healthily adult rat blood (pass and Freeth, 1993).

Plasma is a yellowish fluid in which cells, platelets, organic compounds, and electrolytes are suspended and /or dissolved (Gartner and Hiatt, 2007). It transports nutrients from their site of absorption or synthesis and distributes them to various area of the organism. It also transports metabolic residues to the excretory organs. Plasma is obtained from blood after treatment with an anticoagulant. In contrast, serum is obtained from clotted blood from which serum biochemical parameters (Glucose, Urea. creatinine, total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) and others) are studied to test renal and hepatic function (Krause, 2005, Junqueira and Carneiro, 2012). Rats' serum is also composed of similar components as human serum (Pass and Freeth, 1993; Delaney, 2008).

Glucose is derived from the breakdown of carbohydrates in the diet or in body stores (glycogen) and endogenous synthesis from protein or from the glycerol moiety of triglycerides (Tietz, 2000). The reference value of serum glucose in normal adult rat is about 50-120 mg/dl (pass and Freeth, 1993; CRL, 1998, and Delany, 2008). Urea is formed by catabolism of proteins and amino acids; it is primarily cleared from the body by the kidney (Tietz, 2000). In normal adult rat serum is measured about 15- 45 mg/dl (Pass and Freeth, 1993; CRL, 1998 and Delaney, 2008). Creatinine is synthesized in the kidney, liver and pancreas; its renal clearance is used as diagnostic indicator of kidney function. The normal reference value of creatinine in normal adult rat about is 0.2-0.8 mg/dL (Delaney, 2008). Protein is a nutrient normally broken down by the liver and its enzymes. Total protein in the serum of normal adult rat is about 5.6-7.6 mg/dL (Pass and Freeth, 1993). Liver enzymes such as AST, and ALT, can be very useful in evaluation of liver disease and injury. ALT is found primarily in the liver. AST is found primarily in the red blood cells, cardiac and skeletal muscle and kidney. The normal reference values of AST, and ALT in normal adult rat serum are 45.7-80.8 IU/L, and 17.5-30.2 IU/L respectively (Pass and Freeth, 1993; CRL, 1998, and Delaney, 2008).

However, normal values for some controls and some treated animals sporadically varied from the reference ranges. In such cases, the reference ranges do not replace the need for control animals, comparison of mean values of treated animals were made with mean values of control. Normal reference values may be affected by food intake, age, sex, species, and environmental factors (CRL, 1998 and Dirikolu, *et al.,* 2011).

1.6 The liver

The liver is the largest internal organ, in adult human being average weight of about 1.5 kg or 2% of the body weight. Located in the right upper quadrant of the abdomen just below the diaphragm the liver has major left and right lobes with two smaller inferior lobes, most of which are covered by a thin capsule and mesothelium of the visceral peritoneum. The capsule thickens at the hilum (or porta hepatis) on the inferior side, where the dual blood supply from the hepatic portal vein and hepatic artery enters the organ and where the hepatic vein, lymphatics, and common hepatic (bile) duct exit (Junqueira and Carneiro, 2012). The rat liver is a large multilobulated gland occupying the anterior third of the abdominal cavity. In rats, the liver mass represents approximately 5% of the total body weight. The mean weight is 6-15 g and its transverse diameter measures from 7.5 to 8.0 cm. The superior- inferior diameter measures from 3.8 to 4.2 cm, while the anterior-posterior ranges from 2.2 to 2.5 cm (Martins and Neuhaus, 2007).

The rat liver has 4 lobes, like the human liver, and they are named after the portal branches that supply them. The middle or median lobe (ML) is the largest, and is in continuity with the left lateral lobe (LLL). It is subdivided by main fissure (MF) into a large right median lobe (RM) and into a smaller left medial lobe (LM). The right lateral lobe (RLL) is located on the right of the inferior vena cava (IVC) and is almost covered by the ML; and is divided by a horizontal fissure into the superior right lobe (SRL) and inferior right lobe (IRL). The LLL lies over the anterior surface of the stomach; and its medial portion covered by the part of the median lobe and has no fissure. The caudate lobe (CL) is behind the LLL and the left of the IVC. It is divided into the *caudate process* (CP), which has an anterior caudate (AC) and posterior caudate (PC) portions. The AC is anterior to the esophagus and stomach, while PC is located behind these structures. Rats do not have a gall bladder which is used for storing bile in other animals (Martins and Neuhaus, 2007). The surface of the rat liver covered by a thin serosa from which fine strands of reticular connective tissue project inward to from the supporting frame work for hepatic cells, blood vessels, and bile ducts (Cook, 2008). The rat liver is shown in figure 2 below.

A **B**

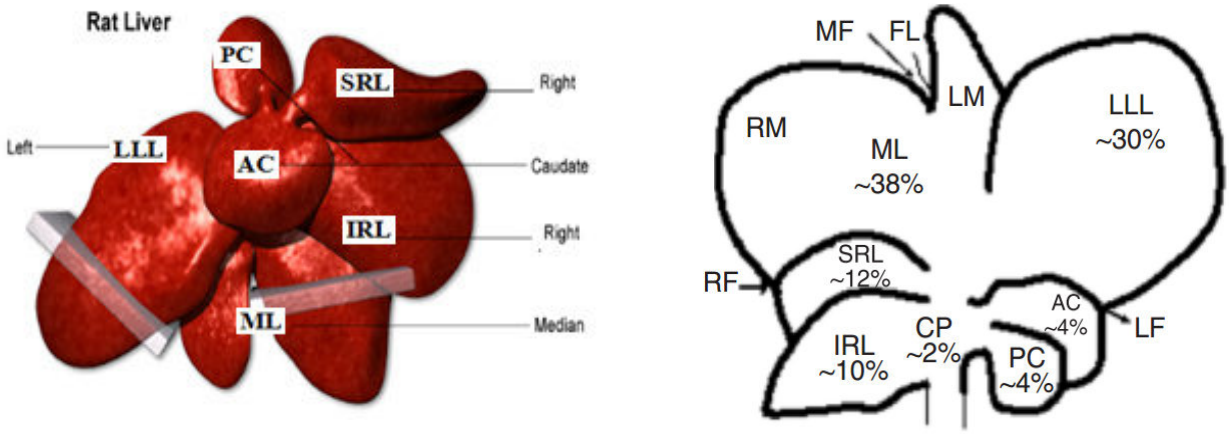


Figure 2; A. Rat liver *in situ*, (A), visceral aspect. (B) Visceral (inferior) view showing approximate percentages of total liver weight for each lobe. *CP, caudate process; AC, anterior caudate lobe: PC, posterior caudate lobe; SRL, superior right lobe; IRL, inferior right lobe; ML, median lobe; RM, right median lobe; RML, right portion of the median lobe; LML, left portion of the median lobe; LLL, left lateral lobe; MF, median fissure; LF, left fissure; RF, right fissure and FL, falciform ligament* (Fehlert *et al.,* 2003 and Martins, *et al.,* 2007).

Despite the gross differences between the liver of humans and rats, their microscopic features are more or less similar. The liver is structurally organized into polygon-shaped lobules surrounded by connective tissue. This lobular organization is most easily appreciated in sections of rat liver. The center of lobule is central vein cords of hepatocytes and the intervening sinusoids extend radially between the central vein and the periphery of the lobule. Portal triads, consisting of branches of a portal vein, a hepatic artery and a bile duct/s, are found at the apices of the lobule (Demetris, 2008).

The hepatic parenchymal cells, in rat liver, are large and polygonal with large central nuclei (sometimes two in a cell) and one or more nucleoli. The cytoplasm is extremely variable in appearance; may be granular, vacuolated, deep staining, or very pale (Cook, 2008, Demetris, 2008).

Liver sinusoids conduct nutrient/hormone-rich portal venous and arterial blood slowly past the hepatocytes. The sinusoids are constructed by Kupffer's cells (that perform phagocytosis)

fenestrated endothelium (that allow easy passage of nutrients), microvilli of hepatocytes (increase the surface area available for exchange and absorption of substance), and space of Disse (a potential space contains several different cell types). The cell present in the space of Disse include Ito cells (which manufacture and secrete a number of important hepatic growth factors and matrix components), and pit cells (also known as natural killer cells, are active against viruses, and tumor cells) (Demetris, 2008).

The hepatocytes synthesize bile and secret it from the cell into the bile canaliculi. From the hepatic cords, the bile flows towards the portal triad at the periphery of the hepatic lobules (Duker, 2007). The origin and course of the major vessels, supplying the rat liver are similar to those of humans (Martins and Neuhaus, 2007). Most of liver blood (70-80%) comes from the portal vein, arising from the stomach, intestines, and spleen; the smaller percentage (20-30%) is supplied by the hepatic artery (Krause, 2005).

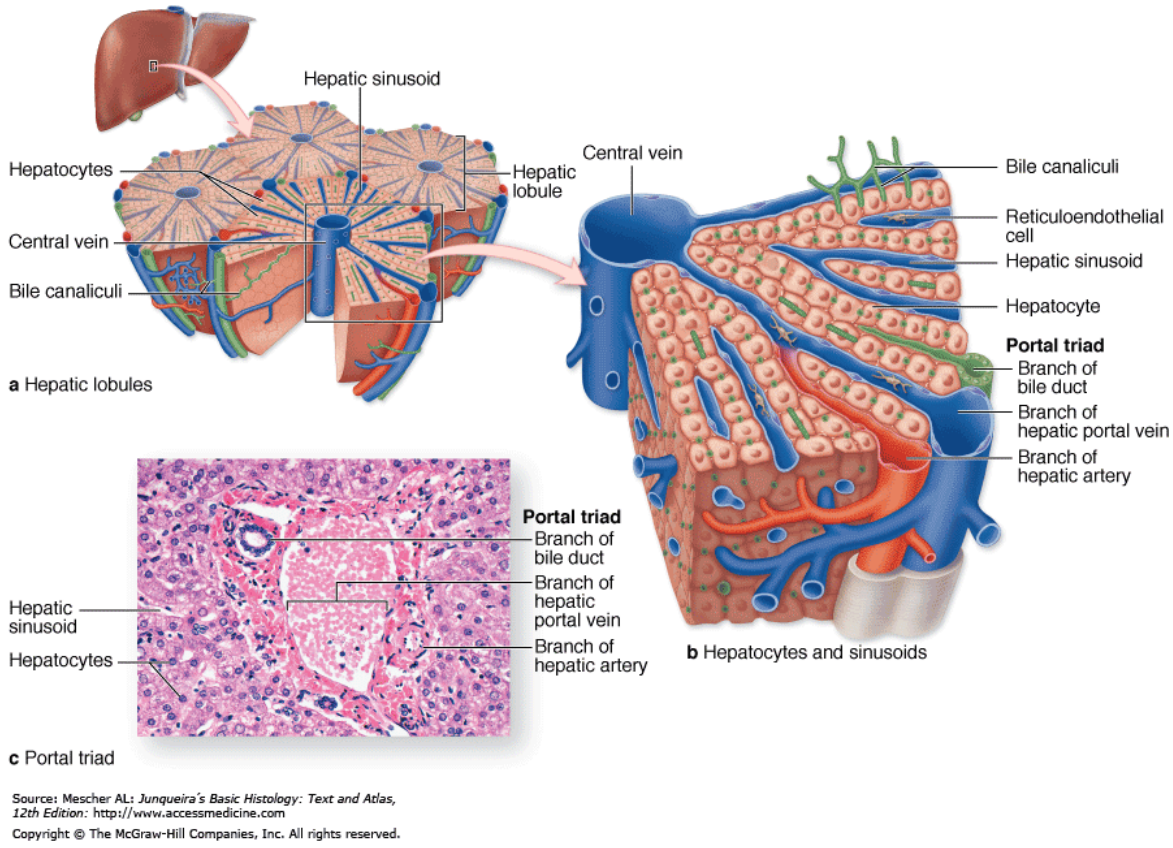


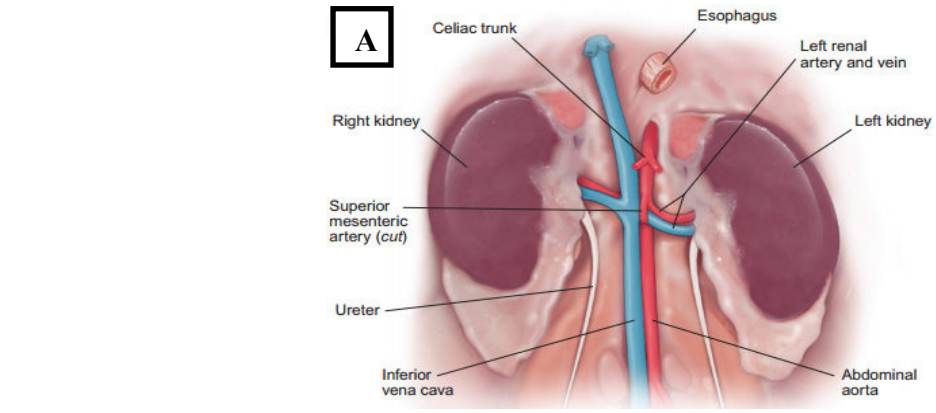
Figure 3: Diagram of human liver (a) central vein and portal triad. (b): Sinusoids, which run between plates of hepatocytes and drain into the central vein. (c): Micrograph showing components of the portal triad. X220. H&E. (Junqueira and Carneiro, 2012)

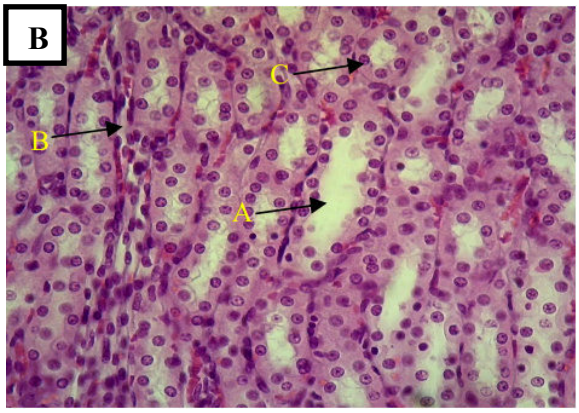
1.7 The kidney

The kidneys are a pair of organ that clear the blood plasma of metabolic wastes, regulate fluid osmolality and volume, maintain electrolyte balance, eliminate foreign chemicals, and help maintain the acid–base balance of the body. In addition, to these, the kidneys have properties of endocrine organs (Gartner and Hiatt, 2007). The rat kidneys are bean-shaped organs that lie in a retroperitoneal position against the posterior abdominal wall, one on either side of the upper lumbar vertebrae. They are not attached to the body wall, but are held loosely in place by adipose tissue. The kidney is dorsoventrally flattened and has an extensive convex lateral and a short concave medial border. The concavity is the hilus blood vessels and the ureters join the kidney (Cook, 2008 and Sowash, 2009).

Each kidney has three major components: the cortex, the medulla, and the collecting system. In the hemi-sectioned kidney the outer pale region is the cortex, which has a granular appearance. The medulla is series of pyramidal structures. Cortical parenchyma extends into spaces between adjacent pyramids and is known as the columns of Bertin. A medullary pyramid with both columns of Bertin as well as the sub-capsular cortex constitutes a renal lobe. The collecting system consists of the renal pelvis (Fogo *et al.,* 2006)

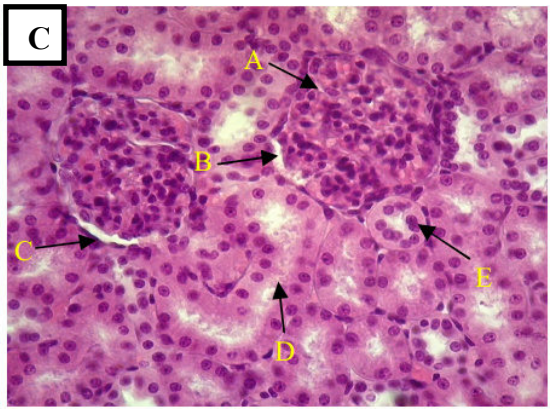
Each pelvis has two or three major branches known as the major calyces. Each major calyx divides further into three or four smaller branches known as minor calyces, each usually receiving one medullary papilla (Fogo *et al.,* 2006). In humans, the medulla is divided into 8 to 18 renal pyramids. The base of each pyramid is positioned at the corticomedullary boundary, and the apex extends toward the renal pelvis to form papilla. In contrast to the human kidney, the rat has a single renal pyramid and is therefore termed “unipapillate.” The unipapillate kidney of the rat is directly surrounded by the renal pelvis. Otherwise, the rat kidneys resemble the human kidney in its gross and microscopic appearance (Webster *et al.,* 1947; Fogo *et al.,* 2006*;* Sowash*,* 2009*).* The rat kidney is shown in figure 4 below.





A-Collecting tubule…B-Thin limb…

C- Thick descending limb. (400 × H & E)



A- Glomeruli. B- Visceral layer.

C- Parietal layer. D- Distal tubule

E- Proximal tubule. (400 × H & E).

Figure 4. A, Diagram of the rat kidneys, B and C histological observations of rat kidney (Piper *et al*., 2012 and Al-Samawy, 2012).

The functional unit of the kidney is the nephron. Each human kidney contains 0.6 to 1.4 X 10^6 nephrons, which contrasts with the approximately 30,000 nephrons in each adult rat kidney. Each nephron is a tubule with a widened end, Bowman's capsule, enclosing the glomerulus. The average diameter of the glomerulus is approximately 200µm in the human kidney and 120µm in the rat kidney. However, glomerular number and size vary significantly with age and gender as well as birth weight. The average glomerular volume has been reported to be 3 to 7 million mm^3 in humans and 0.6 1 million mm^3 in the rat. In the rat, juxtamedullary glomeruli are larger than

glomeruli in the superficial cortex. This is not the case in the human kidney (Webster *et al.,* 1947; Gartner and Hiatt, 2007).

The glomeruli are spherical collection of interconnected capillaries (tuft of blood capillaries) within a space known as Bowman's/urinary space, which is lined by flattened parietal cells (composed of simple squamous epithelial cells). The Bowman's space is continuous with the proximal convoluted tubules at the urinary pole. The region where the vessels supplying and draining the glomerulus enter and exit Bowman's capsule is known as the vascular pole. The outer aspect of the glomerular capillaries covered by a layer of visceral epithelial cells or podocytes (composed of modified epithelial cells). Each visceral epithelial cell has a large body containing the nucleus and cytoplasmic extensions, which divide; forming small finger-like processes that interdigitate with similar structures from adjacent cells cover the capillaries. These interdigitating processes are known as pedicles/foot processes (Webster *et al.,* 1947*;* Durotoye and Gartner and Hiatt, 2007).

The space between adjacent foot processes is known as the filtration slit; adjacent foot processes are joined together by a thin membrane known as the slit-pore diaphragm. Filtrate leaking out of the glomerulus enters the Bowman's space through a complex filtration barrier composed of the endothelial wall of the capillary, the basal lamina, and the visceral layer of Bowman's capsule. The glomeruli are supported by mesangium, which represents the intraglomerular continuation of connective tissue component of the afferent arteriole (Gartner and Hiatt, 2007).

The remaining portion of the nephron is divided into proximal convoluted tubules (PCT), the loop of Henle and the distal convoluted tubule (DCT). The PCT is composed of the cuboidal epithelium, is very tortuous and longer tubule than DCT. The cell apex has abundant long microvilli which form a permanent brush border for reabsorption (Junqueira and Carneiro, 2012). The loop of Henle is a U-shaped structure with a descending limb and an ascending limb, both composed of simple epithelia, cuboidal near the cortex, but squamous deeper in the medulla. The DCT is composed of simple cuboidal cells, which are flatter, smaller and have no brush border as cells of PCT. The initial, straight part of the distal tubule makes contact with the vascular pole of the renal corpuscle of its parent nephron and forms part of a specialized structure, the juxtaglomerular apparatus (JGA). At the point of contact with the arterioles, the cells of the distal tubule become columnar and more closely packed and is called as the macula

densa. Adjacent to the macula densa, the tunica media of the afferent arteriole is also modified into secretory cells known as juxtaglomerular cells. Also at the vascular pole are lacis cells, which have many of the supportive function as cells inside the glomerulus (Fogo *et al*., 2006; Gartner and Hiatt, 2007).

2. Significance of the study

Plants are the oldest known source for human and livestock health care, and an important source of global biodiversity. They produce a diverse range of bio-active molecules, making them a rich source of different types of medicine (Gugu, 2009). With the increasing use of medicinal plants, several concerns regarding the safety and quality of the plant medicines have been observed.

Hence it has become necessary to standardize the safety and quality assurance measures so as to ensure supply of medicinal plant materials of good quality (Thadani, 2006). One of the important components of this study was scientific evaluation of safety and toxicity of *S. guineense.* The outcome of this study may fill the gap of the previous studies on *S. guineense* and also it is hoped to provide some additional evidence for recommending further studies to assess toxicity profiles associated with the use of herbal preparations of this plant.

3. Objectives of the study

3.1 General objective

- To investigate the acute and subacute histopathological effect of leaves of crude 80% methanol extract of *Syzygium guineense* on liver and kidney; and blood parameters of rats.

3.2 Specific objective

- To determine effect of *S. guineense* on body and organ weight of rats.
- To observe the effect of *S. guineense* on general behavior and gross lesion of liver and kidney of rats.
- To estimate the median lethal dose (LD_{50}) of 80% methanol leaf extract of *S. guineense.*
- To asses histopathological effect of *S. guineense* on liver and kidney of rat.
- To evaluate effect of *S. guineense* on blood parameters of rat.

4. Method and materials

4.1 Study Design:

Laboratory based experiment was conducted to evaluate toxic effect of *Syzygium guineense* leaves on liver and kidney; and blood parameters of rats. A total of 18 rats of both sex containing 6 rats per group were used for subacute study and test substance (80% methanol extract of *S. guineense* leaves) was administered for 28 days, at doses of 500 (I) and 1500 (II) mg/kg body weight orally and control group (III) was given distilled water. Whereas, 12 female rats containing four female rats per group were used, which was grouped into two experimental ($Group_1$- 2000 mg/kg & $Group_2$- 5,000 mg/kg) and one control group were used for acute toxicity study (14 days), (OECD 1995, and OECD 2001).

4.2 Location of the study

The study was conducted in the Addis Ababa University department of Anatomy and Traditional and Modern Medicine Research Directorate of Ethiopian Public Health Institute (EPHI).

4.3 Study period:

The study was conducted from May 2015- December 2016.

4.4 Collections of plant materials

The leaves of *S. guineense* were collected from Wondogenet around Shashemene town located about 270 km south of Addis Ababa. Then the leaves were identified and authenticated by a taxonomist at EPHI.

4.5 Extraction of plant materials

The plant materials were cleaned of extraneous materials and crude hydromethanolic extract of *S. guineense* was used for the study. The powdered leaves were macerated with 80% methanol in water for 72hrs with intermittent agitation by orbital shaker DS-500. Then, the supernatant part of agitated materials was separated from the un-dissolved portion of plant. The supernatant portion was filtered with 0.1 mm^2 mesh gauze and 18.5 cm diameter Whatman grade1filter paper with pore size of 11µm. The filtrated extract was then concentrated by evaporating the solvent using a rotary evaporator (BUCHI Rota-vapor type R-205, Switzerland) under reduced pressure

at a temperature of 40 - 45 ºC**.** Then the residue was dried by steam bath at 40 ºC for period of one week to make it dry (Debella, 2002).

4.6 Preparation of experimental animals

The healthy wistar albino rats of both sex with age of 8 weeks old and above and weight of 120g – 140g were obtained from physiology department, Addis Ababa University. Healthy young adult and non-pregnant female animals, which were acclimated to laboratory conditions for 5 days and have not been subjected to previous experimental procedures, were used. The animals were housed in standard cages and kept under standard condition (at a temperature of 22°C (± 3°C), with 12hrs light / 12hrs dark cycle artificially) (OECD, 1995). They were provided with free access to standard diet and tap water *ad-libitum*.

4.7 Grouping of experimental animals

Animals were randomly assigned to a control and two treatment groups. Each animal was assigned a unique identification number. A total of 18 rats of both sex containing 6 rats per group (three female and three male) were used for subacute study. Whereas, 12 female rats containing four female rats per group were used, which was grouped into two experimental and one control group were used for acute toxicity study, (OECD 1995, and OECD 2001).

4.8 Administration of doses

The acute toxicity study was conducted according to OECD 2001/420 guideline or fixed dose procedure. According to this guideline the limit test is used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity only above regulatory limit doses. Using the normal procedure, a sighting study starting dose of 2000mg/kg (or exceptionally 5000mg/kg) followed by dosing of a further four animals at this level serves as a limit test for this guideline. Accordingly, hydromethanol extract of leaves of *S. guineense* administered for rats at a dose of 2000mg/kg and 5000mg/kg. Dose of subacute toxicity chosen based on efficacy study done previously i.e hydroalcoholic extract of *S. guineense* has analgesic and anti-inflammatory effect at dose of 500mg/kg. Accordingly, initial dose 500mg/kg was administered for group I and next dose was chosen based on OECD guideline 1995 i.e triple dose of initial was given for group II (1500mg/kg) for 28 days. And the control group was given 10ml/kg of distilled water.

4.9 Acute and subacute toxicity studies

4.9.1 Acute toxicity study

Acute toxicity test methods measure the adverse effects that occur within a short time after administration of a single dose of a test substance. This testing is performed principally in rodents and is usually done early in the development of a new chemical or product to provide information on its potential toxicity (Ernest, 2004). Acute toxicity study was conducted based on the Organization of Economic Co-Operation and Development (OECD 420) guideline (OECD, 2001). According to this guideline normal females are used, and they should be nulliparous and non-pregnant. Before conducting the experiment the animals were randomly selected and grouped into three group (n=4); and then kept in their cage for 5 days prior to dosing to allow acclimatization to the laboratory conditions. All groups of the rats fasted overnight prior to administration. Following the fasting period, all animals were weighed and the doses were calculated based on their body weight. The doses were prepared in distilled water.

The hydromethanolic extract of the leaves was then administered orally at the doses of 2000mg/kg and 5000mg/kg body weight of rats in the test groups (I and II) respectively. Control group (III) received distilled water. After administering the plant materials, the animals were kept under close observation continuously for 1 hour and intermittently for 4 hours and thereafter once every 24 hours for the next 14 days.

During these periods clinical observations were made for mortality, behavioral, neurological and any other abnormalities and their weight were measured weekly. Finally, on the 15^{th} day, their final weights were measured and gross physical examinations were carried out. The rats were then anesthetized under diethyl ether and sacrificed by cervical dislocation. After sacrificing the rats, gross pathological observation were carried out on vital organs.

4.9.2 Subacute toxicity study

Subacute toxicity study examine toxicity caused by repeated dosing over an extended period. A 28- or 90-day oral study in the rat or dog would be typical of this type of study. Such tests provide information on dose regimens of prolonged chronic studies. They are frequently used as the basis for the determination of the no observed effect level (NOEL). This value is often

defined as the highest dose level at which no deleterious or abnormal effect can be measured, and is often used in risk assessment calculations. Subacute tests are preliminary to long term study and also useful in providing information on target organs and on the potential of the test chemical to accumulate in the organism (Ernest, 2004).

The subacute toxicity study was conducted, based on the OECD 407 guideline (OECD, 1995), for 28 days to examine the toxicity of the extract on some blood parameters and histopathology of the liver and kidneys. For this study healthy adult rats of both sexes were used. Eighteen rats were randomly distributed into three groups (I, II, and III) each consisting of six rats (three female and three male) per group. Group I and II treated with hydromethanolic extract of leaves at doses of 500 and 1500mg/kg body weight per day respectively and Group III served as control group and received distilled water or vehicle. Clinical observation were carried out for 28 days and their weight was measured weekly for four weeks. On the 28^{th} day the final weight of the rats were measured and then they were anesthetized under diethyl ether and blood samples were collected from each animal by cardiac puncture.

The blood was placed in two groups of test tubes; half of the test tubes containing anti-coagulant, ethylene diaminotetraacetic acid (EDTA), and the other half without anti-coagulant. Blood samples in the test tubes containing EDTA were used to determine the hematological parameters (WBCs, RBCs, HGB, HCT, MCH, MCHC, and Platelets) using Automated Hematology Analyzer (Symex- RX, 21, Japan). Blood samples in the test tubes without anti-coagulant were allowed to clot and sera were obtained by centrifuging the blood by an electrical centrifuge (HUMAX-K, HUMAN -Germany) from which blood chemistry (glucose, Urea, Creatinine, total protein, ALT, and AST) was studied to test renal and hepatic functions. Values in the sera were analyzed using Automated Clinical Chemistry Analyzer (AUTO LAB 18, clinical chemistry analyzer Italy). After collection of blood samples, the rats were sacrificed by cervical dislocation and parts of the liver and the kidney were dissected out and gross pathological observation were performed on liver and kidney to check for any gross lesions.

4.9.3 Histopathological studies

The liver and kidney sections taken randomly for tissue processing were fixed in 10% neutral buffered formalin (NBF) overnight at room temperature. After fixation, the tissue sections were

washed with water to remove excess fixatives for about six hours and dehydrated with increased concentration of alcohol of 70% for two hours, 90% for two hours, absolute alcohol-I, II for one and half hours, and III 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, where by each tissue block was labelled and stored at room temperature till sectioned.

The tissue blocks were sectioned in ribbons at a thickness of 5 µm with Leica microtome (Leica RM 2125RT Nussloch GmbH, Germany). The ribbons of the section were collected at every 5^{th} sections and put onto the surface of a warm water bath of temperature of 40^0c. The floating ribbons over the surface of warm water were mounted onto pre-cleaned slides spread with egg albumin. The slides containing paraffin wax were arranged within the slide holder and placed in an oven with temperature of 40^0c for about 20 minutes so as to fix the tissue to the slides and allowed to cool at room temperature for 30 minutes and stained regressively with routine Harris haematoxylin for 6 minutes and then eosin for 17-20 second (H and E).

For routine H and E staining, two series of coupling jars were prepared. One for paraffin removal and hydration and the other was for dehydration and clearing. So sections were placed in xylene-I for 5 minutes and xylene II for 2 minutes again to remove the paraffin from tissue and hydrated with decreasing concentrations of absolute I, II and 95% alcohol for two minutes each, 70% of alcohol for three minutes and 50% alcohol for five minutes. The tissue sections were washed with tap water for five minutes and stained regressively with Harris haematoxylin for 6 minutes, then washed under running tape water for five minutes again. The slides were immersed in acidic alcohol for differentiation and controlling over stained haematoxylin for 1 second and then put in bluing solution (Sodium bicarbonate) until they became blue. After bluing, the slides were counter stained with eosin for 17-20 seconds and then washed in tape water for two minutes. The sections were dehydrated with increasing alcohol concentration of 50%, 70%, 95%, absolute I and II for two minutes each. The dehydrated sections were cleared with xylene I and II for three minutes each and permanently mounted on microscopic slides using DPX and cover slips and

then observed by light microscope for the investigations of any histological change, thereby the histology of the treated groups were compared with histology of the control group.

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

4.9.4 Statistical analysis

Data were presented as mean with 95% confidence interval and analyzed by SPSS version 22 and ANOVA (one way-ANOVA). The difference between groups with respect to variables under investigation was considered to be significant at (P values < 0.05).

4.9.5 Ethical consideration

The study was conducted after having approval by department of Anatomy graduate committee; school of Medicine, college of health sciences, and AAU in line with the highest standard for the humane and compassionate use of animals in biomedical research. Animals used in this study were not subjected to any unnecessary painful and terrifying situations (OECD, 1995). To keep the pain and suffering minimal during any surgical intervention all animals were given diethyl ether anesthetic and analgesic; and the procedure was carried out by a well-trained person. The animals were protected from pathogens; and placed in appropriate environment. The numbers of animals were reduced to the minimum consistent with achieving the scientific objectives of the study.

5. Result

5.1 Acute toxicity study

5.1.1 Effects of hydromethanolic leaf extract of *Syzygium guineense* on the behavior, gross pathology, organ and body weight

The acute toxicity study was carried out by oral administration of single dose of the hydromethanolic extract of the leaves. Treatment groups (I, and II) received 2000, 5000 mg/kg respectively, while group III (the control group) received distilled water (the vehicle). The acute toxicity study did not show toxicity sign and symptom such as clonic or tonic movements as well stereotypes (e.g., repetitive circling) or bizarre behavior (e.g., self-mutilation, walking backwards), changes in gait, posture and response to handling, changes in skin, eyes, pillo erection, lacrimation and pupil size change, salivation, food and water intake, excitement and depression, urination - color change, diarrhea, and change in breathing system at both doses employed. No mortality was observed in the treated groups i.e. at 2000mg/kg and 5000mg/kg during acute toxicity study. As a result the LD_{50} of the extract could be greater than 5000mg/kg body weight.

The gross pathological studies on the liver and kidneys of treated rats showed no significant abnormal changes in color, size, shape, and texture compared with the control.

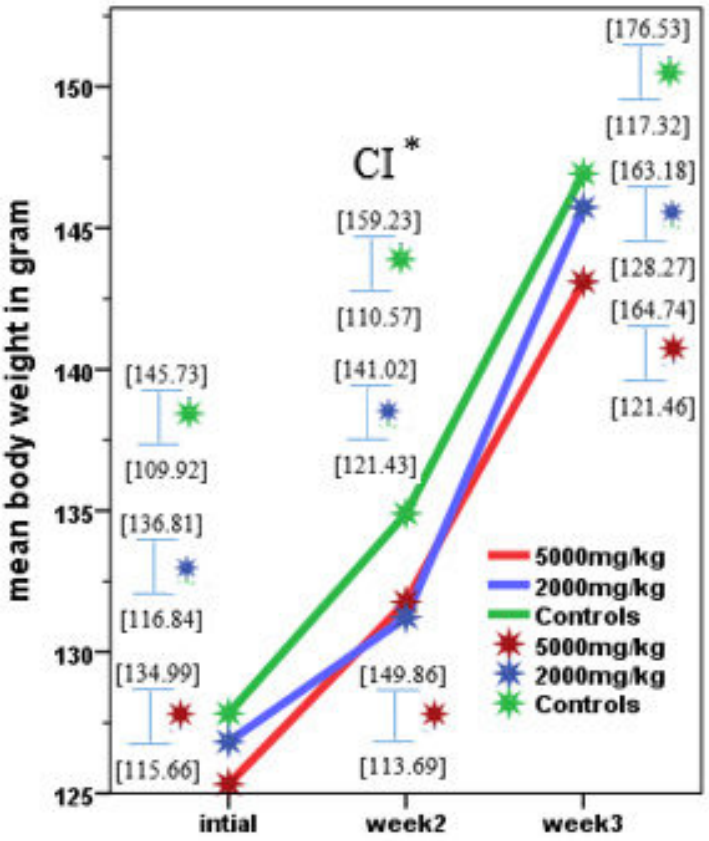
The mean absolute weights of the liver were 5.98 ± 0.82 g (at 2000mg/kg), 5.03 ± 0.55g (at 5000mg/kg) as compared with the control (5.40 ± 1.81) g. The mean absolute weights of the kidneys were 1.10 ± 0.18 g (at 2000mg/kg), 1.15 ± 0.16 g (at 5000mg/kg) as compared with the control (1.15 ± 0.54) g.

Table 1: Comparison of mean absolute organ weights of rats treated with different doses of extract as compared to that of the control rats during the acute toxicity study

Parameters	Doses	Mean	95% Confidence Interval for Mean		*P value
			Lower Bound	Upper Bound	
Liver Weight (in gram)	Controls	5.40	3.59	7.21	.248
	2000mg/kg	5.98	5.16	6.79	
	5000mg/kg	5.03	4.48	5.57	
Kidney weight (in gram)	Controls	1.15	.61	1.69	.932
	2000mg/kg	1.10	.92	1.28	
	5000mg/kg	1.15	.99	1.31	

**One way ANOVA, n= 4/group*

There were a gradual increase in the body weight of both the treated and control rats (Figure 5), which was not statistically significant ($p > 0.05$). The initial mean body weights of rats treated with doses of 2000mg/kg, and 5000mg/kg were 126.83 ± 9.98 g, and 125.33 ± 9.66 g respectively. At the end of the experiment (after 15 days) the final mean body weight of rats treated with 2000mg/kg, and 5000mg/kg were 145.73 ± 17.46 g, and 143.10 ± 21.64 g respectively. The mean body weight gain for rats treated with 2000mg/kg, and 5000mg/kg were 18.90 g and 17.80 g respectively.



***CI:** upper & lower bound *95% confidence interval for mean*

Figure 5: Line graph of mean body weight (in gram) change in rats treated with 2000mg/kg and 5000mg/kg extract as compared to the control group during acute toxicity study.

5.2 Subacute toxicity study

5.2.1 Effects of hydromethanolic leaf extract of *Syzygium guineense* on the behavior, gross pathology, organ and body weight

Subacute toxicity study carried out by oral administration of the hydromethanolic extract of the leaves at 500mg/kg and 1500mg/kg, for group I, and II rats respectively, while the control group (group III) received distilled water. Throughout the study period no sign of toxicity was observed on treated rats such as clonic or tonic movements as well stereotypes (e.g., repetitive circling) or bizarre behavior (e.g., self-mutilation, walking backwards), changes in gait, posture and response to handling, changes in skin, eyes, pillo erection, lacrimation and pupil size change, salivation, excitement (nervous system) and depression, urination - color change, diarrhea, change in breathing system and no mortality occurred in the treated groups.

Gross observation of the liver and kidneys of the treated rats showed no significant changes compared with the control group (figure 6); and no significant difference ($P > 0.05$) were observed in the mean absolute organs weight between control and treated groups (Table 3). The mean absolute weights of the liver were 7.70 ± 1.07 g (at 500mg/kg) and 6.40 ± 0.97 g (at 1500mg/kg), as compared with the control (7.03 ± 1.02 g). The mean absolute weights of the kidneys were 1.47 ± 0.09 g (at 500mg/kg) and 1.28 ± 0.10 g (at 1500mg/kg)

Table 2: Comparison of the effect of methanol extract of the leaves of *S. guineense* on absolute organ weight of treated and control rats during subacute toxicity study.

Parameters	Doses	Mean	95% Confidence Interval for Mean		*P value
			Lower Bound	Upper Bound	
Liver Weight (in gram)	Controls	7.03	6.02	8.05	.100
	500mg/kg	7.70	6.63	8.77	
	1500mg/kg	6.40	5.43	7.37	
Kidney Weight (in gram)	Controls	1.40	1.20	1.60	.082
	500mg/kg	1.47	1.38	1.55	
	1500mg/kg	1.28	1.18	1.39	

**One way ANOVA, n = 6/group*

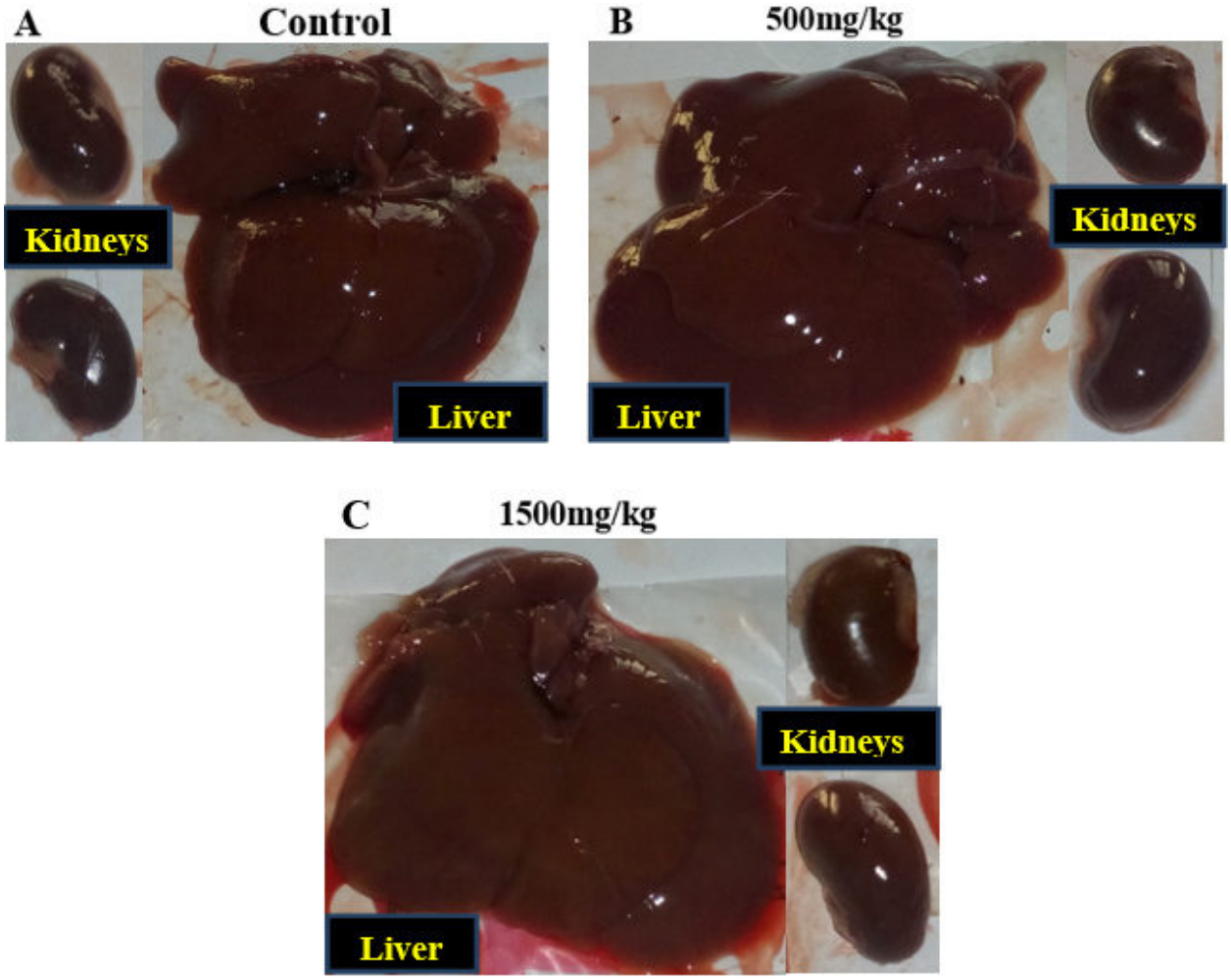
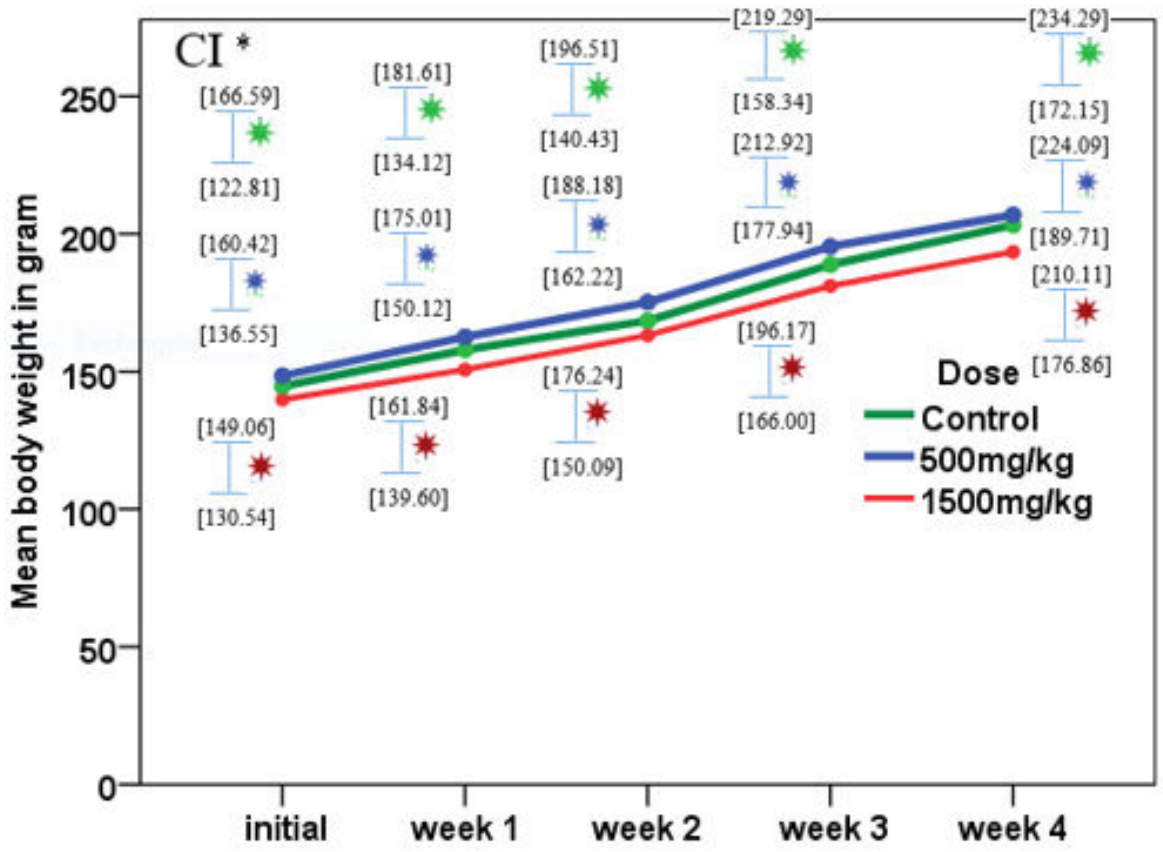


Figure 6: Photograph of liver and kidneys from the subacute toxicity study.

During the subacute experimental period all groups of rats showed gradual increase in their body weight (Figure 7**)**. However, there was no statistically significant ($p > 0.05$) weight difference between the treated and control groups. The initial mean body weight of control group was 144.70 ± 21.89 g, and final mean body was 203.22 ± 31.07 g. The initial mean body weight of rats treated with the dose of 500mg/kg was 148.48 ± 11.94 g, and final mean body weight was 206.90 ± 17.19 g. The initial mean body weight of rats treated with the dose of 1500mg/kg was 139.80 ± 9.26 g, and the final mean body weight was 193.48 ± 16.63 g.



***CI:** upper & lower bound *95% confidence interval for mean*

Figure 7: Line graph of mean body weight (in gram) change in rats treated with 500mg/kg and 1500mg/kg extract as compared to the control group during subacute toxicity study.

5.2.2 Effects of hydromethanol leaf extract of *Syzygium guineense* on hematological parameters

In the subacute toxicity study, the hematological parameters, such as WBC, RBC, PLT, HGB, HCT, MCV, MCH, and MCHC, of the treated groups (500mg/kg and 1500mg/kg) were within the reference range for rats and were not significantly different from the control (Table 3).

Table 3: Comparison of the effect of hydromethanol extract of leaves of *S. guineense* on hematological parameters of treated and controls.

Hematological parameters	Doses	Mean	95% Confidence Interval for Mean		*P value
			Lower Bound	Upper Bound	
WBC x10^3/ µL	Control	8.30	7.09	9.51	.384
	500mg/kg	8.13	7.23	9.03	
	1500mg/kg	9.45	6.67	12.23	
RBC x 10^6/ µL	Control	7.31	6.92	7.70	.859
	500mg/kg	7.30	6.73	7.86	
	1500mg/kg	7.43	6.99	7.86	
HGB (g/dL)	Control	14.98	13.74	16.23	.693
	500mg/kg	15.35	14.75	15.95	
	1500mg/kg	15.40	14.50	16.30	
HCT (%)	Control	48.80	47.14	50.46	.814
	500mg/kg	49.18	45.90	52.47	
	1500mg/kg	49.80	46.55	53.05	
MCV (fL)	Control	66.80	64.26	69.34	.887
	500mg/kg	67.47	64.59	70.34	
	1500mg/kg	67.05	65.10	69.00	
MCH (pg)	Control	20.50	19.10	21.90	.618
	500mg/kg	21.12	19.99	22.25	
	1500mg/kg	20.75	19.97	21.53	
MCHC (g/dL)	Control	30.73	28.08	33.39	.894
	500mg/kg	31.30	29.50	33.10	
	1500mg/kg	30.98	29.02	32.95	
PLT x 10^3/ µL	Control	697.17	603.01	791.32	.768
	500mg/kg	671.67	577.80	765.54	
	1500mg/kg	713.17	591.87	834.47	

**One way ANOVA, n = 6/group*

5.2.3 Effects of hydromethanol leaf extract of *Syzygium guineense* on biochemical parameters.

In the subacute toxicity study, the biochemical parameters (except glucose) of the treated groups (500mg/kg and 1500mg/kg) were within the reference range for rats and were not significantly different from the control group (Table 4). The level of urea in both treated grouped increased even though not statistically significant ($p > 0.05$). The mean values of serum glucose were 53.83 ± 4.11 mg/dl at 500mg/kg and 61.83 ± 10.77 mg/dl at 1500mg/kg, while 80.83 ± 6.62 mg/dl for the controls. The mean serum glucose level showed significant change/decrease ($p < 0.05$) at both doses as compared with the controls. Post Hoc Test of effect of extract on blood glucose (mg/dl) level of rats (multiple comparisons by using Dunnett T3) indicated that the mean difference of serum glucose was significant at 500mg/kg ($p < 0.0001$) which was decreased by 27 mg/dl and decreased by 19.0 mg/dl at 1500mg/kg ($P = 0.013$) when compared to controls. However, the two dose difference (500 and 1500mg/kg) was not significant ($p = 0.294$).

Table 4: Comparison of the effect of hydromethanol extract of leaves of *S. guineense* on biochemical parameters of treated and controls.

Biochemical parameters	Dose	Mean	95% Confidence Interval for Mean		[a*] P value
			Lower Bound	Upper Bound	
AST (IU/L)	Controls	287.83	213.56	362.10	.181
	500mg/kg	325.83	296.77	354.90	
	1500mg/kg	257.50	181.82	333.18	
ALT (IU/L)	Controls	198.17	170.56	225.78	.257
	500mg/kg	228.50	181.83	275.17	
	1500mg/kg	193.67	150.80	236.54	
Urea (mg/dl)	Controls	51.33	46.25	56.42	.055
	500mg/kg	58.00	54.62	61.38	
	1500mg/kg	60.17	51.15	69.18	
Creatinine (mg/dl)	Controls	.72	.64	.80	.307
	500mg/kg	.78	.68	.89	
	1500mg/kg	.80	.69	.92	
Total Protein (mg/dl)	Controls	6.32	5.55	7.09	.891
	500mg/kg	6.28	5.73	6.84	
	1500mg/kg	6.43	6.04	6.83	
Glucose(mg/dl)	Controls	80.83	74.22	87.45	
	500mg/kg	53.83	49.72	57.95	(.0001)[b*]
	1500mg/kg	61.83	51.06	72.60	(.013)[c*]

[a] **One way ANOVA, n = 6/group*

[b* & c*] $P < 0.05$ - using Post Hoc Test (multiple comparisons by using Dunnett T3)

5.2.3 Effects of hydromethanol leaf extract of *Syzygium guineense* on histology of the liver

Histopathological studies of the liver sections in the control group (Figure 8a A and B) showed normal appearance of central vein (CV) and hepatic sinusoids (S) lined by endothelial cells (EC) with normal radiating hepatocytes. There was also normal appearance of the portal triad including hepatic portal vein, interlobular bile duct, and branches of hepatic artery. Rats treated with hydromethanolic extract of the leaves of *S. guineense* at both doses of 500mg/kg (Figure 8b C and D) and 1500mg/kg (Figure 8c G and H) showed normal appearance of the central veins (CV) and hepatic sinusoids (S) lined with endothelial cells (E) with normal radiating hepatocytes.

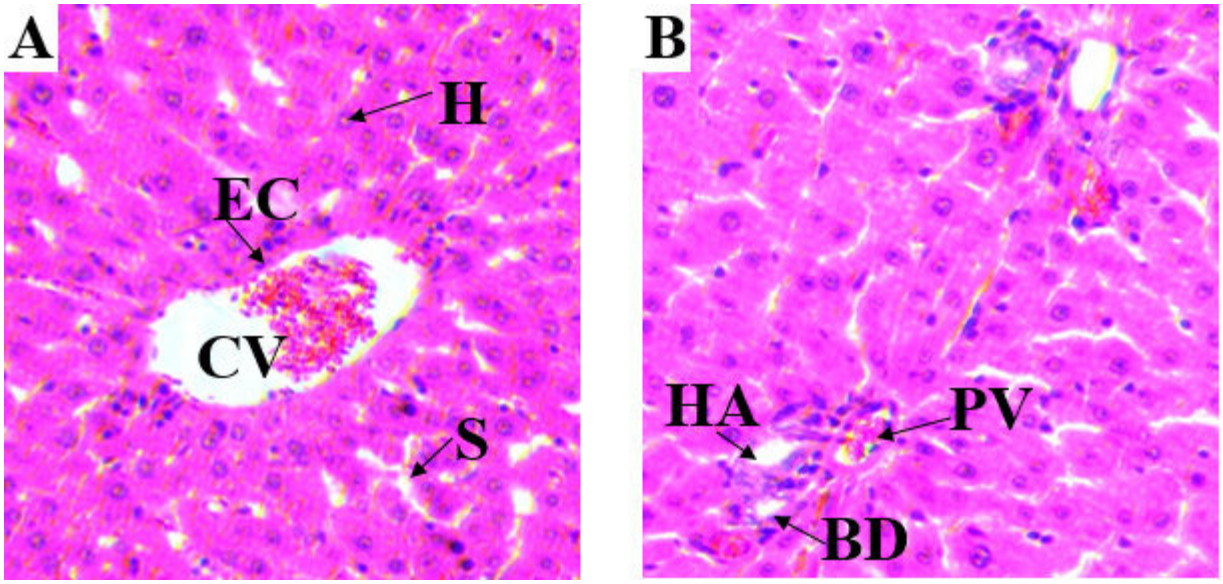


***Figure 8a: A and B - Photomicrographs of liver section of control rats (H and E, X400). CV**= Central vein, **EC**=Endothelial cells, **H**= Hepatocytes, **KC**=Kupffer cells, **BD**= Bile duct, **HA**= Hepatic artery **PV**= portal vein*

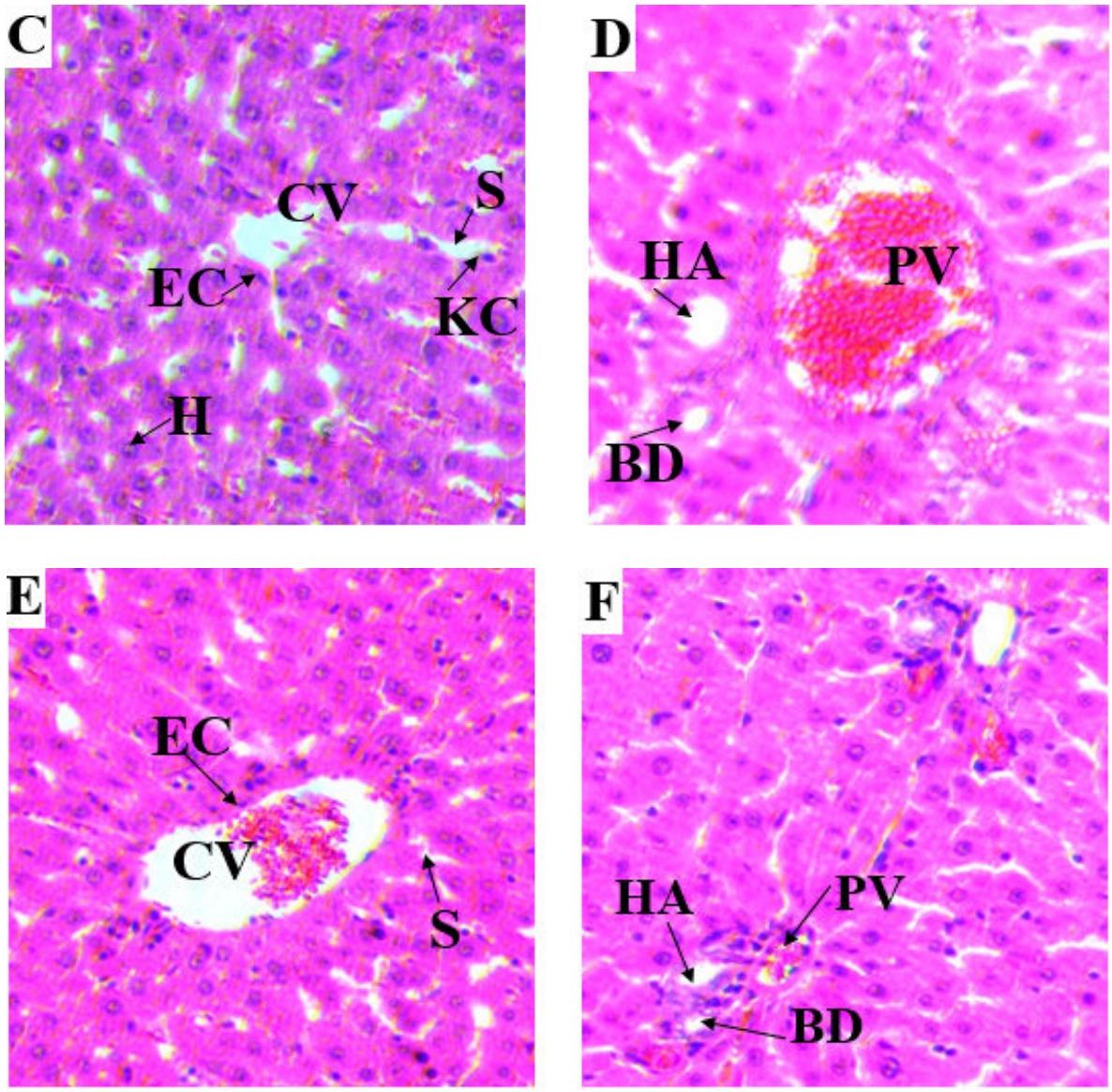


Figure 8b: C and D– Photomicrographs of liver section of rats treated with 500mg/kg of hydromethanol extract of* S. guineense*). E and F- liver section of control rats (H and E, X400). ***CV****= Central vein,* ***EC****=Endothelial cells,* ***H****= Hepatocytes,* ***KC****=Kupffer cells,* ***S****=Sinusoids* ***BD****= Bile duct,* ***HA****= Hepatic artery* ***PV****= portal vein*

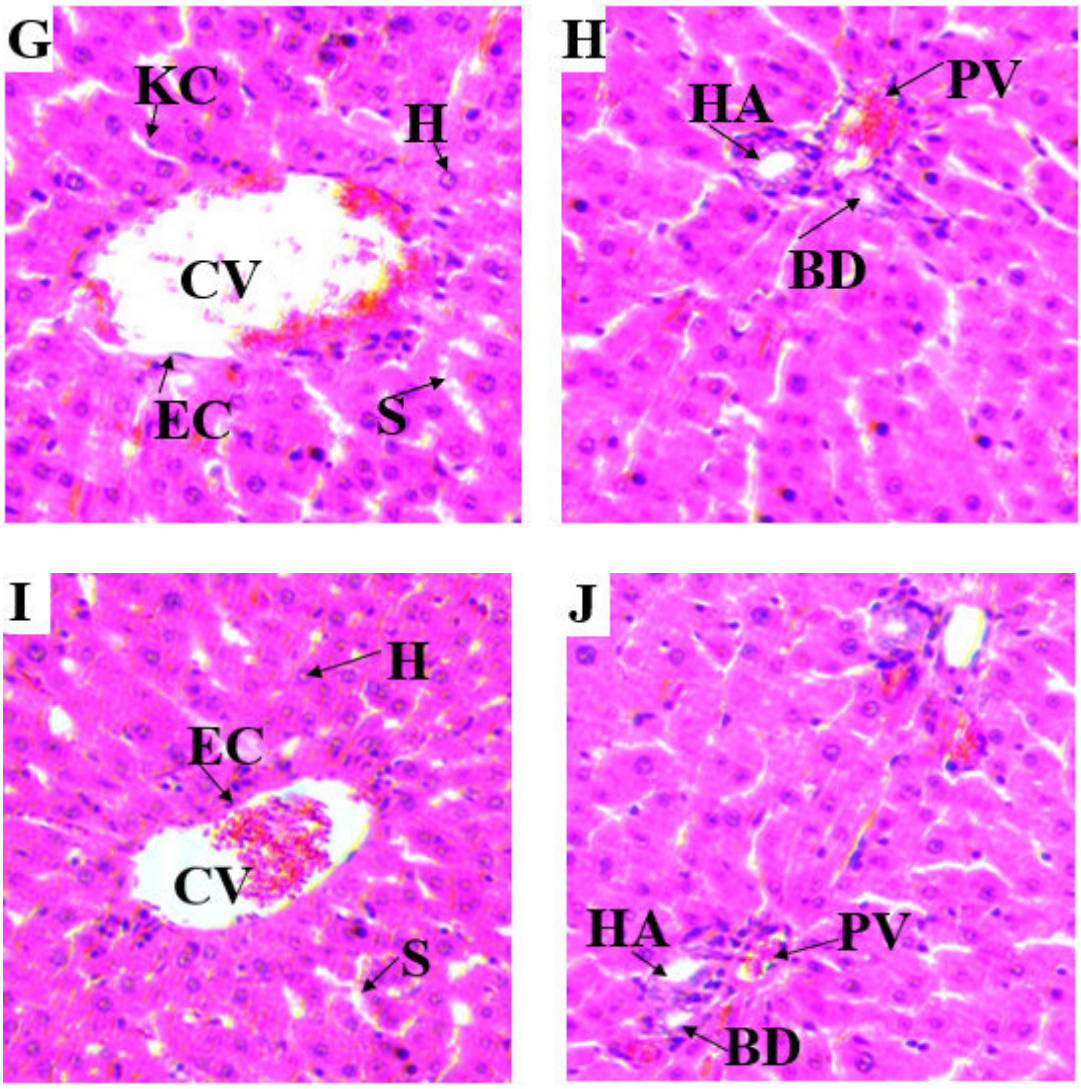


Figure 8c –G and H- Photomicrographs of liver section of rats treated with 1500mg/kg of hydromethanol extract of S. guineense. I and J - liver section of control rats (H and E, X400). ***CV****= Central vein,* ***EC****=Endothelial cells,* ***H****= Hepatocytes,* ***KC****=Kupffer cells,* ***S****=Sinusoids* ***BD****= Bile duct,* ***HA****= Hepatic artery* ***PV****= portal vein*

5.2.4 Effects of hydromethanol leaf extract of *Syzygium guineense* on histology of the kidneys

Histopathological studies of the kidneys sections of rats treated with doses of 500mg/kg (Figure 9b -C and D) and 1500mg/kg (Figure 9c- E and F) showed no significant microscopic changes as compared with the controls (Figure 9a- A and B). In the treated rats of kidney sections revealed normal glomerulus (G), Bowman's capsule lined with outer parietal layer/squamous cells (SC) and inner visceral layer/podocytes (P), urinary space (US), proximal convoluted tubules (PCTs) lined by simple cuboidal epithelium with brush border, distal convoluted tubules (DCTs) lined by simple cuboidal epithelium with more nuclei per cross-section, macula densa (MD) with taller cells around the vascular pole.

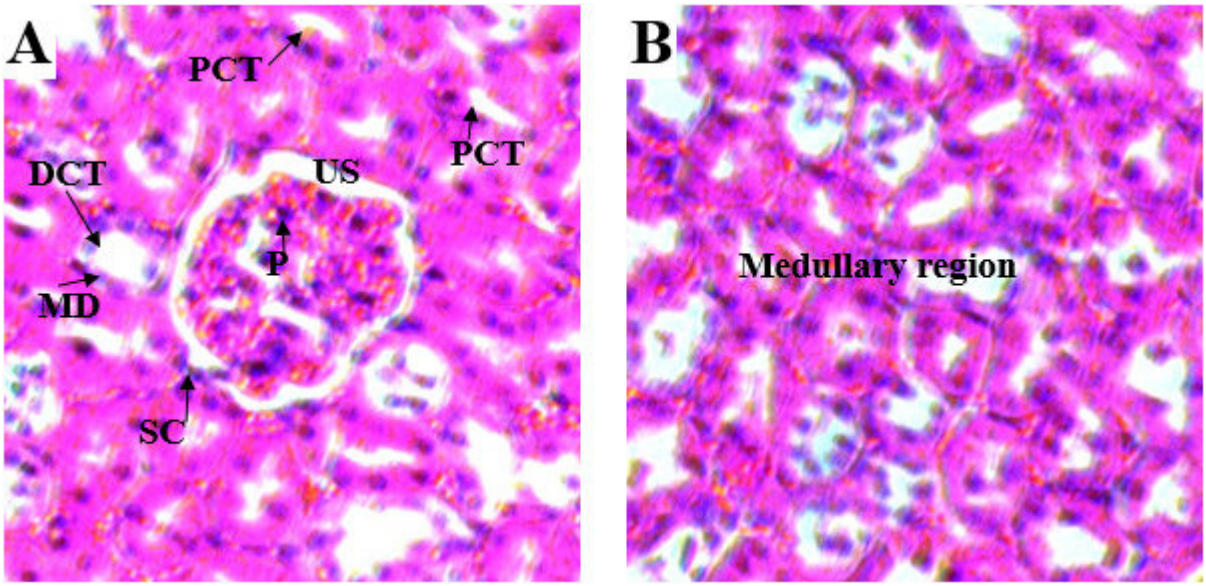


Figure 9a: ***A and B - Photomicrographs of the kidney sections of control rats (H & E x 400)*** ***PCT****=Proximal convoluted tubule,* ***DCT****= Distal convoluted tubule,* ***MD****=Macula densa,* ***G****=glomerulus,* ***US****=Urinary space,* ***SC****=Squamous cell,* ***P****=Podocyte.*

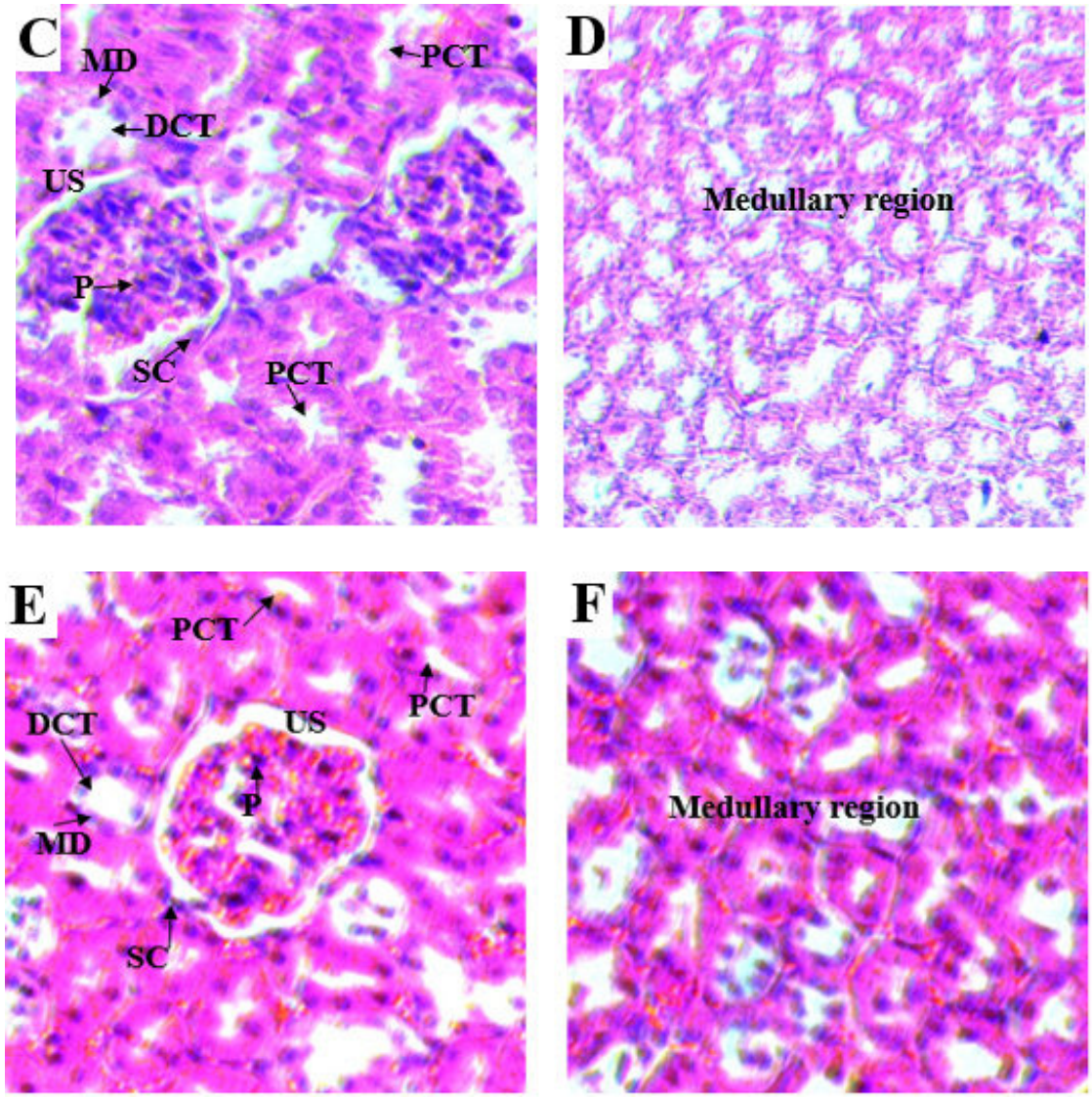


Figure 9b: C and D- ***Photomicrographs of the kidney sections of rats treated with 500mg/kg of hydromethanol extract of the leaves of*** **S. guineense (C-H&E-x400 & D-H&E-x200)** ***and E & F - kidney sections of control rats (H&E-x400). PCT****=Proximal convoluted tubule,* ***DCT****= Distal convoluted tubule,* ***MD****=Macula densa,* ***G****=glomerulus,* ***US****=Urinary space,* ***SC****=Squamous cell,* ***P****=Podocyte.*

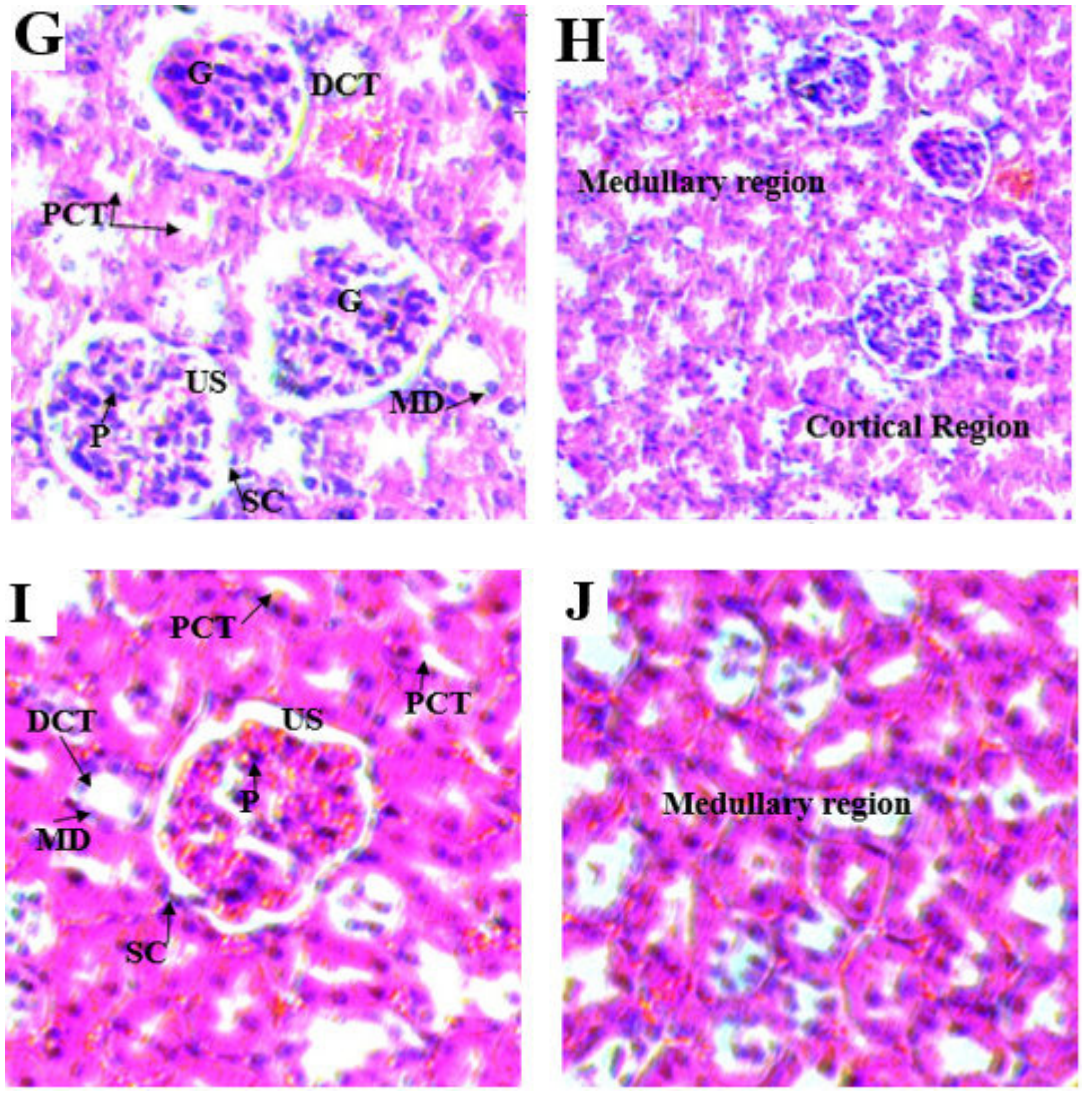


Figure 9c: ***G and H- Photomicrographs of the kidney sections of rats treated with 1500mg/kg (G- H & E x400 & H-H&E x200). I & J- kidney sections of control rats (H&E-x400).*** **PCT**=Proximal convoluted tubule, **DCT**= Distal convoluted tubule, **MD**=Macula densa, **G**=glomerulus, **US=**Urinary space**, SC=**Squamous cell, **P=**Podocyte.

6. Discussion

Syzygium guineense is included among the African plant species that are active against malaria (Ssegawa and Kasenene, 2007). Traditionally, many morphological parts of this plant have been utilized in the management of various ailments in many Ethiopian communities. For example, its fruits and bark are used for the treatment of dysentery and diarrhea and infusion prepared from its leaves, fruits or bark is used for treatment of hypertension (Abebe *et al*., 2003). The present study therefore attempted to evaluate the hydromethanol extract of leaves of *S. guineense* on histopathology of liver and kidneys and on some blood parameters in rats.

For acute toxicity study hydromethanol extract of leaves of *S. guineense* administered for rats at a dose of 2000mg/kg and 5000mg/kg. Administration at both dose group did not produce significant toxicity sign and symptom such as clonic or tonic movements as well stereotypes (e.g., repetitive circling) or bizarre behavior (e.g., self-mutilation, walking backwards), changes in gait, posture and response to handling, changes in skin, eyes, mucous membranes, pillo erection, lacrimation and pupil size change, salivation, food and water intake, excitement and depression, urination - color change, diarrhea, and change in breathing system at both doses employed. No mortality was observed in the treated groups i.e. at 2000mg/kg and 5000mg/kg during acute toxicity study. This indicate that the LD_{50} of the extract could be greater than 5000mg/kg body weight. The hydromethanol extract may, therefore, be considered relatively safe on acute exposure.

Similar results were reported by other researchers and it was suggested that, acute toxicity study showed the non-toxic nature of the aqueous extract of *S. guineense* up to 6000mg/kg (Worku, 2009). The study conducted on antimalarial activity of 80 % methanol extract of the stem bark of *S. guineense* (Wild.) DC. (Myrtaceae) in mice infected with *Plasmodium berghei* indicated that the acute toxicity study caused no mortality, at 2000mg/kg dose within the first 24h as well as on the following 14 days, and physical and behavioral observations of the experimental mice also revealed no visible signs of overt toxicity like lacrimation, loss of appetite, tremors, hair erection, salivation and diarrhea (Zeleke, 2015). Another study by Nigatu, (2004) showed that the LD_{50} of leaf tips aqueous extracts, twigs 80% methanolic extracts, stem bark aqueous extracts, stem bark 80% methanolic extracts, and fruit 80% methanolic extracts were 14.10, 2.91, 5.12, 8.77 and >10.0g/kg respectively.

Similarly, the study conducted in Brazil to evaluate toxicity of *S. cumini* leaves in rodents show that no sign of acute toxicity to rats and mice, at doses up to 2 and 6 g/kg respectively, or death up to the 14thday of observation, even at doses above 5 g/kg when administered orally (Silva *et al.,* 2012). The study on anti-inflammatory and analgesic activities of the ethanolic extract of the leaf of *S. Guineense* in rats and mice in Nigeria showed that the intraperitoneal LD_{50} of the ethanolic extract of *S. guineense* was found to be 3.807 g/kg (Ior *et al.,* 2012). Another study conducted on islet regenerative potential of purified fraction of *S. cumini* seeds *on* streptozotosin (STZ) induced diabetic mice showed that lethal concentration (LC_{50}) analysis SC2 (active constituent of *S. cumini*) of fraction was 12.33mg/ml. The total dosage of 2.1 mg of SC2 fraction was administered to mice within 21 days which is very minimal to show any toxicity (Menakshi and Bimba, 2011).

In the 14 days acute toxicity study, there was a gradual increase in the mean body weight of the treated groups though the differences were not statistically significant ($p > 0.05$) as compared with the control group (figure 5). Body weight change is an important index for assessment of toxicity (Vahalia *et al.,* 2011). The mean body weight gain for the control rats was 19.10 g. The mean body weight gain for rats treated with 2000mg/kg, and 5000mg/kg were 18.9 g and 17.8 g respectively. The lower mean body weight gain observed with the hydromethanolic extract as compared with the control, suggests that the extract might have harmful effect on body growth patterns.

Liver and kidneys of rats are used by many researchers to assess the safety or toxicity of drugs or plant materials (Graaf, 1995 and Satyapal *et al.,* 2008). In this acute toxicity study, gross pathological examination of the liver and kidneys did not show any significant difference in size shape, and color and texture upon treatment with the extract. The result show that no significant difference in the absolute weight of liver and kidneys of treated rats as compared to control group (table 1). The variation of organ weight between the control and treated groups may be caused by age, size, and rate of growth of rats.

During the 28 days subacute toxicity study the rats that were treated with hydromethanol extract of leaves at doses 500mg/kg and 1500mg/kg showed no signs of morbidity and mortality. During the experimental period no death or no apparent behavioral changes were observed as compared

with the control group. The current result was in disagreement with findings of study done on oral treatment of the aqueous leaf extract at dose of 200, 400 and 600mg/kg body weight daily for six weeks for the albino mice at which two mice died at day 32 and 40 from groups treated with 600 and 200mg/kg body weight of the extract, respectively (Amare, 2009).

In the gross pathological examination of the liver and kidneys of the treated rats showed no change in color, shape, size, and texture, as compared to the control group (Figure 6). According to Lu (1996), remarkable change in relative organ weight between treated and untreated animals is an indicator of toxicity as organ weight is affected by the suppression of body weight. In the present study there was no significant change in liver and kidneys weight of both treated and control group ($P>0.05$). This was inconsistent with the finding of Amare (2009), who reported a significant increase in the liver weight ratio in mice treated with 200mg/kg and a significant increase in the right kidney weight ratio was observed at the highest dose level, i.e., 600mg/kg.

In the subacute toxicity study rats treated with hydromethanol extract of the leaves of *S. guineense* showed gradual increase in their body weight (Figure 7) and the mean body weight gain of control, group I (500mg/kg), and group II (1500mg/kg) was 58.52g, 58.42g and 53.68g respectively which was statistically insignificant ($p>0.05$). This was consistent with the study conducted in Brazil to evaluate toxicity of *S. cumini* leaves in rodents in which no significant difference in body weight gain was noted between the control and the treated groups (Silva *et al.,* 2012). Increment in body weight determines the positive health status of the animals (Heywood, 1983). The lower mean body weight gain observed in the rats treated with highest dose i.e. 1500mg/kg as compared with the control and low dose (500mg/kg), suggests that the extract might have harmful effect on body growth patterns. Similar study done by Amare (2009) showed that the weight gain at 200 and 600mg/kg body weight was significantly lower than that of the control and decreased weight gain was also observed at 400mg/kg body weight of the extract comparing with the control though not significant ($P > 0.05$).

Assessment of hematological parameters can be used to determine the extent of harmful effect of foreign compounds including plant materials on blood (Yakubu *et al.,* 2008). In the present

subacute toxicity study, the hematological parameters (RBC, WBC, PLT, HGB, HCT, MCV, MCH, and MCHC) were within the reference range for rats (Table 3).

The reference values of RBCs, HGB, HCT, MCH, and MCHC for rats are 7 – 10 x 10^6/ µL, 11 – 19.2g/dl, 35 – 64%, 14.3 – 19.5 pg and 26.2 – 40g/dL, respectively (Pass and Freeth, 1993; Delaney, 2008). In the current study, the changes were not statistically significant ($p>0.05$) as compared with the controls. The absence of significant effect of the extract may indicate that the extract may not possess toxic substance that can cause anemia or other abnormalities. This is in disagreement with Amare's work in which a significant decrease in RBC and HGB count was observed and in the remaining hematological results MCV, MCH, and MCHC there were no statistically significant differences between the treated and control groups.

Blood of normal adult rat possesses about 6 – 18 x 10^3 WBC per µL of blood (Pass and Freeth, 1993). In this study, the mean WBCs count were within normal range for rats and were not significant ($p>0.05$). Although it was not statistically significant there was slight increment in mean WBC count at dose of 1500mg/kg were observed (Table 3). This was inconsistent with the finding of Amare, 2009, who reported a significant increase WBC count in mice treated with 400mg/kg body weight of the extract and statistically insignificant increase in the WBC count was observed in mice treated with 200mg/kg ($P>0.05$) and on the contrary the WBC count appears to decrease at 600mg/kg body weight.

Normal value of platelets in adult rats is 500 – 1,300 x 10^3/µL (Delaney, 2008). In this study, even though there was slight increment in mean platelets count at dose of 1500mg/kg, the change was not statistically significant ($p>0.05$). This was also in agreement with the work of Amare, (2009) showed non-significant decrease with mice treated at doses of 200, 400 and 600mg/kg.
In toxicological evaluation, biochemical parameters have significant roles because of their response to clinical signs and symptoms produced by toxicants. Evaluation of hepatic and renal function is of prime importance to assess the toxic properties of drugs (Rahman *et al.,* 2001). In the present study, the biochemical parameters did not show significant changes except for the mean values of glucose (Table 4). The mean serum glucose level showed significant change/decrease ($p < 0.05$) at both doses as compared with the controls. Post Hoc Test - multiple

comparisons by using Dunnett T3 indicated that, the mean amount of glucose levels decreased by mean difference of 27.0 mg/dl at dose of 500mg/kg ($p<0.0001$), and 19.0 mg/dl at 1500mg/kg ($P = 0.013$) when compared to control group. However, the two dose difference (500 and 1500mg/kg) was not significant ($p = 0.294$).

The decrease in serum glucose level with the extract observed in this study may be due to presence of insulin like substance in the hydromethanol extract, either promote glucose uptake and metabolism by the liver or inhibit hepatic gluconeogenesis, supporting the traditional use of the leaves for treatment of diabetes. The result was in agreement with the works of Worku, (2009) who reported that the aqueous extract reduced the blood glucose level of both normoglycemic and diabetic mice at all doses tested. This is also in consistent with another study conducted on administration of active constituent of seeds of *S. cumini* fraction (SC2) in mice that showed sustained reversal in experimental diabetes as evidenced by restoration of normoglycemia, increase in glycolytic enzyme glucose – 6 – phosphate- dehydrogenase (G6PD) and hepatic glycogen contents of diabetic mice were significantly lower than those of the normal control mice ($p<0.01$) (Menakshi and Bimba, 2011). In contrast to these study, the study conducted in Nigeria on anti-venom studies on *Olax viridis* and *S. guineense* extracts studied against *Naja katiensis* venom rats reveal that plant extracts has no significant change in blood glucose level in those group taking only extracts of *O. viridis* and *S. guineense* without envenomation (there was no significant increase in their blood glucose before and after envenomation) (Omale *et al.,* 2013). Similarly, the study conducted in Brazil to evaluate toxicity of *S. cumini* leaves in rodents show that, there was no change in the serum concentration of glucose in animals treated for 30, 90 and 180 days with the *S. cumini* (Silva *et al.,* 2012). Inconsistence with these study may due to difference in dose employed, and the environment where plant grown.

Measurement of plasma urea has been used for many years as an indicator of kidney function. Urea is usually increased in acute and chronic renal diseases. Urea clearance falls as the kidney fails and as a result, urea tends to accumulate with diseased kidneys that are unable to excrete these substances at normal rate; this will raise blood urea level (Tietz, 2000; Feres *et al.,* 2006). In normal adult rat serum is measured about 15- 45 mg/dl (Pass and Freeth, 1993). In this study, the mean values of urea showed a slight increment at dose of 500 and 1500mg/kg even though

p>0.05 and this was not associated with the histopathological changes of the kidney. The result was in disagreement with the work of Silva *et al.,* 2012, conducted in Brazil to evaluate toxicity of *S. cumini* leaves in which the serum levels of urea from the animals treated at doses of 0.05, 0.1 and 0.25 g/kg were reduced by 19.4, 13.8 and 18.4%, respectively, after 30 days.

Creatinine is produced endogenously and released in to body fluids at a constant rate and its plasma concentration is maintained predominantly by glomerular filtration. Consequently, both plasma concentration and its renal clearance have been used as markers of the glomerular filtration rate (Tietz, 2000). In the current study, the mean amounts of creatinine showed slight increment but not significant (p>0.05). The reference value of creatinine in adult rat is about 0.2 – 0.8 mg/dL (CRL, and Delaney, 2008). In this study the changes were within reference value for rat and was supported by the absence of histopathological changes of the kidney. The result was similar with the finding of Silva *et al.,* (2012) in which no differences in the serum level of creatinine was found between extract-treated groups and the control groups after 90 and 180 days of administration. However, the result was inconsistent with Silva *et al.,* (2012) in which creatinine showed a reduction of 15.4% with a dose of 0.25 g/kg, when compared to the control group after 30 days of administration.

Serum total protein change is caused by a change in the volume of plasma water and a change in the concentration of one or more specific proteins in the plasma. Decrease in the volume of plasma water (hyperproteinemia) is noted in cases of dehydration due to inadequate water intake or excessive water loss, as the case in severe vomiting or diarrhea (Tietz, 2000). The reference value of total protein in the serum of adult rat is in the range of 5.6 – 7.6 mg/dL (Pass and Freeth, 1993). In the current study, the amounts of total protein showed a slight increment at dose of 1500mg/kg compared to control but not significant (p>0.05). The mean values of total protein were within the reference range for rats, which was also supported by the absence of histopathological changes in the kidneys of treated rats. The result was in agreement with the finding of Silva *et al.,* (2012), who asserted that no differences in the serum levels of total protein was found between extract-treated groups and the control groups.

The abnormal elevation of the liver enzymes (ALT, and AST) may indicate liver damage or alteration in bile flow. ALT is found primarily in the liver and is the most sensitive marker for liver cell damage. When a cell is damaged, it leaks this enzyme into the blood. AST is found primarily in the red blood cells, cardiac and skeletal muscles and kidney. AST is not specific to liver as ALT. These parameters are elevated whenever disease processes affect liver cell integrity. In this study, the mean values of AST and ALT at dose 500mg/kg showed increment, while at dose 1500mg/kg showed decrement as compared with the controls, but it was not significant ($p>0.05$). This was supported by the absence of histopathological changes in the liver of treated rats. The result was in consistent with the studies of Silva *et al.,* 2012 in which no differences in the serum level of AST and ALT was found between extract-treated groups and the control groups.

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The liver and kidneys have fundamental roles in the metabolism and excretion of drugs or plant products. However, during their biotransformation process, generation of reactive metabolites and presence of secondary metabolites in the plant materials might result in toxicity or cell damage on these organs (Marcela *et al.,* 2001; Belay, 2008). In the current histopathological examination of the liver, rats treated with doses of 500mg/kg and 1500mg/kg of the hydromethanol extract of the leaves of *S. guineense* showed no change in the microscopic structure of the liver (Figure 8 C, D, E and F). The general architecture of the liver, appearance of the hepatocytes, the hepatic sinusoids, portal triads and central veins are normal as compared with controls (Figure 8 A and B). The result were also accompanied by the non-adverse effects of the extract in any of the biochemical markers (such as ALT, and AST), which showed statistically insignificant changes compared with the control group. This finding was in agreement with work of Amare, (2009) who reported that, mice treated at a dose of 200 and 400mg/kg body weight of the extract showed no histopathological changes, as compared to the control group. The current study was also in agreement with Silva *et al.,* (2012), in which microscopic examination of the selected organs (liver, lungs, kidneys, stomach, intestine, heart and pancreas) did not reveal any treatment-related effects. However, the finding was in disagreement with Amare's work who reported that, tissue morphology of mice treated with 600mg/kg body weight of the extract showed hemmorhegic centrilobular necrosis and fatty cytoplasmic vacuolation of the hepatocytes (Amare, 2009). Inconsistence with the Amare's

finding may due to difference in dose employed, solvent used during extraction and environmental difference where plant grown.

In the histopathological study of the kidney, rats treated at both doses (500 and 1500mg/kg) of the extract showed no difference (Figure 9 C, D, E and F) as compared to controls (Figure 9 A and B). The present finding of sections of the kidneys of treated rats showed normal general structure of the kidney, the normal appearance of glomeruli and tubules. The proximal convoluted tubules, distal convoluted tubules and macula densa are intact. The result can be further supported by the values of biochemical parameters of the blood (such as urea, creatinine, and total protein), which are main indicator of kidney damage. This was in line with the work Amare (2009), who observed no difference in tissue morphology between control group and mice treated with 200mg/kg body weight. The current study also in agreement with Silva *et al.,* -(2012), in which microscopic examination of the selected organs (liver, lungs, kidneys, stomach, intestine, heart and pancreas) did not reveal any treatment-related effects. On the contrary the current finding was disagreement with work of Amare (2009), in which tissue morphology differences were observed in the kidney of mice treated with 400, and 600mg/kg body weight. Inconsistence with the Amare's finding may due to difference in dose employed, solvent used during extraction and environmental difference where plant grown.

7. Conclusions

- The oral LD_{50} of the hydromethanol extract of the leaf of *S. guineense* was above 5000mg/kg.
- The hydromethanol extract of the leaves did not produce adverse effects on the behavior and gross pathology of the rats at treated doses.
- The hydromethanol extract of the leaves did not adversely affect the body weight, hematological and biochemical parameters of tested doses.
- There were no signs of toxicity observed in the kidney and liver sections of treated rats.

8. Recommendations

1. Repeat the subacute study;
    - ✓ Using increased sample size (at least minimum number required).
    - ✓ Use pair-matching instead of randomization
    - ✓ Use increase dose groups which could provide “relative dose response curve”.
    - ✓ Operationalize discrete variables/observational variable.

2. Well-designed subchronic and chronic toxicity studies of the extract should be carried out on body weight, hematological and biochemical parameters and histopathology of the liver and kidney.

3. Well-designed subacute, subchronic and chronic toxicity studies should be carried out on the effect of the extract at ultramicroscopic level of the liver and kidney.

4. Further subacute, subchronic and chronic toxicity studies should be carried out on other organs such as the brain, lungs, heart, pancreas, stomach, and intestines and others.

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Appendices

Appendix- I: Preparation of working chemicals or solutions

1. **10% Neutral Buffered Formalin:**
   - 40% formaldehyde……..100 ml
   - Distilled water ……....900 ml
   - Sodium dehydrogenate phosphate…….……. 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. **Alcoholic Eosin (E):**
   - Eosin, both water and alcohol soluble……...1 gm
   - 95% Ethanol……..100 ml
   - Glacial acetic acid……..0.5 ml
4. **1% Acidic alcohol:**
   - 70% alcohol……...500 ml
   - Hydochloric acid, concentrated……..0.5 ml
5. **Bluing solution:**
   - Sodium bicarbonate……2.5 gm
   - Distilled water……1000 ml

Appendix II: Tissue fixation protocol

Fixation	
10% buffered formalin	overnight
Washing	
Running tap water	several times
Dehydration	
50% alcohol	2hrs
70% alcohol	2hrs
90% alcohol	2hrs
Absolute alcohol- I	1 1/2hrs
Absolute alcohol-II	1 1/2hrs
Absolute alcohol-III	1 1/2hrs
Absolute alcohol-IV	overnight
Clearing	
Xylene-I	1 1/2hrs
Xylene-II	1 1/2hrs
Infiltration	
Wax-I	1 1/2hrs
Wax-II	2 1/2hrs
Wax-III	overnight

Appendix III: Tissue staining protocol (H & E staining)

Deparaffinization	
Xylene-I	4 minutes
Xylene-II	4 minutes
Rehydration	
Absolute alcohol- I	4 minutes
Absolute alcohol-II	4 minutes
95% alcohol	2 minutes
70% alcohol	2minutes
Wash in distilled water	5 minutes
Stain in Harris Hematoxylin	8 minutes
Rinse in running tap water	5 minutes
Decolorize 1% acid alcohol	30 sec
Rinse in running tap water	1 minutes
Immerse in Sodium carbonate solution	(30sec- 1minutes)
Rinse in running tap water	5 minutes
Counter stain in Eosin	(30 sec – 1 minutes)
Dehydration	
70 % Ethanol- I	5 minutes
95% Ethanol - II	5 minutes
Absolute alcohol- II	4 minutes
Absolute alcohol-I	4 minutes
Clearing	
Xylene-I	5 minutes
Xylene-II	5 minutes