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Toxicity study of anti-ectoparasiticformulation comprising *Eucalyptus globulus* and *Jatrophacurcas* oils blended using industry based emulsifier on the histopathology of liver, kidneys and some blood parameters in mice.

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List of Abbreviations

^{0}C……Degree Celsius

AAU……Addis Ababa University

ALP ……Alkaline Phosphatase

ALT ……...Alanine Aminotransferase

ANOVA ……....Analysis of Variance

AST ……Aspartate Aminotransferase

DPX …….. Dibutyl phthalate in xylene

EDTA……Ethylene Diamine Tetra-acetic Acid

EPHI ……Ethiopian public Health institution

FAO……Food and agricultural organization

H&E ……....Hematoxylin and Eosin

HCT……..Hematocrit

HDL……...High density fat level

Hgb. ……Hemoglobin concentration

LD_{50}……..Lethal dose that kills 50% of animals

LDL……Low density fat level

MCH……Mean Corpuscular Hemoglobin

MCHC ……Mean Corpuscular Hemoglobin Concentration

MCV ……Mean Corpuscular Volume

OECD……Organization of Economic Co-operation and Development

PCV ……Packed Cell Volume

RBC ……..Red Blood Cell

Rpm ……. Revolution per minute

SEM ……. Standard Error of the Mean

SPSS …… Statistical Package for Social Sciences

WBC ……....White Blood Cell

WHO ……...World Health Organization

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Abstract

Background: Agriculture delivers a livelihood for the people of many African countries. Accordingly, animal agriculture provides strong base for Ethiopian economy. However, the presence of ectoparasites and secondary infections caused by their infestations decrease the quantity and quality of livestock production leading to low income. A lot of chemicals such as organophosphates, carbamates, acaricides, and chlorinated hydrocarbons are widely applied to regulate these ectoparasites and their adverse effects. However, most synthetic anti-ectoparasites are toxic and the relatively safe chemicals are expensive. Utilization of affordable and safer products from natural sources is, therefore, highly commendable.

Objectives: The present study was carried out to evaluate the acute and sub-chronic toxic effects of the formulation.

Methods: The experiments were performed on a total of 72 healthy male and female (54 for acute and 18 for sub-chronic) Swiss Albino mice. Grouping was done randomly based on the OECD guideline. The treatment groups were orally administered with 1.25%, 1.9%, 2.9%, 4.4%, 6.6%, 9.9%, 14.9% and 20% ml/kg body weight doses of the formulation for the acute toxicity studies. 1.25% and 3.75% ml/kg body weight doses of the formulation were used for the sub-chronic toxicity studies. The control groups were administered with distilled water during both acute and sub-chronic toxicity studies.

Results: There was no change in the general behavior of treatment groups as compared to the control during acute toxicity study. No death was recorded. The LD_{50} was found higher than 20% ml/kg body weight dose of the formulation. In the sub-chronic studies, no significant biochemical, hematological and body weight changes were observed, except for LDL, which was found increased in both treatment groups. Generally, histological architecture of liver and kidneys were normal. However, liver of animals treated with dose of 3.75% ml/kg showed small number of mononuclear leukocytic infiltrations around portal areas. Minor tubulointerstitial leukocytic infiltrations were also observed in the kidney sections of these animals.

Conclusion and Recommendations: Results of the acute and sub-chronic toxicity studies revealed that the formulation is relatively safe. Further studies in other organs and animals are recommended toward establishing the safety of the formulation.

Keywords: *Eucalyptus globulus, Jatropha curcas*, Ectoparasites, Toxicity, Swiss albino mice

1. Introduction

1.1. Background of the study

Agriculture delivers a livelihood for the people of many African countries. More than 80% of Ethiopian population is dependent on agriculture, which is the core economic activity that contributes to 47% of the country's GDP and more than 80% of the export. The sector employs over 85% of the population in which livestock plays key role (Bureau of African Affairs, 2006). Livestock is the second major source of foreign currency through export of live animals, skin and hide (FAO, 2010).

Animal agriculture provides strong base for Ethiopian economy directly or indirectly, through crop production and soil fertility management as fertilizer, input as row material for industries, food, fuel and other social functions. The livestock sectors also support and sustain initiatives and interest groups which are related and linked with the livestock value chains such as the livestock traders, transporters, slaughter facilities/processors, feed manufacturers, veterinary drug suppliers, etc. who also create employment opportunities. Furthermore, Ethiopia is currently considered the tenth largest livestock producer and chief exporter of livestock in Africa. In a legal way Ethiopia exports approximately 200,000 livestock per year (Yacob and Catley, 2010).

The economic benefits of livestock populations through providing milk, meat, skin and manure, however, remain marginal due to the prevailing livestock diseases. Animal health problems are among the principal bottlenecks for very poor livestock performance and cause of high economic losses, in particular to the resource-poor farmers (Mesfine and Lemma, 2001).

Diseases and parasite infestations, lack of suitable livestock extension facilities, malnutrition, mishandling and mismanagement of the animals, etc. decrease the quantity and quality of livestock production. These factors bring huge production loss in the livestock industry leading to low income (Kedija *et al*., 2008). Ectoparasites and secondary infections caused by their infestation hold significant rank of the factors that lead to low productivity on the livestock industry causing measurable mortality and low-income. Ectoparasites are organisms that live on the surface of bigger animals upon which they depend for nourishment, housing and other

rudimentary needs to survive (Rechav and Nutall, 2000). Ticks, fleas, lice, mite and sheep keds are selected external parasites which feed on body tissues such as blood, skin and hair of ruminants. These organisms are culpable for reduction of meat and milk production by sucking animal blood, low weight gains, skin irritation and discomfort. Additionally, they are the most chief vectors of protozoan, bacterial, viral and rickettsial infections (Radostits *et al.*, 2007). Due to faults caused by external parasites, 35% of sheep and 56% of goat skins are rated lower; decreased in value and rejected in various tanneries (Kassa, 2005). Study done for assessment of major factors that cause skin rejection at Modjo export tannery, Ethiopia, revealed that ectoparasites play key role in the rejection of skin (Berhanu *et al.*, 2011).

External parasites are controlled commonly by strips, baits, sprays, pour-on products aimed at the parasite, foggers and dust bags. Nowadays great numbers of chemicals such as organophosphates, carbamates, acaricides, chlorinated hydrocarbons etc. are widely applied to regulate ectoparasites and their adverse effects as ectoparasiticides (Njoroge and Bussmann, 2006). However, some of the conventional chemicals are toxic and do not deliberate economic capacity of developing countries such as Ethiopia. Synthetic pyrethroids, such as Fenvalerate and Deltamethrin even though, considered as safer and very effective chemicals they are very expensive (Njoroge and Bussmann, 2006). Since most synthetic antiectoparasites are hazardous and toxic and the relatively safe chemicals are expensive, searching for more affordable and safer products from natural sources is highly commendable. Therefore, the primary concern of this research was to investigate safety of the anti-ectoparasitic formulation comprising *Eucalyptus globulus* and *Jatropha curcas* oils blended using industry based V497 emulsifier in mice after acute and sub-chronic treatment.

1.2. Medicinal plants

Traditional medicine is defined as health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to diagnose, treat and prevent illnesses and maintain well-being (WHO, 2001). It is known that many countries in the world use traditional medicine to meet some of their primary health care needs. In Africa, up to 80% of the population uses traditional medicine for primary health care (WHO, 2003). In recent years, worldwide

interest in medicinal plants has increased significantly not only for humans but also for animals. Cattle, horses, sheep, goats and pigs represent about 70% of the animals treated with herbal remedies (Viegi *et al*., 2003).

In Ethiopia, medicinal plants play significant role in primary health care delivery, where 80% of human and 90% of livestock population depend on traditional medicine similar to many developing countries (Kassaye *et al*., 2006). Great varieties of medicinal plants are used for livestock healthcare system, particularly by the pastoral and agro-pastoral communities. However, although, traditional medicine has cultural acceptance and employed by the majority of the population, there are limitations. Some commonly used herbal medicines are toxic and have chemical compositions as their own "defense mechanism" which induces adverse consequences on the well-being of humans and livestock population (Abebe *et al*., 2001; Nwafor, 2004). Their effects range from allergic reaction to cardiovascular, hepatic, renal, neurologic and dermatologic problems (Dasgupta, 2003; Steenkamp *et al.*, 2006). Therefore, since medicinal plants are widely used in formulation of drugs, as food staffs and in cosmetic industries in addition to those of traditional medicine, toxicity assessment of those herbs needs to be investigated (Haq, 2004).

1.3. *Eucalyptus globulus*

1.3.1. Botanical distribution of *Eucalyptus globulus*

Eucalyptus globulus is one of the early and widely studied *eucalypt* species. It is native for Australia and exotic for Eritrea, Ethiopia, France, Guyana, Zimbabwe, Haiti, India, Japan, Kenya, Lesotho, Mozambique, Namibia, New Zealand, Paraguay, Peru, South Africa, Spain, Surinam, Swaziland, Tanzania, Uganda, Uruguay, Venezuela, Zambia, Zimbabwe etc. in its species distribution. This plant species is also most extensively cultivated in cool zones of tropical mountains and Mediterranean regions including Spain and Morocco. *Eucalyptus globulus* also known as Tasmanian blue gum or blue gum is one of the species most broadly introduced overseas. It belongs to Myrtaceae family and is long evergreen tree cultivated for paper, pharmaceutical and cosmetics industry (Hasegawa *et al*., 2008; Rassaeifar *et al*., 2013).

About 70 species of *Eucalyptus* exist in Ethiopia, most of which are extensively spread in several regions of the country, mostly in central highlands where there is higher population density. Planting of *Eucalyptus globulus* has been increasing from state owned forestry enterprises and projects to community woodlots, household and farm field boundaries (Yirdaw and Luukkanen, 2003). Growing up 100 m in height in favorable conditions, *Eucalyptus globulus* is known by the local name Nech Bahir zaf (in Amharic), Baarzaafii adii (in Afaan Oromoo) and Tsaeada-kelamitos (in Tigrigna). Farmers in diverse agro ecological zones sustained to plant the species for several intentions such as fuel wood, transmission poles and construction material for their own use and for income generation (Yirdaw and Luukkanen, 2003).

1.3.2. *Eucalyptus globulus* essential oils

Essential oils derived from *Eucalyptus globulus* are widely applied as natural pesticides (Lee *et al*., 2000; Lee *et al*., 2004; Papachristos and Stamopoulos, 2004; Haouel *et al*., 2010). Among the 700 species of Eucalyptus; more than 300 of them contain volatile oils in their leaves. Leaves of *Eucalyptus globulus* are the most widely used parts and appreciated for the withdrawal of eucalyptol, the commercially important eucalyptus oil. The oil content is 54-61% 1,8-cineole, 19.5-24.3% α-pinene, 6.7-9.1% limonene and 2.1-5.4% α-terpinyl acetate. Juvenile leaves of *Eucalyptus globulus* have higher oil content than mature ones. Cineole is the major composition in most *Eucalyptus* species (Lucia *et al*., 2012). The leading methods for the extraction of Eucalyptus essential oils are water and steam-distillation, hydrodistillation (water-distillation) and steam-distillation (Boland *et al*., 1991).

1.3.3. Medicinal value of *Eucalyptus globulus*

Eucalyptus globulus is widely used in traditional medicines. Leaves extract of this plant have been applied to treat influenza, chest problems, and skin rashes while their vapor is inhaled to fight inflammation (Musyimi and Ogur, 2008). Eucalyptus oil is also useful for alertness, antifungal, antimicrobial, antiviral, aromatherapy, arthritis, astringent, back pain, burns, cancer prevention, cancer treatment, deodorant, diabetes, diarrhea, ear infections, emphysema, fever, flavoring, hookworm, insect repellant and leukemia (Vankar *et al*., 2006; Ponte *et al*., 2008). It is also applied for liver protection, muscle spasm, nerve pain, rheumatoid arthritis, runny nose,

scabies, shingles, sinusitis, snoring, tuberculosis, urinary difficulties and urinary tract infection (Ponte *et al*., 2008). In addition, *Eucalyptus globulus* delivers inevitable advantages for anthelmintic activities, as food additives due to its antioxidant activity, in the form of formulation as sprays and lozenges mostly in developed countries, act as antimalarial and highly active against bacteria, for allergies such as bronchitis, sinus and asthma, congestion and for the treatment of parasitic skin infections. It has also anti-inflammatory, healing and antimicrobial actions (Sakai *et al*., 2006). *Invivo* evaluation of *Eucalyptus globulus* oil on infested goats resulted complete elimination of mange mites in goats, suckling and biting sheep lice besides complete recovery of the lesion. This showed that the essential oil could serve as an alternative for treatment of scroptic mange in goats (Yasine, 2010; Wondimu, 2010; Negese, 2011).

Figure1: Photographs of *Eucalyptus globulus* showing the whole tree (A) leaves and flowers (B).

1.4. *Jatropha curcas*

Jatropha curcas is a perennial small tree or shrub belonging to family Euphorbiaceae (Carels, 2009). The genus *Jatropha* contains approximately 170 known species (Katembo and Gray, 2007). It is native to the American tropics mostly Mexico and Central America. *Jatropha curcas* is exotic to Ethiopia, Eritrea, French Guiana, Gabon, Gambia, Ghana, Grenada, Guadeloupe, Guinea, Guinea-Bissau, Haiti, India, Angola, Antigua and Barbuda, etc. It can grow in poor soils and areas of low rainfall (from 250 mm a year) hence it is being promoted as the ideal plant, where degradation of agricultural land is widespread (Foidl *et al*., 1996; Sarin *et al*., 2007; Gressel, 2008).

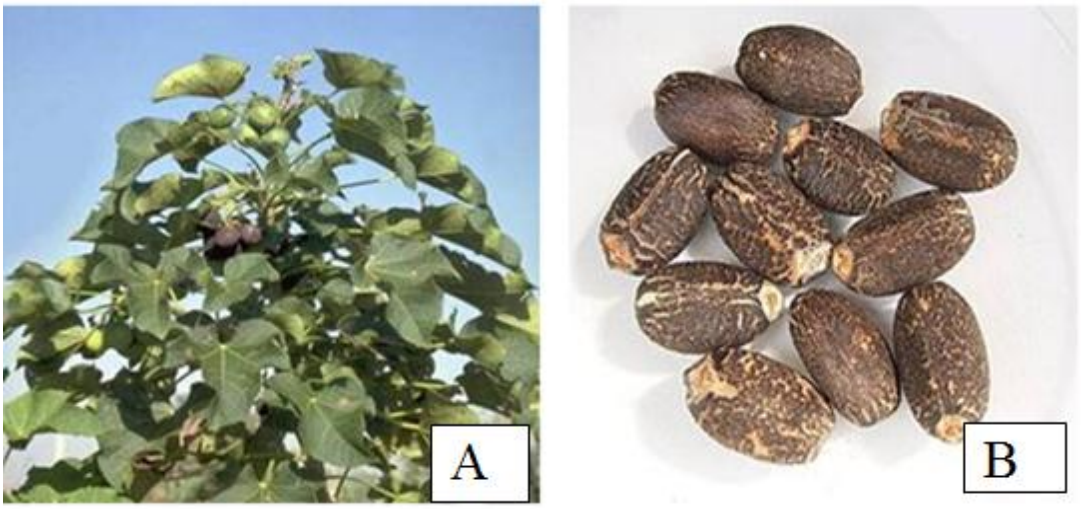

Figure 2: Photographs of *Jatropha curcas* (pig nut) showing tree (A) and dry seed (B).

Jatropha curcas is a multipurpose tree having several industrial and medicinal uses. Extracts from its leaves, bark, roots, seeds, and latex present different and important medicinal properties for animals and humans. *Jatropha curcas* is widely used in traditional and folk medicine and veterinary purposes (Duke, 1988). Traditionally it is applied to treat skin infections, fever, constipation, cervical cancer, inflammation, inhibit bleeding, diarrhea, fungal and bacterial infections (Akintayo, 2004). The leaves, stem, roots and all other parts of *Jatropha curcas* show also insecticidal properties. Its latex is used as an external application for skin diseases, rheumatism and for sores on domestic livestock. The seeds of the *Jatropha* contain 30 - 40% oil (Gubitzet *et al*., 1999; Akintayo, 2004).

Safety of medicinal plants could be determined through evaluation of histopathology; biological markers synthesized by vital organs such as liver and kidney as well as different blood parameters. Studying their normal architecture prior to administration of plant extracts or formulations provides a clue to the welfare of these products.

1.5. Liver structure and functions

Liver is the largest single organ in the body next to the skin. It weighs approximately 1.5 kg or 2.5% of the body weight in adult humans. In mice, it weighs approximately 2 g and accounts nearly 6% of the body weight (Harada *et al*., 1999). Liver is located in the right upper quadrant

of the abdomen beneath the diaphragm, surrounded by the Glisson's capsule. It is protected by the thoracic rib cage and occupies large portion of the mouse abdominal cavity (Piper and Suzanne, 2012).

Mouse liver has four lobes with variable lobation pattern as; median lobe, two lateral lobes (one right and one left) and caudal lobe (Cook, 2008). While, human liver has right and left anatomical lobes. Caudate and quadrate lobes are parts of right anatomical lobe (Moore and Dalley, 2006).

The liver gets dual blood supply: oxygen rich blood by the hepatic artery (20-30%) and nutrient rich blood (70-80%) via the portal vein through the hilum where the bile duct also exits (Krause, 2005). Portal vein is formed by the splenic and superior mesenteric veins draining the gastrointestinal tract as well as from the spleen, pancreas and gallbladder (Moore and Dalley, 2006).

Attached to the gallbladder via extra hepatic bile ducts, mice liver is dedicated for vital functions such as bile production and secretion, neutralization and elimination of a variety of toxic substances, drug metabolism, and secretion of blood clotting plasma proteins (Yamada *et al*., 1990). It is also committed for production of urea, preparation of vital amino acids that are the building blocks of proteins, blood filtration and storage, maintaining blood glucose level and metabolism of lipids (Stöhr *et al*., 1978; Bartok *et al*., 1982; Yamada *et al*., 1990; Naito *et al*., 1997; Sigal *et al*., 1999; Longmuir *et al*., 2007; Robertson *et al*., 2008). In addition to its metabolic activity, the liver actively participates in defensive response, inactivating toxins and xenobiotics absorbed by the intestine and eliminating foreign particles from the body (Robertson *et al*., 2008). Currently, several studies in research laboratories depend on mice to answer certain questions of liver structure and functions. Human liver is also the most toxin-exposed organ performing excretory, storage, synthesis and metabolic functions with no recent artificial organ or equipment that compensates its life serving action (Guyton and Hall, 2006).

1.5.1. Histology of liver

Septas from the capsule subdivide the liver in to lobules: the classic lobule, portal lobule, and liver acinus. Classic lobule is hexagonal mass of tissue composed of plates of hepatocytes

radiating away from the central vein (Junqueira and Carneiro, 2005; Ross and Pawlina, 2011). The central vein is located at center. Classic lobule is the basic architectural unit of the liver. At the periphery of each lobule are regularly distributed portal canals (also known as portal tracts). These are areas of connective tissue between lobules containing portal triads composed of branches of portal vein, hepatic artery and bile duct. Lymphatic vessels are also present along with the portal triads (Junqueira and Carneiro, 2012).

Liver portal lobule is the area confined within imaginary lines connecting three adjacent central veins. It contains portions of three adjacent classic liver lobules with portal canals at the center. Exocrine functions of the liver are emphasized with this lobule (Junqueira and Carneiro, 2012).

Liver acinus is a diamond shaped region bordered by two central veins and portal triads. It is divided into zones I, II, and III corresponding to the periportal, mid-, and pericentral zones of the lobule, respectively (Crawford *et al*., 1998; Suriawinata and Thung, 2007). Many Pathological changes, blood perfusion, and metabolic activities of the liver are understood from this acinus. Zone I receives the most oxygenated blood and is least sensitive to ischemic injury while susceptible to toxins. Conversely, zone III has the poorest oxygenation. It is the most affected during a time of ischemia and less vulnerable to toxins. Zone II is intermediate in between zone I and III (Braeuning *et al*., 2006).

The liver comprises parenchymal and non-parenchymal cells. Hepatocytes, where most of the metabolic and synthetic functions of the liver take place are the parenchymal cells (Tortora and Derrickson, 2009). They account about 80% of the volume of the organ and 60% of total liver cells. They are highly polarized, 13 - 30 μm in diameter and polygonal in shape. Their sides are in contact either with sinusoids (sinusoidal face) or neighboring hepatocytes (lateral faces). Each hepatocyte has both microvillar and straight surfaces. The microvilli face the perisinusoidal space and extend into the pericellular space (Tortora and Derrickson, 2009). Their basolateral surfaces face fenestrated sinusoidal endothelial cells, facilitating the exchange of materials between hepatocytes and blood vessels in a persinusoidal space called space of Disse. Fat storing cells, also known as cells of Ito are present also in this space (Ross and Pawlina, 2011). Tight junctions formed between hepatocytes create canaliculi that surround each hepatocyte. Bile secreted from mature hepatocytes is exported sequentially through bile canaliculi surrounded by the apical membrane of neighboring hepatocytes, intrahepatic bile ducts, extrahepatic bile ducts,

and, finally, the duodenum. Bile moves in opposite direction of blood to the portal triads (Junqueira and Carneiro, 2012).

Kupffer cells, natural killer (pit) cells, endothelial cells and Ito cells are the non-parenchymal liver cells. Kupffer cells are macrophages located inside the liver sinusoids. These cells phagocytize ingested pathogens and function as the immunity cells (Stockert and Wolkoff, 2001). In addition to the endothelial or Kupffer cells are also liver-associated lymphocytes (pit cells) killing infected or tumorigenic liver cells (Ross and Pawlina, 2011). The fat storing cells of Ito control sinusoidal blood flow and fibrogenesis. Endothelial cells line the liver sinusoids and control the exchange of materials between the blood and hepatocytes (Schuppan *et al.,* 1998; Stockert and Wolkoff, 2001)

The endocrine and exocrine functions of mice liver are carried out by hepatocytes. Most of them are binucleated cells arranged in cords (Gupta, 2000). Their basolateral portions facing the sinusoidal capillaries display rich microvilli. This microvillous border occupies much of the space of Disse. Their apical portion is associated with bile canaliculi. Fenestrated endothelial cells form 'sieve plates' for the exchange of materials between blood and hepatocytes. Mice liver Kupffer cells are found more frequently around the portal areas for high phagocytosis (Bouwens *et al*., 1986; Bouwens *et al*., 1992). Fat storing cells of Ito are also commonly encountered in peri-portal areas than in peri-central areas (De Bleser *et al*., 1991).

1.6. Kidneys structure and functions

The kidneys in both humans and mice are bean shape bilateral retroperitoneal organs. They are located in the posterior abdominal wall beside the vertebral column (Moore and Dalley, 2006; Piper *et al*., 2012). The renal fascia of the kidney encloses the perinephric fat, suprarenal glands and the kidneys internal to the paranephric fat. In humans the kidney extends from T12 to L3 vertebra. The right kidney is crowded by the liver and lies slightly lower than the left. However, the right kidney of the mice is positioned most cranially than the left kidney in the right renal fossa of the caudate lobe of the liver (Piper *et al*., 2012).

Each kidney has concave medial and convex lateral surfaces. Vessels, nerves, lymphatics, and pelvis of ureter enter and exit the renal sinus through the renal hilum at the medial margin

(Moore and Dalley, 2006; Piper *et al*., 2012). Hemisected kidney reveals an outer light red region cortex, and an inner medulla. Cortex gets 90% to 95% of the blood passing through the kidney and is the site for most of blood filtration. It contains the glomeruli and a heterogeneous population of renal tubule segments. The medulla is located deep to the cortex and extends from the arcuate vessels to the papillary tip (Guyton and Hall, 2006). It is formed from collecting tubules, thick and thin parts of the loops of Henle.

The kidneys coordinate homeostasis in the body. They perform basic processes of urine formation: filtration, reabsorption and secretion. Kidneys regulate blood pH and ion levels especially Na^{+}, K^{+}, Ca^{2+}, and phosphate ions. Excretion of nitrogenous wastes and other hydrophillic toxins, hormone production, controlling blood and extra cellular fluid volume are also the main functions of kidneys (Vainio and Lin, 2002).

1.6.1. Histology of kidneys

The functional units of the kidneys are the nephrons numbering about a million in each kidney in human beings (Nyengaard and Bendtsen, 1992; Tortora and Derrickson, 2009). Each mice kidney contains over 14,000 nephrons (Piper and Suzanne, 2012). Each nephron contains the renal corpuscle, proximal convoluted tubule, thin and thick limbs of Henle's loop and the distal convoluted tubule.

The renal corpuscle has two components: the glomerulus and Bowman’s capsule (Tortora and Derrickson, 2009). The glomerulus is tuft of fenestrated capillaries between afferent and efferent arterioles (Junqueira and Carneiro, 2005). Intra-glomerular mesangial cells provide structural support for the glomerulus. They are modified smooth muscle cells having phagocytic properties. The Bowman's capsule (glomerular capsule) surrounds the glomerulus. It has inner visceral and outer parietal epithelial cell layers. Specialized cells known as podocytes are found adhering to the external surface of the glomerulus (visceral layer of Bowman’s capsule). Podocytes exhibit primary processes and pedicles that form filtration slits around capillaries (Quaggin and Kreidberg, 2008).

The proximal convoluted tubule is formed by simple cuboidal epithelial cells with prominent microvilli on the luminal surface, forming a brush border (Barrett *et al.*, 2010). It is the site where most of solute and water reabsorption takes place. The Henle's loop is a U-shaped tube that consists of a descending limb and an ascending limb. It's descending and ascending thick portions consists of simple cuboidal epithelium while, the descending and ascending thin portions are composed of simple squamous epithelium (Junqueira and Carneiro, 2012). The distal convoluted tubule, like the proximal convoluted tubule, is very tortuous and is lined by simple cuboidal epithelium, but it is shorter and cells are smaller, having no brush border and more empty lumens (Junqueira and Carneiro, 2012).

The juxtaglomerular apparatus is a specialized structure formed by the distal convoluted tubule and the glomerular afferent arteriole. It is located near the vascular pole of the glomerulus and its main function is to regulate blood pressure and the filtration rate of the glomerulus. juxtaglomeruar apparatus consists of juxtaglomerular cells of afferent arteriole, macula densa cells and extra-glomerular mesangial cells. At the point of contact with the arterioles, the cells of the distal tubule become columnar and more closely packed known as macula densa (Junqueira and Carneiro, 2012).

1.7. Blood: Composition and functions

Blood is a fluid connective tissue that circulates through the cardiovascular system. It is "the river of life" that supplies necessary substances to the body cells such as nutrients and oxygen and transports waste products away from those cells in both the mice and human beings. Blood is composed of about 45% formed elements and 55% non-formed elements. The formed elements, which are produced in the bone marrow, include red blood cells, white blood cells and platelets (Junqueira and Carneiro, 2012). The non-cellular component, the blood plasma as liquid extracellular matrix, is primarily water.

The most prevalent blood cells are the red blood cells numbering 3.9 to 5.5 million/mm^3. They have shape of biconcave disks, resulting in a high surface-area-to-volume ratio. Their diameter

ranges 4–7 μm in mouse and 7.5 μm in human binges (Junqueira and Carneiro, 2012; Piper and Suzanne, 2012). RBCs undergo extensive deformation, which permits them to bend and adapt to the irregular turns and small diameters of capillaries (Mohandas and Gallagher, 2008). They lose their nuclei upon maturation and have no other organelles. Erythrocytes have red colored, iron containing complex protein called hemoglobin filling their cytoplasm. They transport oxygen and carbon dioxide to and from tissues. RBCs circulate in the body for about 120 days after which they are removed by macrophages of the spleen, liver, and bone marrow.

WBCs participate in the immune response of organisms. They are of two types, namely granulocytes and agranulocytes according to the type of cytoplasmic granules and their nuclear morphology. Neutrophils, eosinophils and basophils are grouped as granular leukocytes while lymphocytes and monocytes are agranulocytes (Junqueira and Carneiro, 2012). Together with megakaryocytes, WBCs form the buffy coat (located at the red blood cells-plasma interface) which accounts almost 1% of the centrifuged blood. WBCs are capable of ameboid movement and diapedesis to leave the circulatory system in reaction to triggered immune response (Tortora and Derrickson, 2009). Elevated number of leukocytes indicates an exposure to infection (Galli *et al*., 2011).

Neutrophils constitute 60–70% of circulating WBCs (Junqueira and Carneiro, 2012). In mice they comprise 20–25% of peripheral blood leukocytes (Piper and Suzanne, 2012). They have short life span with a half-life of 1.5 hours in mice and 8 hours in humans in the circulation (Galli *et al*., 2011). Neutrophils are 12-15 μm in diameter and have 2-5 lobed nuclei. They contain specific and azurophilic granules in their cytoplasm. Azurophilic granules are specialized to kill ingested bacteria. Neutrophils are important for primary antimicrobial host defense, combating an invading microorganism and quickly responding to sites of infection during acute inflammation (Ley *et al*., 2007; Phillipson and Kubes, 2011; Amulic *et al*., 2012).

Eosinophils are 2–4% of human leukocytes. In mice 0-3% of the leukocytes are eosinophils. These leukocytes modulate inflammatory responses by releasing chemokines, cytokines, and lipid mediators. They also remove antigen-antibody complexes from interstitial fluid (Junqueira and Carneiro, 2012). Eosinophilia, increase in the number of eosinophils in blood, is associated with allergic reactions and helminthic (parasitic) infections (Rothenberg and Hogan, 2006). They have bilobed nuclei and abundant coarse cytoplasmic granules. Eosinophils are 12-17 μm in

diameter. Mouse eosinophils from peripheral blood can take on different shapes and the granules are smaller and less refractile than human eosinophils (Doyle *et al*., 2013). Eosinophils persist in the circulation for 8-12 hours and additional 8-12 days in tissues in the absence of stimulation (Young, 2006).

Basophils are 12–15μm in diameter. Their nucleus has two or more irregular lobes (Junqueira and Carneiro, 2012). They gained their name from the affinity of their cytoplasmic granules for basophilic staining. Basophils are of the least frequent granulocytes that comprise less than 1% of blood leukocytes in humans or mice (Marone *et al*., 2002; Galli *et al*., 2005). They play significant role in defense against certain parasites and have short half-life of only a few days.

Lymphocytes are the most numerous agranulocytes and account for about 30% of blood leukocytes (Ross and Pawlina, 2011). They constitute 70–75% of the peripheral blood leukocytes in mice. With respect to the amount of cytoplasm and size, lymphocytes are divided into small, medium and large. Most lymphocytes in the blood are small with 6–8 μm in diameters (Junqueira and Carneiro, 2012). They are also, divided into B and T cells and the natural killer cells functionally (Ross and Pawlina, 2011). Lymphocytes are the main soldiers in immune system battles by direct cell attack or via antibodies.

Monocytes are the largest leukocytes having the diameter of 12 to 20 μm (Junqueira and Carneiro, 2012). They have distinctly indented or C-shaped nucleus with basophilic cytoplasm. In mice, monocytes comprise 2–6% of the peripheral blood leukocytes. In humans, monocytes comprise 1–6% of blood cells (Piper and Suzanne, 2012). They act as tissue macrophages and antigen-presenting cells in the immune system. Monocytes circulate for 2–3 days in the bloodstream before they migrate and enter into different tissues to replenish the tissue macrophage populations (Sköld *et al.*, 2000; Gordon and Taylor, 2005).

Platelets (or thrombocytes) are 2–4 μm in diameter in both humans and mice (Piper and Suzanne, 2012). They are non-nucleated, disk-like cell fragments formed in the bone marrow from megakaryocytes (Guyton and Hall, 2006; Junqueira and Carneiro, 2012). Platelets are responsible for blood clotting and repair of damaged tissue. They have a life span of about 10 days (Junqueira and Carneiro, 2012).

1.8. Significance of the study

Besides to their wide range of application, safety of medicinal plants still remains as a question to be answered. Safety of different medicinal plant species that play important role in managing various health problems have been investigated by different researchers. Some herbs, such as, *Silybum marianum, Ginkgo biloba* and *Aristolochia fangchi* contain harmful chemicals that cause neuropathy, hepatic failure and spontaneous hemorrhage (Nortier *et al.,* 2000*;* Rowin and Lewis, 1996). Therefore, toxicity investigation of local medicinal plants that are applied for therapeutic or diagnostic purposes is mandatory.

Sub-chronic toxicity study on the laboratory based formulated product of *Eucalyptus globulus* was undertaken using *Jatropha curacus* as fixer and 2% tween-80 as emulsifier by G/Mickael (2014). Accordingly the effective and higher doses of the formulation administered orally to laboratory animals did not significantly change the gross and microscopic structures of liver and kidneys. In addition, the general behavior, blood parameters, organ and body weights of the treated mice were not significantly affected (G/Mickael, 2014). As a next step to this, developing effective anti-ectoparastic drug from these plants was aimed. Towards this goal, the anti-ectoparastic formulation comprising *Eucalyptus globulus* and *Jatropha curcas* oils blended using industry based V497 emulsifier has been developed by EPHI. The safety of this formulation should also be investigated. Therefore, the rationale of this study was to investigate the toxic effects of the formulation on the histopathology of liver, kidney and some blood parameters in mice following acute and sub-chronic administration.

2. Objectives of the study

2.1. General objective

- To investigate the acute and sub-chronic toxic effect of the anti-ectoparastic formulation comprising *Eucalyptus globulus* and *Jatropha curcas* oils blended using industry based V497 emulsifier on the histopathology of liver, kidneys and some blood parameters in mice.

2.2. Specific objectives

- To determine the oral LD_{50} of the formulation in mice.
- To assess the acute and sub-chronic effects of the formulation on general behavior, body weight and gross pathology of liver and kidneys.
- To assess the acute effect of the formulation on weight of liver and kidneys.
- To investigate any histopathological changes of liver and kidneys following sub-chronic treatment of the formulation.
- To evaluate sub-chronic effects of the formulation on some haematological and biochemical parameters of blood.

3. Material and Methods

3.1. Study design

Laboratory-based experiment on Swiss albino mice

3.2. Study area

The study was conducted at Traditional Medicine and Modern Research Directorate Laboratory of EPHI and Addis Ababa University, College of Health Sciences, School of Medicine, Department of Anatomy (Histology Laboratory).

3.3. Study period

The study was conducted from March 2016-August 2017.

3.4. Experimental animal preparation

Healthy adult male and female Swiss albino mice used in both the acute and sub-chronic experiment were obtained from the animal house of EPHI and transported to the Traditional and Modern Medicine Research Directorate Laboratories of EPHI. A total of 72 healthy male and female *Swiss Albino* mice, comprised of 63 females and 9 males, were used in this experiment. Female mice were nulliparous and non-pregnant. Males and females were kept in separate aluminum cages. Grouping was done randomly and animals were marked with permanent marker for individual identification. Prior to the start of dosing, animals were kept for one week to allow for acclimatization (OECD, 2001). Light was set for 12 hours on and 12 hours off throughout the study period. At the commencement of dosing, the animals were between 8-12 weeks old (OECD, 2008).

3.5. Preparation of the stock solution

The antiectoparasitic formulation comprising *Eucalyptus globulus* and *Jatropha curcas* oils blended using industry based V497 emulsifier was collected from Wondogenet agricultural and research center. It has the odor of fresh *Eucalyptus* leaves and white milky appearance. The stock solution contains active ingredient of 20% *Eucalyptus globulus*, 3% fixative oil of *Jatropha curcas*, 15% V497 emulsifier and 100 ml water. Doses of 1.25%, 1.9%, 2.9%, 4.4%, 6.6%, 9.9%, 14.9% and 20% of 1ml/kg body weight of the formulation were prepared for

administration for the acute toxicity, while 1.25% and 3.75% of 1ml/kg body weight were prepared for administration for the sub-chronic toxicity studies (appendex IV).

3.6. Acute toxicity study and LD_{50} determination

Following a week of acclimatization period animals were fasted from food but not water for 3-4 hours prior to administration of the formulation. Each animal was weighed before dosing (OECD, 2001). A total of fifty four female *Swiss Albino* mice were used for the acute toxicity study and LD_{50} determination. The animals were divided into nine groups as GI, GII, GIII, GIV, GV, GVI, GVII, VIII and IX each group contained six animals. The first eight groups (GI-GVIII) were treatment groups taking the formulation at different doses, while; the last one (GIX) was control group given the vehicle, distilled water.

Dose for each animal in a group was calculated according to their body weight taken following the fasting period, while selections of doses for toxicological investigation were based on the prior efficacy study of the formulation. The calculated dose for each animal was administered using intragastric catheter (gavage). Single doses of 1.25%, 1.9%, 2.9%, 4.4%, 6.6%, 9.9%, 14.9% and 20% ml/kg body weight were orally administered for group I, II, III, IV, V, VI, VII, and VIII respectively.

All animal groups were observed for any mortality and signs of toxicity for two hours daily, throughout the consecutive fourteen days. Observations for the different signs of toxicity included increased motor activity, tremors, arching and rolling, ptosis, lacrimation, exophthalmos, piloerection, salivation and depression. Body weight of each animal and differences from the initial weight, was recorded at the 7^{th} day and end of the experiment at 14^{th} day after 3-4 hours of fasting period (OECD, 2001).

At the end of the 14^{th} day of the study, all mice in each group (experimental and control) were sacrificed by cervical dislocation after taking body weight. Careful gross pathological examination of the external surface of the body, abdominal cavities and their contents with special attention for the liver and kidneys was performed. The liver and kidneys were taken out and subjected to further observation for any gross pathological lesions. Then their weights were taken after debridement of all fats and other soft tissues.

3.7. Sub-chronic toxicity study

A total of eighteen, nine male and nine female Swiss albino mice were used for this study. They were grouped into six groups (I, II, III, IV, V and VI) of three mice per group. Groups I, III and V were female mice, while the remaining groups were comprised of male mice. Males and females were kept in separate cages. Administrations of the test chemical to the treatment groups and the vehicle to the control group were began seven days after acclimatization (OECD, 2001).

The first two groups, groups I (females) and II (males) were given 1.25% ml/kg of the formulation while group III (females) and IV (males) treated with 3.75% ml/kg of the formulation in accordance to their body weight. The 5^{th} (females) and 6^{th} (males) groups were control groups receiving the vehicle, distilled water. Weight was measured initially at the beginning of dosing and every 7^{th} day throughout the study period following 3-4hrs of fasting before administration. The minimum dose was taken from the antiectoparasite efficacy of the formulation and the higher dose was taken triple of the minimum dose. Throughout the study period animals were administered every 24hrs with cautious cage side observation for any signs of toxicity.

3.8. Gross pathologic observations

At the end of treatment period each mouse was anesthetized using diethyl ether, blood samples were collected for hematological procedures and humanely sacrificed by cervical dislocation. The whole liver and both right and left kidneys were excised. Detailed gross pathological observation was made for any lesion. The organs were then thoroughly cleaned in distilled water and preserved in a container with 10% neutral buffered formaldehyde solution over 24 hours.

3.9. Histopathological studies

Tissue samples from the right lobe of liver and coronal section of both kidneys were immediately fixed in 10% neutral buffered formalin overnight at room temperature to preserve the cellular structure of the tissue. The following morning tissues were washed with tap water several times, dehydrated in ethanol in step-wise manner passing them through a series of increasing concentrations of 70% and 90% for 120 minutes, followed by absolute alcohol I, absolute alcohol II and absolute alcohol III, each for one and half hours, and absolute alcohol IV overnight.

Xylene-I and xylene-II were then applied to clear alcohol for one and half hours and for two and half hours, respectively. The specimens were then infiltrated with three changes of paraffin wax (I, II and III) for one and half hours, two and half hours, and overnight respectively. Finally the tissues were embedded in paraffin wax in square metal plates forming tissue blocks, whereby each tissue block was labeled and stored at room temperature till sectioned.

Tissue blocks were sectioned with a thickness of 5μm using Leica rotary microtome (LEICA RM 2125 RT, Germany). The ribbons of the tissue sections were gently collected and placed onto the surface of a water bath heated at 40^0C. After the sections were appropriately spread on the water bath, they were mounted over tissue slides. The slides were arranged in slide racks and were placed in an oven with a temperature of 60^0C for 15 minutes to facilitate the adhesion of the specimens onto the glass slides. Specimens were then allowed to cool at room temperature and stained using Hematoxylin and Eosin staining method. The procedures used are also shown in appendix III.

3.10. Hematological and biochemical analyses

Blood samples were withdrawn from the jugular vein at the end of the experiment following anesthetization and prior to scarification of each mouse. Parts of the blood samples obtained from each mouse were collected in separate test tubes with an anti-coagulant substance, EDTA (ethylene diamine tetra-acetic acid) and the remaining parts in plain test tubes with no EDTA. Blood samples from EDTA containing test tubes were immediately processed for hematological parameters using Automated Hematological Analyzer, Sysmex xt-1800i (Sysmex Corporation, Japan). White blood cell count, red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet count were determined. For biochemical analysis, the blood samples in the plain test tubes were allowed to stand for 3 hours for complete clotting and then centrifuged at 5000 rpm for 15 minutes using a bench top centrifuge (Humax-k, Human-Gmbh, Germany). The sera were withdrawn and transferred into other clean vials and kept at -20°C until analysis for clinical biochemistry measurements. The concentrations of alanine aminotransferase, aspartate aminotransferase, total bilirubin, urea, uric acid and creatinine were automatically determined using Cobasintegra 400 plus Analyzer (Rochdiagnostics, Japan).

3.11. Light microscopy and photomicrography

Stained tissue sections of the liver and kidney were carefully examined under binocular compound light microscope (Olympus cx41, Japan). Tissue sections from the treated groups were examined for any evidence of histopathological changes with respect to those of the controls. After examination, photomicrograph of selected slides from both the treated and control groups were taken using (Evos XI, China) automated built-in digital photo camera under a magnification of x20 objective lens.

3.12. Statistical analysis

All data were organized and analyzed using SPSS version 23 statistical software. The values of body and organ weight changes, different hematological and biochemical parameters were analyzed and the results were expressed as M ± SE(x) (standard error of the mean). Differences between experimental and control groups were compared using one-way analysis of variance (ANOVA), followed by Dunnett's T-test to determine their level of significance. Differences at $p<0.05$ were considered statistically significant.

3.13. Ethical consideration

The study was conducted after approval of the study proposal by the Department of Anatomy, School of Medicine, College of Health Sciences, Addis Ababa University and a recommendation letter was sent to Ethiopian Public Health Institute from where ethical clearance letter was obtained. Animals used in this study were kept from any unnecessary painful and terrifying situations (OECD, 2008). To minimize suffering during blood collection prior to sacrification, all animals were anesthetized using diethyl ether.

3.14 Communication of the results

The results of the study will be compiled in the form of a thesis, and communicated to all concerned institutions including the Department of Anatomy (AUU), Ethiopian Public Health Institute (EPHI) and to advisers. It will also be published in reputable journal and disseminated in scientific forums.

4. Results

4.1. Acute toxicity

4.1.1. LD_{50} determination

There was no mortality and death in all treatment groups throughout the study period of acute toxicity. This indicated that, the LD_{50} is greater than 20% ml/kg body weight dose of the formulation.

4.1.2Effects of the formulation on physical signs of toxicity and gross pathology

All treated animals showed no sign of physical toxicity as compared with the controls. However, mice treated with the dose of 20% ml/kg body weight showed sign of toxicity such as hypo-activity, faster breathing, piloerection, loss of appetite and dizziness following administration of the formulation. There were no necropsy and other pathologies on the internal organs including liver and kidneys in all groups of animals, which were sacarified and subjected for gross pathological observations at the end of 2 weeks of observations.

4.1.3. Effects of the formulation on body and organ weights

All animals administered with the formulation showed no statistically significant body weight change ($P>0.05$) as compared with the control groups (table 1). However, there were progressive mean body weight increments in all groups of animals at the 1^{st} and 2^{nd} weeks of measurements.

The mean body weight of mice treated with the formulation at 1.25%ml/kg body weight at the commencement of dosing was 23.95±0.59g. This weight was increased to 25.25±0.84g and 27.11±0.69g at the end of 1^{st} and 2^{nd} week, respectively. The initial mean body weight of mice treated with the formulation at 1.9%ml/kg body weight was 23.36±0.28g and increased to 25.45±0.80g at the end of week one and 27.51±0.41 at the end of week two. The mice in the group treated with 2.9%ml/kg mean body weight was initially 23.98±0.79g and increased to 25.50±0.63 at the end of week one and 26.05±0.51g on the last day of observation period. The mean body weight of mice treated with the formulation at 4.4%ml/kg body weight at the onset of dosing was 25.28±0.56g. This weight was increased to 26.55±0.91g and 28.64±0.82 at the end of week one and week two, respectively. The mice in the group treated with 6.6%ml/kg mean body

weight was initially 24.11±0.54g and increased to 24.75±0.79g at the end of week one and increased to 26.16±0.79g on the last day of observation period. The initial mean body weight of mice treated with the formulation at 9.9%ml/kg body weight was 25.30±0.67g and increased to 26.81±0.60g on day 7 and 29.09±0.79g on day 14. Mean body weight of mice treated with 14.9%ml/kg body weight was 23.23±1.39g. This weight increased to 25.38±0.98g and 26.97±0.92g on the 7^{th} day and at the end of 2^{nd} week, respectively. Initial mean body weight of mice treated with the formulation at 20%ml/kg body weight was 24.08±0.57g and increased to 26.21±1.19g at the end of week one and 28.44±0.84g at the end of week two. Mean body weight of mice in the control group was 24.55±0.20g initially and increased to 25.38±0.98g at the end of week one and increased to 28.03±0.45g on the last day of observation period.

Table1: Mean body weight of female mice treated with the formulation as compared to the controls during the two weeks of observation period (expressed as mean ± Standard Error of the Mean, n = 6)

Group	Dose (ml/kg)	Initial mean body weight (gm)	Mean Body weight at the end of week 1 (gm)	Mean Body weight at the end of week 2 (gm)
I	1.25%	23.95±0.59(0.99)	25.25±0.84(0.98)	27.11±0.69(0.92)
II	1.9%	23.36±0.28(0.76)	25.45±0.80(0.89)	27.51±0.41(0.99)
III	2.9%	23.98±0.79(0.99)	25.50±0.63(0.99)	26.05±0.51(0.27)
IV	4.4%	25.28±0.56(0.97)	26.55±0.919(0.99)	28.64±0.82(0.99)
V	6.6%	24.11±0.54(0.99)	24.75±0.79(0.85)	26.16±0.79(0.32)
VI	9.9%	25.30±0.67(0.97)	26.81±0.60(0.97)	29.09±0.79(0.86)
VII	14.9%	23.23±1.39(0.68)	25.38±0.98(0.99)	26.97±0.92(0.85)
VIII	20%	24.08±0.57(0.99)	26.21±1.19(1)	28.44±0.84(0.99)
IX	Distilled water	24.55±0.20	26.00±0.46	28.03±0.45

The figures under brackets indicate p-value, n – number of mice per group

Examination of the kidneys and liver showed no gross pathology. No necropsy and color changes were observed in both treated and control mice. Weight of these organs showed no significant changes compared with the control groups ($p>0.05$) (table 2).

Mean organ weight of mice treated with the formulation at 1.25% ml/kg, 1.9% ml/kg, 2.9%ml/kg and 4.4%ml/kg body weight was1.53±0.07g, 1.80±0.03g, 1.72±0.07g and 1.81±0.04g for liver and 0.18±0.01g, 0.16±0.01g, 0.17±0.00g and 0.18±0.01g for kidney. Mean organ weight of liver and kidney mice treated with the formulation at doses of 6.6% ml/kg, 9.9% ml/kg, 14.9%ml/kg and 20%ml/kg body weight was 1.78±0.06g, 1.75±0.05g, 1.72±0.08g, 1.80±0.04g and 0.17±0.00g, 0.19±0.01g, 0.17±0.01g and0.18±0.01g respectively.

Table 2: Mean organ weight of female mice treated with the formulation as compared to the controls during two weeks observation period (values are expressed as mean ± Standard error of the mean, n = 6)

Groups	Dose (ml/kg)	Liver (gm)	Kidney (gm)
I	1.25%	1.53±0.07(0.20)	0.18±0.01(0.98)
II	1.9%	1.80±0.03(0.51)	0.16±0.01(0.98)
III	2.9%	1.72±0.07(0.99)	0.17±0.00(0.92)
IV	4.4%	1.81±0.04(0.49)	0.18±0.01(0.58)
V	6.6%	1.78±0.06(0.77)	0.17±0.00(0.83)
VI	9.9%	1.75±0.05(0.95)	0.19±0.01(0.12)
VII	14.9%	1.72±0.08(0.99)	0.17±0.01(0.83)
VIII	20%	1.80±0.04(0.54)	0.18±0.01(0.71)
IX	Distilled water	1.69±0.02	0.17±0.00

The figures under brackets indicate p-values, n –number of mice per group

4.2. Sub chronic toxicity study

4.2.1. Effects of the formulation on physical signs of toxicity, gross pathology and body weight

During the three months of study period no death was observed. Animals which received oral repeated doses of 1.25% ml/kg and 3.75% ml/kg body weight showed no signs and symptoms of toxicity including loss of appetite, diarrhea, dizziness, salivation, restlessness, partial hypo-activity and change in their general behavior in comparison with the control groups. Liver and

kidney from all the test animals and control groups evaluated for gross pathological changes suggest no necropsy and lesions.

As shown in table 3 throughout the study period no significant ($p>0.05$) body weight changes were observed in all male and female treatment groups as compared to the control groups. There was, however, body weight gain in both the male experimental as well as control groups.

The initial mean body weight of male mice treated with the formulation at 1.25% ml/kg body weight was 23.03±0.59g. This weight increased to 25.70±0.64g, 29.17±2.01g, 32.00±1.58g, 33.17±2.50g, 33.97±2.65g, 34.27±2.92g, 35.03±2.98g, 35.40±2.95g, 36.53±2.88g, 37.40±2.68g, 38.53±2.54g and 39.93±2.15g from the first to the twelfth week respectively. The mean body weight of male mice treated with the formulation at 3.75%ml/kg body weight was 23.43±0.29g initially. This weight showed insignificant ($p>0.05$) increment to 25.50±0.80g, 29.73±0.48g, 33.27±1.08g, 33.83±1.08g, 34.37±1.21g, 34.23±0.84g, 35.63±0.80g, 36.37±0.80g, 38.03±1.21g, and 39.57±1.78g from first to tenth week. However, insignificant decrement observed through the last two weeks to 38.53±2.33g and 39.13±2.13g. Mean body weight of male mice in the control group was 23.30±0.95g at the onset of the study. This weight increased to 26.06±0.54g, 34.10±0.60g, 35.23±0.49g, 36.37±1.26g, 39.73±0.06g, 38.40±0.12g, 40.40±0.30g, 41.47±0.42g, 42.00±0.47g, 43.30±0.28g, 43.73±0.20g and 44.63±0.47g progressively from the first week of the study to the last week of observation period. The mean body weight of female mice treated with the formulation at 1.25%ml/kg body weight at the commencement of dosing was 23.06±1.43g. This weight gradually increased to 22.67±0.33g, 23.70±0.84g, 26.06±0.67g, 26.70±0.70g, 27.33±0.87g, 28.33±0.43g, 28.77±0.66g, 29.17±0.44g, 29.93±0.55g, 30.70±0.67g, 31.07±0.67g and 31.93±0.64g from the first to the twelfth week respectively. Mean body weight of female mice in the control group was 23.06±1.43g during the onset of the study. This weight increased correspondingly to 23.06±1.43g, 25.37±1.56g, 28.23±2.43g, 27.77±2.39g, 32.43±4.05g, 30.20±2.59g, 30.60±2.69g, 35.93±2.28g, 31.23±2.49g, 31.83±2.44g, 32.97±2.66g, 34.17±2.62g and 30.73±2.79g.

Table 3: Mean body weight of male and female mice treated with the formulation as compared to the controls during the three months administration period (values are expressed as mean ± Standard error of the mean, n = 3)

			Treatment groups (ml/kg body weight/day)	
Weeks	Sex	Control	1.25%	3.75%
Initial	Male	23.30±0.95	23.03±0.59(0.94)	23.43±0.29(0.99)
	Female	23.06±1.43	22.67±0.33(0.94)	22.83±0.67(0.98)
1	Male	26.06±0.54	25.70±0.64(0.89)	25.50±0.80(0.78)
	Female	25.37±1.56	23.70±0.84 (0.56)	24.37±1.19 (0.79)
2	Male	34.10±0.60	29.17±2.01(0.06)	29.73±0.48(0.08)
	Female	28.23±2.43	25.03±0.98 (0.41)	27.03±1.74 (0.86)
3	Male	35.23±0.49	32.00±1.58(0.16)	33.27±1.08(0.42)
	Female	27.77±2.39	26.06±0.67 (0.69)	27.37±1.33 (0.98)
4	Male	36.37±1.26	33.17±2.50 (0.38)	33.83±1.08 (0.52)
	Female	32.43±4.05	26.70±0.70 (0.26)	27.90±1.55 (0.40)
5	Male	39.73±0.06	33.97±2.65 (0.09)	34.37±1.21 (0.11)
	Female	30.20±2.59	27.33±0.87 (0.48)	28.40±1.65 (0.73)
6	Male	38.40±0.12	34.27±2.92 (0.24)	34.23±0.84 (0.23)
	Female	30.60±2.69	28.33±0.43 (0.58)	29.60±1.25 (0.89)
7	Male	40.40±0.30	35.03±2.98 (0.13)	35.63±0.80 (0.18)
	Female	30.73±2.79	28.77±0.66 (0.69)	29.80±1.36 (0.91)
8	Male	41.47±0.42	35.40±2.95 (0.09)	36.37±0.80 (0.15)
	Female	31.23±2.49	29.17±0.44 (0.62)	30.30±1.49 (0.89)
9	Male	42.00±0.47	36.53±2.88 (0.13)	38.03±1.21 (0.29)
	Female	31.83±2.44	29.93±0.55 (0.65)	31.00±1.37 (0.91)
10	Male	43.30±0.28	37.40±2.68 (0.11)	39.57±1.78 (0.33)
	Female	32.97±2.66	30.70±0.67 (0.58)	31.90±1.24 (0.88)
11	Male	43.73±0.20	38.53±2.54 (0.12)	38.53±2.33 (0.12)
	Female	34.17±2.62	31.07±0.67 (0.55)	31.50±2.77 (0.63)
12	Male	44.63±0.47	39.93±2.15 (0.18)	39.13±2.13 (0.12)
	Female	35.93±2.28	31.93±0.64 (0.17)	33.70±0.93 (0.49)

The figures under brackets indicate p-value, n – number of mice per group

4.2.2. Effects of the formulation on hematological and biochemical blood parameters

Treatment with the formulation for 12 weeks did not produce significant ($p>0.05$) change in any of the hematological parameters in the experimental animals as compared to the control groups (table 4). The value of WBC was 4.61±0.53, 25±0.38 and 4.28±0.64 for mice treated with the formulation at 1.25% ml/kg, 3.75% ml/kg body weight and control groups respectively. Mice treated with 1.25%ml/kg body weight of the formulation appeared to have 16.00±1.04 HGB, 47.33±2.01 HCT, 52.47±0.39 MCV, 17.55±0.19 MCH, 34.50±0.38 MCHC and 743±139.52 PLT. Value of these parameters mice treated with 3.75%ml/kg body weight was 9.62±0.29, 16.17±0.31, 50.03±1.11, 52.57±0.46, 17.35±0.22, 35.13±0.22 and 734±122.66 respectively. Hematological parameters of the control mice was 9.90±0.32, 16.83±0.18, 50.50±0.56 53.10±0.54, 17.18±0.69, 34.11±0.44 and 919±137.83 correspondingly for RBC, HGB, HCT, MCV, MCH, MCHC and PLT.

Table 4: Effects of the formulation on hematological parameters of treated mice as compared to the controls during the three months' administration period (values are expressed as mean ± Standard error of the mean, n = 6)

Hematological Parameters	1.25% (ml/kg)	3.75% (ml/kg)	Control
WBC ($x10^3/\mu L$)	4.61±0.53(0.87)	4.25±0.38(0.99)	4.28±0.64
RBC ($x10^6/\mu L$)	9.00±0.61(0.27)	9.62±0.29(0.86)	9.90±0.32
HGB (g/dL)	16.00±1.04(0.57)	16.17±0.31(0.69)	16.83±0.18
HCT (%)	47.33±2.01(0.21)	50.03±1.11(0.96)	50.50±0.56
MCV (fL)	52.47±0.39(0.54)	52.57±0.46(0.64)	53.10±0.54
MCH (pg)	17.55±0.19(0.77)	17.35±0.22(0.94)	17.18±0.69
MCHC (g/dL)	34.50±0.38(0.67)	35.13±0.22(0.11)	34.11±0.44
PLT (x103/iL)	743±139.52(0.56)	734±122.66(0.53)	919±137.83

The figures under brackets indicate p-value, n – number of mice per group

Administration of the formulation showed no significant ($p>0.05$) change in the biochemical parameters of blood in the treatment groups compared to the controls (table 5). The value of ALT, AST, ALP, Urea, Creatinine, Uric Acid and total Bilirubin was 338.30±43.92,

64.43±12.68, 83.85±2.59, 56.52±3.69, 0.15±0.004, 4.15±0.48 and 0.23±0.1 respectively for mice treated at 1.25%ml/kg body weight dose of the formulation. In addition, for mice treated with the formulation at 3.75% ml/kg body weight value of these parameters was , 370.25±61.64, 62.70±10.01, 79.53±15.99, 68.06±12.04, 0.13±0.02, 4.67±0.99 and 0.10±0.01 respectively. The control values of these biochemical parameters were 98.83±14.95, 389.13±32.82, 66.00±10.48, 54.30±2.97, 0.13±0.01, 2.97±0.53 and 0.18±0.01 respectively.

Table 5: Effects of the formulation on biochemical parameters of treated mice as compared to the controls during the three months administration period (Expressed as mean ± Standard error of the mean, n = 6

Parameters	1.25% (ml/kg)	3.75% (ml/kg)	control
ALT (IU/L	64.43±12.68(0.13)	62.70±10.01(0.11)	98.83±14.95
AST(IU/L)	338.30±43.92(0.68)	370.25±61.64(0.94)	389.13±32.82
ALP(IU/L)	83.85±2.59(0.66)	79.53±15.99(0.78)	66.00±10.48
Urea (mg/dL)	56.52±3.69(0.97)	68.06±12.04(0.35)	54.30±2.97
Creatinine (mg/dL)	0.15±0.004(0.59)	0.13±0.02(0.87)	0.13±0.01
Uric Acid	4.15±0.48(0.41)	4.67±0.99(0.19)	2.97±0.53
Total Bilirubin	0.23±0.1(0.26)	0.10±0.01(0.99)	0.18±0.01

The figures under brackets indicate p-value, n – number of mice per group

4.2.3. Effects of the formulation on triglyceride, cholesterol, HDL & LDL levels

As shown in table 6, serum levels of cholesterol, HDL and triglyceride showed no significant difference between the test animals and the control groups. However, the LDL values in the 1.25% ml/kg and 3.75% ml/kg body weight doses treated mice were found significantly ($p>0.05$) increased by 115% and 153% respectively as compared to the controls. The value of cholesterol, HDL and Triglyceride for mice treated with the formulation at 1.25% ml/kg body weight was respectively 139.33±11.96, 95.28±9.72, and 120.37±10.26; and for mice treated with the formulation at 3.75% ml/kg body weight were correspondingly 136.92±13.26, 88.35±7.22 and

118.93±11.32. The control values for these parameters were 109.57±1.8, 89.83±2.05 and 123.40±8.96 respectively.

Table 6: Effects of the formulation on lipid profile of treated mice as compared to the controls during the three months' administration period (Expressed as mean ± Standard error of the mean, n = 6)

Parameters	1.25% (ml/kg)	3.75% (ml/kg)	Control
Cholesterol	139.33±11.96(0.11)	136.92±13.26(0.14)	109.57±1.8
HDL	95.28±9.72(0.81)	88.35±7.22(0.98)	89.83±2.05
LDL	39.80±5.66(0.01)*	46.77±6.23(0.002)*	18.50±0.65
Triglyceride	120.37±10.26(0.97)	118.93±11.32(0.93)	123.40±8.96

The figures under brackets indicate p-value, *: significant, n – number of mice per group

4.2.4. Effects of the formulation on histology of liver

Light microscope examination of liver sections obtained from treated animals showed no significant histopathological architectural differences as compared with the controls. The liver of control groups showed normal histological features with hepatic lobules, formed by cords of hepatocytes separated by hepatic sinusoids (figure 3A&B). Similarly liver of treated animals revealed the typical interanastomosing network of hepatocytes with eosinophilic cytoplasm and basophilic central nuclei arranged in single cell thick plates separated by vascular sinusoid (figure 3B,C&D). However, liver of animals treated with 3.75% ml/kg body weight dose showed small number of portal mononuclear leukocytic infiltration (figure 4F).

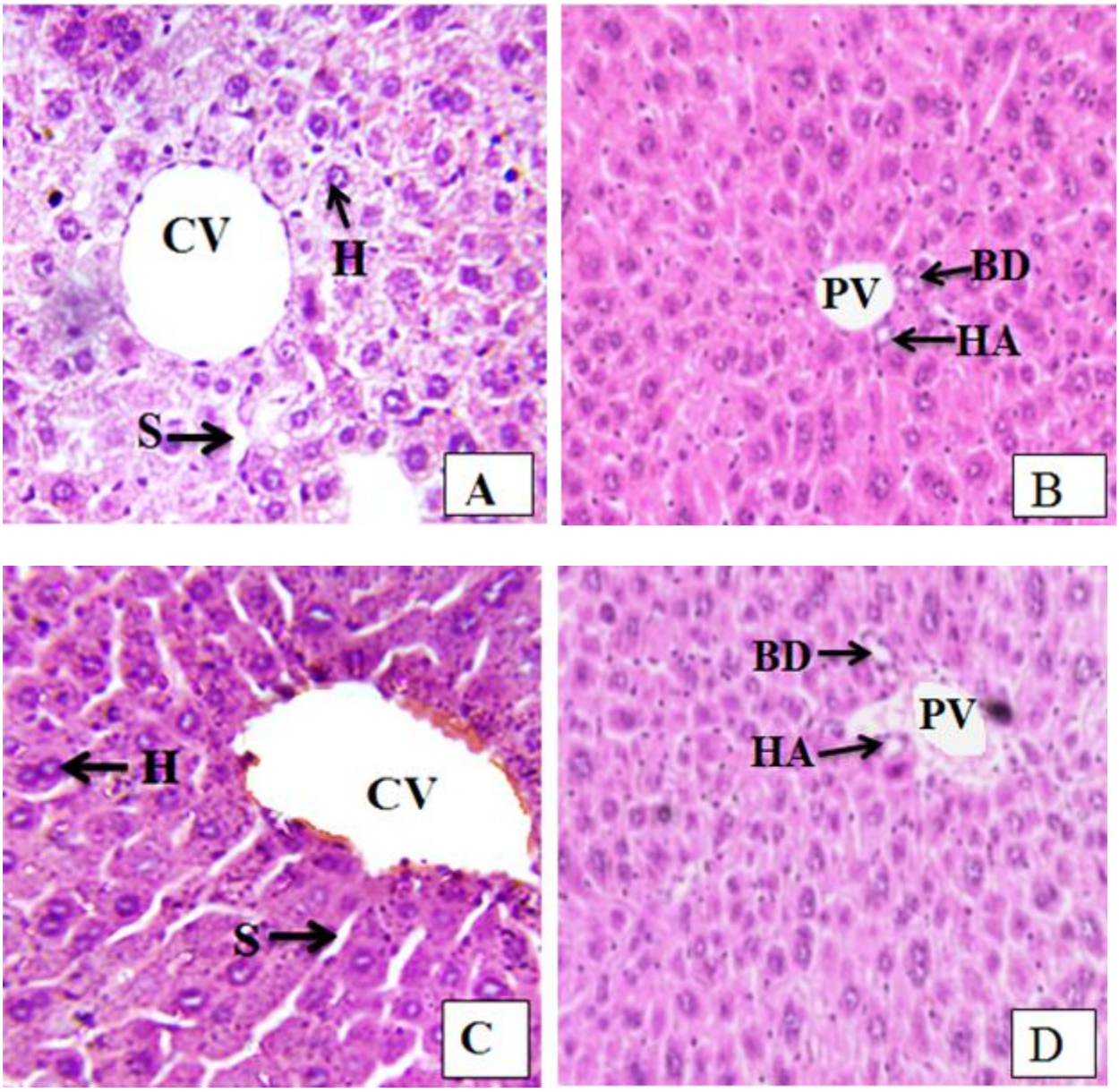

Figure 3: Photomicrographs of liver sections of control mice (A&B), and mice administered with the formulation at 1.25% ml/kg doses (C&D). Sections are from female mice. CV=Central vein, PV = Portal vein, HA = Hepatic artery, H= Hepatocyte, S= sinusoids, BD= Bile duct. (Sections were stained with H&E, X200).

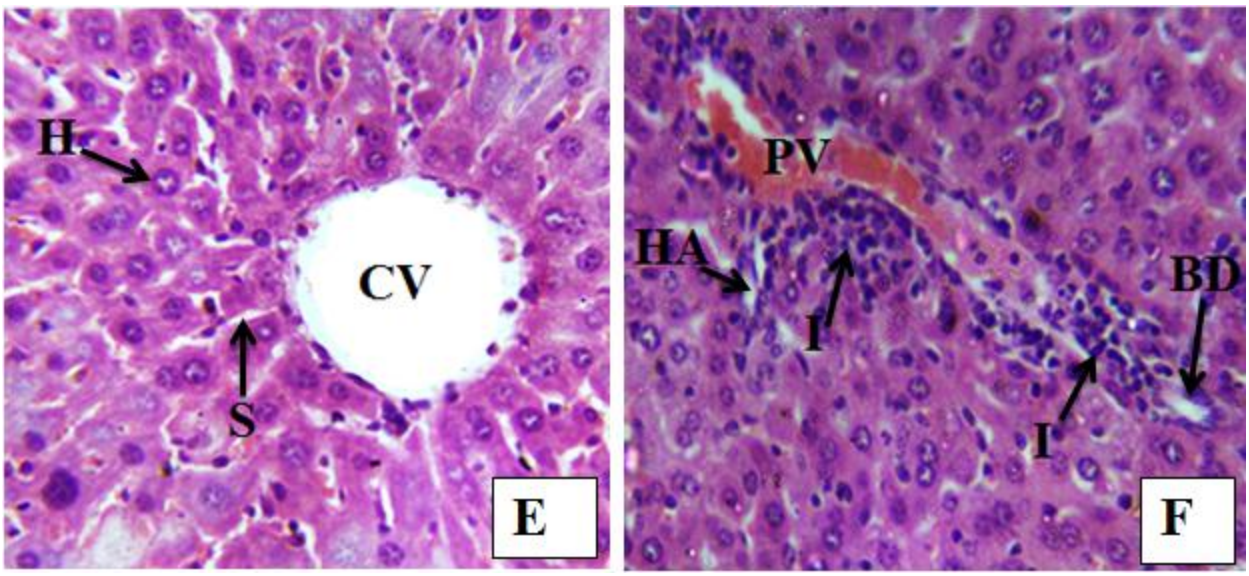

Figure 4: Photomicrographs of liver sections of mice administered with the formulation at 3.75% ml/kg doses (E&F). Sections are from female mice. CV=Central vein, H= Hepatocyte, S= sinusoids, PV = Portal vein, BD = Bile duct, HA = Hepatic artery, I= Leukocytic infiltration. (Sections were stained with H&E, X200). Note: Leukocytic infiltration (I), (F).

4.2.5. Effects of the formulation on histology of kidneys

Histological examination of kidney sections of control mice (figure 5A) showed the normal histological structures of the glomeruli, Bowman's capsule, proximal tubules and distal tubule. The kidneys of treated animals also showed the typical outer cortex and inner medulla, renal corpuscle with normal size of urinary space, normal back to back tubular arrangement with no sign of congestion (figure 5B). However, kidneys of animals treated with 3.75% ml/kg body weight dose of the formulation showed minor tubulointerstitial leukocytic infiltration (Figure 5C).

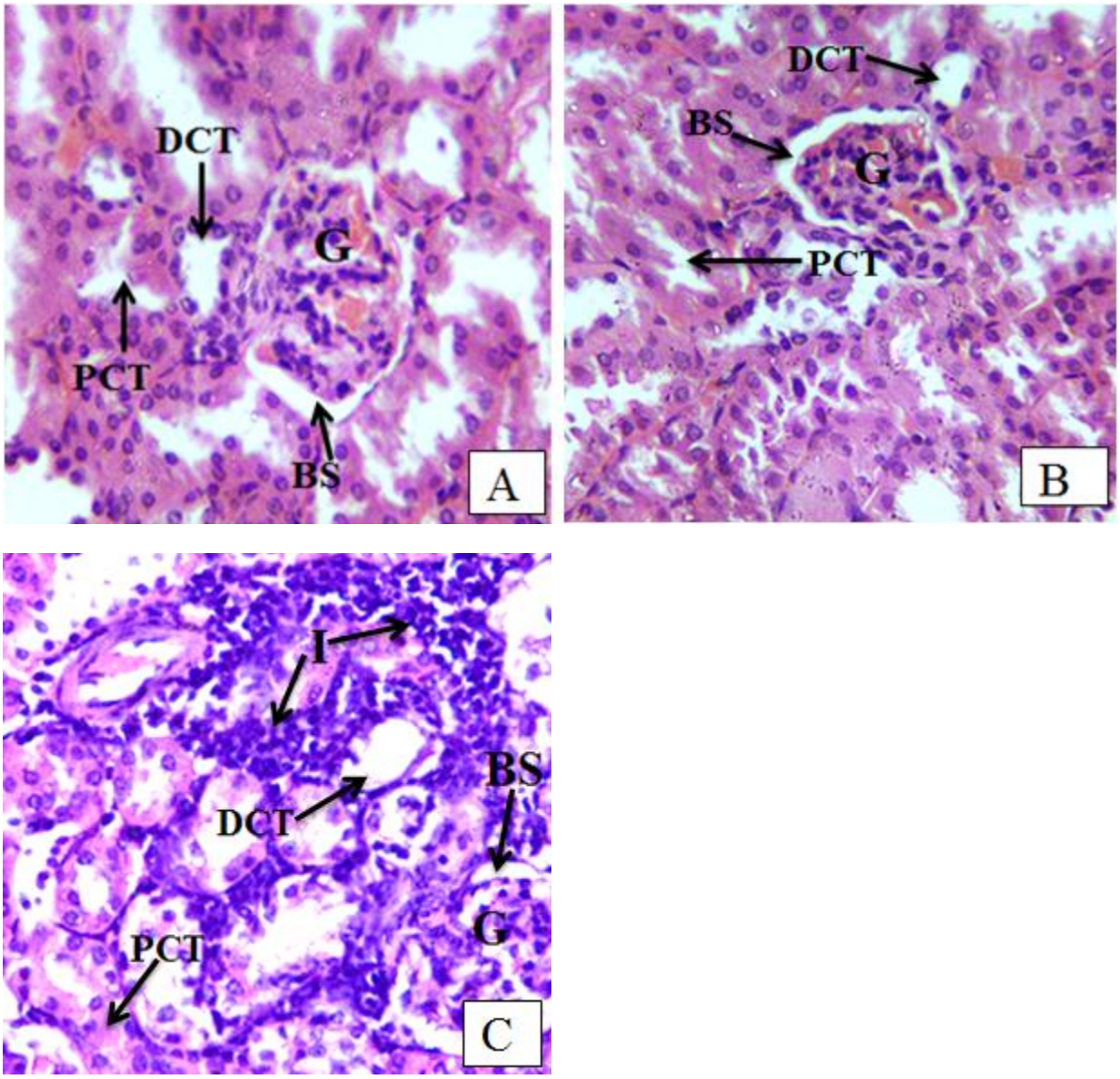

Figure 5: Photomicrographs of kidney sections of control mice (A), and mice administered with 1.25% ml/kg (B), and 3.75% ml/kg (C) of the formulation. Sections are from female mice. G = Glomerulus, BS= Bowman‟s space, DCT = Distal convoluted tubule, PCT = Proximal convoluted tubule, I = Leukocytic infiltration. (Sections were stained with H&E, X200). Note: Tubulointerstitial leukocytic infiltration in the section of mice kidney administered with the formulation at dose of 3.75% ml/kg (C).

5. Discussion

Medicinal plants as a whole or in parts have been commonly utilized to prepare both human and animal medicines throughout the world. Their miraculous value in treatment of ectoparasites acquires great emphasis at the expense of conventional drugs as they are simply accessible, non-expensive, biodegradable, produce no residue, easy for preparation and administration and kill most of the targeted insect pests (Mwale *et al*., 2005). However, not causing harm under the proposed dose and time of therapeutic use admits their practical overutilization. For effective and safe results, application of medicinal plants for treatment of human and animal diseases should not be haphazard. Employing them at the right amount with the right time duration stays mandatory. Addressing to safety questions of natural plant formulations, drugs and reagents performing standard laboratory investigation and implementation of general preclinical toxicity experiments could uncover any potential poisonous effects that may exist. During the toxicity screening of chemicals on test animals investigating changes in general behaviors, body weight and internal organ weight are critical since such deviations are the primary signs of toxic effect of those substances (Carol, 1995). In the present study, acute and sub-chronic toxicity experiments were conducted to investigate the effect of the anti-ectoparasitic formulation comprising *Eucalyptus globulus* and *Jatropha curcas* oils blended using industry based V497 emulsifier on Swiss albino mice.

Acute toxicity test is usually the initial step in the appraisal and evaluation of the toxic characteristics of a substance, supplying information on health hazards. Changes in skin and fur, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, convulsions, tremors, salivation, diarrhea, and other behavioral patterns are measured as toxicity signs in the acute toxicological investigation of substances (OECD, 2008). In the present acute toxicity study animals treated with 1.25%, 1.9%, 2.9%, 4.4%, 6.6%, 9.9%, and 14.9% ml/kg doses of the formulation showed no toxicity signs. Behavioral changes such as hypo-activity, faster breathing, piloerection, loss of appetite and dizziness appeared only at the dose of 20% ml/kg body weight. This result suggests that administration of the formulation lower than 20% ml/kg doses did not strike the general behaviour of the test animals relative to the control groups.

Similar results were reported by G/Mickael (2014) in mice treated with the formulation of oils blended using 2% tween-80 as emulsifier.

The most usual test of short-term toxicity is the LD_{50} test: the dose that can be expected to cause death in half (i.e. 50%) of a group of some particular animal species, usually rats or mice, when administered by a particular route (Randhawa, 2009). Smaller LD_{50} value shows that the more toxic is the chemical. Throughout the fourteen days of observation there were no mortality records in all the treatment groups confirming that the LD_{50} of the formulation is greater than 20% ml/kg. Previous toxicity study conducted by G/Mickael (2014) reported that the LD_{50} for the formulation was 2.5 ml/kg. Results reported by Whitman & Ghazzadeh (1994) also have shown that the oral LD_{50} of eucalyptus oil was 4.44 g/kg for rat and 3.32 g/kg for mice.

Necrosis, distortion in appearance, inflammation, change in color and texture, atrophy or hypertrophy were not observed on the internal organs of the treated animals in comparison with the control groups. Significant changes in organ and body weights between treated and untreated animals are sensitive indices of toxicity after exposure to a toxic substance and imperative indicator of physiological and pathological conditions in animals (Dybing *et al*., 2002; Witthawaskul *et al*., 2003). In this study, administration of the formulation did not cause change in the body weight as well as weight of liver and kidneys. This indicated the good health status of the treated animals suggesting the non-toxicity and non-lethality effect of the formulation.

Administration of any formulation, chemical compounds and drugs for long-term duration may bring significant toxic effects with time. Further investigation was therefore applied to substantiate the sub-chronic effect of the formulation on general behaviour, histopathology of liver and kidney, body weight, hematological and biochemical parameters of blood by administration of the formulation for three months. Shortness of breath, loss of appetite with consequent reduction in feeding (anorexia) and decreased activity, dullness, general behavior changes and body weight deviations are the critical arguments for the assessment of the first signs of toxicity (Sirearatawong *et al*., 2008). In the present sub-chronic toxicity study, no such gross toxicity signs and mortality were documented when the formulation was administered daily at 1.25% ml/kg and 3.75% ml/kg doses for 90 days to the test animals. These observations indicate the safety of the formulation at both doses throughout the study time.

Body weight changes are strongly associated with toxic effects of drugs and medications, stress, feeding phenomena and general health conditions (Vahalia *et al.,* 2011). In the present experiment, body weight of treated animals was not significantly affected compared to the control groups. In line with Ryu (1988) the formulation did not result in loss of appetite and concomitant decrease in food intake or have no any weight affecting toxicological effects.

Hematological parameters deliver blood related activity and toxicity of drugs, formulations and plant extracts (Olson *et al*., 2000). Abnormalities in body metabolic processes, hematopoietic disorders, deprivation and stress can be easily estimated by assessing hematological parameters (Harper, 1993; Raza *et al*., 2002). Reference values of these parameters are also commonly applied in evaluating different changes of internal organs, in response to toxic chemicals and inflammation (Schneck *et al*., 2000). In the present experiment investigation of total WBC count, RBC count, platelet count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) hemograms were performed. Normal values of these parameters vary with intake of toxic plant extracts, formulations and treatments (Ajagbonna *et al*., 1999).

RBC, Hgb, and PCV, are correlated to the total population of red blood cells while MCV, MCHC and MCH are related to individual red blood cell. Anemia may result with the decrease of RBC, Hgb and PCV count as a signal of either the destruction of RBC or their reduced production (Adebayo *et al*., 2005). In contrast, high value of these indices suggest their enhanced production and polycythemia due to disorders in the bone marrow (Iranloye, 2002; Mansi and Lahham, 2008; Kuppast *et al*., 2009; Okpuzor *et al*., 2009). Sub-chronic administration of the formulation for 90 days did not affect values of all the above red blood cells indices in the treated animals as compared with the controls. This suggests that the formulation is relatively safe and does not induce haemolysis, affect the morphology and osmotic fragility of the red blood cells (Adebayo *et al*., 2005). In agreement with Uhmacher *et al*., (2010) these findings also suggest that the formulation has no blood toxicity effect and does not cause anemia. However, this is not in agreement with the findings of Agar *et al*., (1998) which stated that Eucalyptus oil induces oxidative damage of koala red blood cells.

Leukocytes are important blood cells in the immune responses and the disposal of damaged and aged tissues. Their activities increase or decrease in response to toxic environment. WBCs count

detects immune system suppression effect of infections and toxic biochemical materials. Too high or too low WBC counts indicate disease conditions that result in disorder of their production (Pillai and Suresh, 2011; Pillai *et al.*, 2011). The present hematological analysis indicate no significant ($p>0.05$) reduction or rise in the white blood cells count compared to the control groups. These observations demonstrate that the sub-chronic administration of the formulation do not cause suppression in the normal production of WBCs or leukocytosis.

Platelets play key role in homeostasis and thrombosis. They stop bleeding by causing clotting during vascular damage (Saengkhae *et al*., 2008). Under normal conditions, they circulate freely in blood and variation in their values indicate the state of thrombosis. In the present study no significant ($p>0.05$) thrombocytosis or thrombocytopenia was observed. This suggests that the formulation is fair and neither cause toxic effects on thrombopoiesis nor result in platelet destruction (Mdhluli, 2003).

Because of their versatile functions, the liver and kidneys are vulnerable organs to toxic ingredients. Blood tests assist to estimate how well these organs are doing their functions and evaluate potential side effects of medications. Indicators of liver functions, measure how well the organ is performing its normal tasks (Adeoye and Oyedepo, 2004). Liver is more exposed organ for orally ingested drugs and toxic substances through its portal circulation. Liver function tests monitor possible side effects of medications and formulations, check the levels of certain enzymes and proteins in blood and assess the general state of the liver or biliary system. Inflamed or injured liver cells may leak certain chemicals and enzymes into the blood stream higher than normal amounts. The most common serum liver chemistry tests include gamma glutamyl transferase, alanine transaminase, aspartate aminotransferase, alkaline phosphatase, albumin and bilirubin (Mengel *et al*, 2005).

Since AST is also found in other parts of the body and not specific to the liver, it is measured together with alanine transaminase as (ALT-to-AST) ratio to assess liver problems. ALT is hepatic enzyme employed to metabolize proteins in the body and most commonly used pointer of hepatocellular injury. Higher levels of these enzymes suggest damage of liver cells for leaking out into the bloodstream (Edwards *et al*., 1995). In the present test for the serum levels of ALT and AST no significant changes were observed between the treated and control groups. This revealed that the formulation is not a hepatotoxic drug at 1.25% ml/kg and 3.75 ml/kg (Kumar *et*

al, 2004). However, this result is not in agreement with the finding of Arise *et al*. (2009) who stated that frequent administration with aqueous extract of *Eucalyptus globulus* leaves result in a significant rise in acid and alkaline phosphatase activities in the liver and serum of albino rats.

Bilirubin is primarily formed as a metabolic breakdown product of heme derived during destruction of RBCs. Total bilirubin is further subdivided into a water-soluble or ‘‘direct/conjugated bilirubin’’ and lipid-soluble or ‘‘indirect/ unconjugated bilirubin.’’ Serum bilirubin, primarily in an unconjugated form, reflects stability between production and hepatobiliary excretion (Panda, 1989). Due to its small molecular size and water-soluble properties, direct bilirubin appears in urine (Thapa and Walia, 2007). Sub-chronic administration of the formulation cause no significant ($p>0.05$) change in the value of bilirubin suggesting that the formulation is safe.

ALP elevates with the exposure to toxic substances and drugs, with metastatic liver disease, primary biliary cirrhosis and obstruction of the bile ducts. ALP is also found in other organs such as bone, kidneys, placenta, and intestine (Dufour *et al*., 2000). Therefore, to confirm the origin of significant elevation in alkaline phosphatase, measure of other enzymes either the gamma-glutamyl transpeptidase or 5'-nucleotidase and alkaline phosphatase fractionation need to be performed. An elevated gamma-glutamyl transpeptidase suggests that the alkaline phosphatase elevation is of hepatic source (Amacher, 2002). The formulation investigated in the present study does not cause significant ($P>0.05$) change on this parameter suggesting that it does not interfere with the normal functions of the liver.

Kidney function tests check how well the organs regulate and balance the amount of salt and water content in the body, remove wastes and excess fluid from the blood, maintain the blood creatinine level and control the buildup of blood urea nitrogen (Hanisa *et al*., 2011). These tests are also widely applied to assess toxicological effects of formulations, extracts and medications. Levels of serum creatinine, urea, certain dissolved salts and glomerular filtration rate are of the usual blood tests applied to the kidneys (Hanisa *et al*., 2011).

Creatinine is the chemical by-product generated from muscle metabolism as the result of creatine degradation (Heymsfield, 1983). Creatinine passes through the bloodstream to the kidneys to be filtered out and disposed in the urine. The serum creatinine level is comparatively produced at a

relatively constant rate under stable kidney functions. Its value is widely applied to assess glomerular filtration rate (Nematbakhsh *et al.*, 2013). Toxic chemicals, medications and drugs that interfere with the proper activity of kidneys however, affect its normal range. A higher level of creatinine is an indicative of renal deficiency and impairment (Tietz, 2000). In the present investigation the formulation caused no significant ($P>0.05$) increment or decrement in the level of creatinine implying it is not toxic.

Urea and uric acid are other indicators of kidney functions. Urea is a waste product formed from the breakdown of proteins. High level of blood urea ('uraemia') suggests kidney function disease and decline in filtration (Smith *et al*., 2006). In cases of acute or chronic renal toxicity, serum urea usually significantly increases to four or five times higher than the normal values. However reduced blood flow to the kidneys as in various heart cases, dehydration and other problems can also elevate the level of blood urea. Uric acid is byproduct of purine metabolism. Although low values can be related with some kinds of liver or kidney diseases and are unusual, higher levels of uric acid indicate inability and injury of the kidneys to remove it (Smith *et al*., 2006). In the present study the effect of the formulation on the serum urea and uric acid levels of the treated animals were insignificant ($P>0.05$) as compared to the control groups suggesting that the formulation caused no effect in the normal functions of the kidneys.

Lipid profile levels provide useful information on the metabolism of lipids, susceptibility of the heart to atherosclerosis and coronary heart diseases (Toyin *et al*., 2008). Increased values of cholesterol, LDL and triglyceride but not HDL are associated with heart atherosclerosis. In the present study, the formulation administered to the treated animals resulted in no significant deviation of all the parameters, except LDL in all the treated groups compared to the control groups. On the other hand, LDL showed significant ($P<0.05$) increment in the animals treated with both 1.25% ml/kg and 3.75% ml/kg doses of the formulation compared to the control groups. Such effect on LDL may indicate impairment of the transportation of plasma cholesterol (Chia, 1991).

Ingestion of toxic substances distorts not only gross appearance of structures and alters gross behavior but also can cause significant changes in the basic histological architecture of organs and cells. Due to the liver's interaction and communication with digestive system through the hepatic portal circulation and its responsibility for detoxification of harmful substances, it is one

of the primarily vulnerable organs for ingested poisonous drugs, formulations and certain plant extracts (Michalopoulos and Defrances, 1997). Measuring liver function test enzymes assist to investigate healthy liver performance. In addition to these, investigation of its microscopic structures help to further evaluate and quantify liver diseases (Rockey *et al*., 2009; Cui *et al*., 2010).

Toxic chemicals cause heavy necrosis of hepatic cells, hypertrophy and loss of the radial arrangement of hepatocytes, pycnotic nuclei and dilation of central vein with significant infiltration of cells, and apoptosis of hepatocytes resulting in disrupted sinusoidal space and focal necrosis (Kumar *et al*., 2002). Sub-chronic administration of the formulation in this study caused no significant histopathological changes in the liver. Although small number of infiltrations were observed around the portal area, hepatocytes were normal with intact cell margins and normal nuclei. The lobular architecture, portal triad, central vein and sinusoids appeared normal and there were no congestion of sinusoids. These points suggest that the formulation is relatively safe and does not affect the histological configuration of the organ. Nevertheless, the observed small number of the mononuclear leukocytic infiltration may be associated with mild inflammation at higher dose of the formulation.

Similarly, the kidneys are prone organs to many diseases and toxicants because of their role in the filtration, metabolism, and excretion of compounds. Microscopic examinations of damaged kidney sections express variable pathological changes in glomeruli and urinary tubules. Toxic plant extracts, drugs and formulations lead to degeneration of cells lining the kidney tubules, dilatation of Bowman's capsule, accumulation of inflammatory exudates within the lumen of tubules and glomerular shrinkage (Farah *et al*., 2011). Significant focal perivascular lymphocytic infiltrations may result in response to parenchymal cell death with causes ranging from infectious agents, exposure to toxicants, generation of toxic metabolites, and tissue anoxia (Thoolen *et al*., 2010). In the present sub-chronic investigation, the formulation did not cause significant change in kidney architecture in all of the animals treated with 1.25% ml/kg and 3.75% ml/kg doses. This finding suggests that the formulation is safe and does not cause toxicity of these vital organs.

6. Conclusion

Acute administration of the formulation produces no significant general behavior, body and organ weight changes. The LD_{50} was higher than 20% ml/kg dose of the formulation.

In the sub-chronic toxicity experiment mice treated with 1.25% ml/kg and 3.75% ml/kg doses of the formulation showed no significant changes in the general behavior and body weight. In addition, hematological and biochemical parameters as well as the gross and microscopic structures of liver and kidneys were not significantly affected. Among the lipid profile parameters investigated only low-density lipoprotein of treated mice was significantly increased, while others did not change compared to the controls.

From the findings of both acute and sub-chronic toxicity, it can be concluded that the formulation is relatively safe.

7. Recommendations

- Further toxicological investigation of the formulation is recommended on other vital internal organs of the body.
- Further sub-chronic and chronic toxicity studies in other animal model should be carried out.
- The reason for the elevated levels of LDL at both doses, and the small number of infiltrated cells in the organs at the higher dose of the formulation should be further investigated.

8. References

Abebe, D., Urga, K., Debella, A., Ambaye, C. and Dejene, A. 2001. Survey of poisonous plants in southern Ethiopia. *Ethiopian Journal of Health Development*, **15(3)**:209-221.

Adebayo, J.O., Adesokan, A.A., Olatunji, L.A., Buoro, D.O. and Soladoye, A.O. 2005. Effect of ethanolic extract of *Bougainvillea spectabilis* leaves on haematological and serum lipid variables in rats. *Biokemistri*, **17**:45.

Adeoye, B.A. and Oyedapo, O.O. 2004. Toxicity of *Erythrophleum guineense* stem-bark: Role of alkaloidal fraction. *African Journal of Traditional, Complementary and Alternative Medicines*, **1(1)**:45-54.

Agar, N.S., Ogawa, E., O'callaghan, S.S. and Hume, I.D. 1998. The effect of eucalyptus oils on the erythrocytes of koalas. *Comparative Haematology International*, **8(4)**:225-229.

Ajagbonna, O.P., Onifade, K.I. and Suleiman, U. 1999. Haematological and biochemical changes in rats given water extract of *Calotropis procera*. *Sokoto Journal of Veterinary Sciences*, **1(1)**:36-42.

Akintayo, E.T. 2004. Characteristics and composition of *Parkia biglobbossa* and *Jatropha curcas* oils and cakes. *Bio-resource Technology*, **92**: 307-310.

Amacher, D.E. 2002. A toxicologist's guide to biomarkers of hepatic response. *Toxicology*, **21**:253-262.

Amulic, B., Cazalet, C., Hayes, G.L., Metzler, K.D. and Zychlinsky, A. 2012. Neutrophil function: From mechanisms to disease. *Annual Review of Immunology*, **30**:459-489.

Arise, R.O., Malomo, S.O., Adebayo, J.O. and Igunnu, A. 2009. Effects of aqueous extract of *Eucalyptus globulus* on lipid peroxidation and selected enzymes of rat liver. *Journal of Medicinal Plants Research*, **3(2)**:77-81.

Bartok, I., Toth, J., Remenar, E. and Viragh, S. 1982. Fine structure of perisinusoidal cells in developing human and mouse liver. *Acta Morphologica Hungarica*, **31(4)**:337-352.

Berhanu, W., Negussie, H., Alemu, S. and Mazengia, H. 2011. Assessment on major factors that cause skin rejection at Modjo export tannery, Ethiopia. *Tropical Animal Health and Production*, **43(5)**:989-993.

Boland, D.J., Brophy, J.J. and House, A.P.N. 1991. Eucalyptus leaf oils: Use, chemistry, distillation and marketing. Inkata Press, Melbourne/Sydney:23.

Bouwens, L., Baekeland, M., de Zanger, R. and Wisse, E. 1986. Quantitation, tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver. *Hepatology*, **6(4)**:718-722.

Bouwens, L., De Bleser, P., Vanderkerken, K., Geerts, B. and Wisse, E. 1992. Liver cell heterogeneity: Functions of non-parenchymal cells. *Enzyme*, **46(1-3)**:155-168.

Braeuning, A., Ittrich, C., Köhle, C., Hailfinger, S., Bonin, M., Buchmann, A. and Schwarz, M. 2006. Differential gene expression in periportal and perivenous mouse hepatocytes. *The Federation of European Biochemical Societiesis Journal*, **273(22)**:5051-5061.

Bureau of African Affairs. 2006. http:// www.state.gov/r/pa/bgn/2859.htm.

Carels, N. 2009. *Jatropha curcas*: A review. *Advances in Botanical Research*, **50**:39-86.

Carol, S.A. 1995. Acute, subchronic and chronic toxicology. In: Michael JD, Mannfred AH, eds. CRC handbook of toxicology. U.S.A.: CRC Press, Inc.:51-104.

Chia, B.L. 1991. Cholesterol and coronary artery disease- issues in the 1990s. *Singapore Medical Journal*, **32**:291-294.

Cook. 2008. The anatomy of the laboratory mouse. Academic press, London:149-182.

Crawford, A.R., Lin, X.Z. and Crawford, J.M. 1998. The normal adult human liver biopsy: A quantitative reference standard. *Hepatology*, **28(2)**:323-331.

Cui, D.X., Yin, J.Q., Xu, W.X., Chai, F., Liu, B.L. and Zhang, X.B. 2010. Effects of different bile duct flush solutions on biliary tract preservation injury of donated livers for transplantation. *Transplantation Proceedings*, **42(5)**:1576-1581.

Dasgupta, A. 2003. Review of abnormal laboratory test results and toxic effects due to use of herbal medicines. *American Journal of Clinical Pathology*, **120(1)**:127-137.

De Bleser, P., Geerts, A. and Wisse, E. 1991. Role of fat-storing cells in hepatic fibrogenesis. Retinoids as possible therapeutic agents. *Alcohol and Alcoholism (Oxford, Oxfordshire). Supplement*, **1**:345-350.

Doyle, A.D., Jacobsen, E.A., Ochkur, S.I., McGarry, M.P., Shim, K.G., Nguyen, D.T., Protheroe, C., Colbert, D., Kloeber, J., Neely, J. and Shim, K.P. 2013. Expression of the secondary granule proteins major basic protein 1 (MBP-1) and eosinophil peroxidase (EPX) is required for eosinophilopoiesis in mice. *Blood*, **122(5)**:781-790.

Dufour, D.R., Lott, J.A., Nolte, F.S., Gretch, D.R., Koff, R.S. and Seeff, L.B. 2000. Diagnosis and monitoring of hepatic injury. II. Recommendations for use of laboratory tests in screening, diagnosis, and monitoring. *Clinical Chemistry*, **46(12)**:2050-2068.

Duke, J.A. 1988. CRC Handbook of Medicinal Herbs. CRC Press, Boca Raton, FL:253-254.

Dybing, E., Doe, J., Groten, J., Kleiner, J. and O'Brien, J. 2002. Food chemistry. *Toxicology*, **42**:237-282.

Edwards, C.R.W., Bouchier, I.A.D., Haslet, C. and Chilvers, E.R. 1995. Davidson's Principles and Practice of Medicine, 17th edn. Churchill Livingstone:488-490.

FAO. 2010. Food and agriculture organization statistical yearbook, Statistics division. www.fao.org/economic/ess-publications/essyearbook/en/.

Farah, D.P., Hazilawati, H., Rosly, S.M., Shanmugavelu, S. and Noordin, M.M. 2011. Expression of circulating CD146 associated with endovascular dysfunction in adenine-50 induced chronic renal failure in rats using an EvaGreen real-time RT-PCR assay. *Pertanika Journal Tropical Agricultural Sciences*, **34(2**):381-391

Foidl, N., Foidl, G., Sanchez, M., Mittelbach, M. and Hackel, S. 1996. *Jatropha curcas* L. as a source for the production of biofuel in Nicaragua. *Bioresource Technology*, **58(1)**:77-82.

G/Mickael, A. 2014. Toxicity study of anti-ectoparasitic formulation comprising of essential oils obtained from the leaves of *Cymbopogon citratus* and *Eucalyptus glubulus* using *Jatropha curacas* seed oil as a fixer on some blood parameters of liver and kidney in mice. MSc thesis, Addis Ababa University, College of Health Sciences, School of Medicine, Department of Anatomy.

Galli, S.J., Borregaard, N. and Wynn, T.A. 2011. Phenotypic and functional plasticity of cells of innate immunity: Macrophages, mast cells and neutrophils. *Nature Immunology*, **12(11)**:1035-1044.

Galli, S.J., Metcalfe, D.D., Arber, D.A. and Dvorak, A.M. 2005. Basophils and mast cells and their disorders. Williams Hematology. New York*:* McGraw-Hill:538-592.

Gordon, S. and Taylor, P.R. 2005. Monocyte and macrophage heterogeneity. *Nature Reviews Immunology*, **5(12)**:953-964.

Gressel, J. 2008. Transgenics are imperative for biofuel crops. *Plant Science*, **174(3)**:246-263.
Gubitzet, G.M, Mittelbach, M. and Trabi, M. 1999. Exploitation of the tropical oil seed plant *Jatropha curcas* L. Bio-resources. *Technology*, **67**:73-82.

Gupta, S. 2000. Hepatic polyploidy and liver growth control. Seminars in cancer biology, Academic Press, **10(3)**:161-171).

Guyton, A.C. and Hall, J.E. 2006. Textbook of Medical Physiology, 11th edition, Elsevier Saunders:307-309, 859-862.

Hanisa, H., Hadijah, H., Rasedee, A. and Tarmizi, A.S. 2011. Sub-acute oral administrations of *Cymbopogon citratus* stem infusion and its effects on blood biochemical parameters, body and organ weights in rats. *Journal of Tropical Agriculture and Food Science*, **39(1)**:1-2.

Haouel, S., Mediouni-Ben Jemâa, J. and Khouja, M.L. 2010. Postharvest control of the date moth *Ectomyelois ceratoniae* using eucalyptus essential oil fumigation. *Tunisian Journal of Plant Protection*, **5**:201-212.

Haq, I. 2004. Safety of medicinal plants. *Pakistan Journal of Medical Research*, **43(4)**:203-210.

Harada, T., Enomoto, A., Boorman.F. and Maronpot.O. 1999. Liver and gallbladder. In pathology of the mouse (R. R. Maronpot, ed.). *Cache River Press, Vienna, IL*:119-126.

Harper, H.A. 1993. Review of physiological chemistry, 14th ed. California, Lange medical publications:185-402.

Hasegawa, T., Takano, F., Takata, T., Niiyama, M. and Ohta, T. 2008. Bioactive monoterpene glycosides conjugated with gallic acid from the leaves of *Eucalyptus globulus*. *Phytochemistry*, **69(3)**:747-753.

Heymsfield, S.B., Arteaga, C., McManus, C., Smith, J. and Moffitt, S. 1983. Measurement of muscle mass in humans: Validity of the 24-hour urinary creatinine method. *The American Journal of Clinical Nutrition*, **37(3)**:478-494.

Iranloye, B.O. 2002. Effect of chronic garlic feeding on some haematological parameters. *African Journal of Biomedical Research*, **5**:81-82.

Junqueira, L. and Carneiro, J. 2012. Basic histology text and atlas. 12th ed. McGrawHill Medical publishing division, U.S.A:223-224.

Junqueira, L.C. and Carneiro, J. 2005. Basic histology, 11th edition, The McGraw-Hill Companies, Inc.:223-237, 323-324.

Kassa, B. 2005. Pre-slaughter defects of hides/skin and intervention options in east Africa: Harnessing the leather industry to benefit the poor. Regional Workshop Proceedings, April 18-20, Addis Ababa, Ethiopia:71-84.

Kassaye, K.D., Amberbir, A., Getachew, B. and Mussema, Y. 2006. A historical overview of traditional medicine practices and policy in Ethiopia. *Ethiopian Journal of Health Development*, **20(2)**:127-134.

Katembo, B.I and Gray, P.S. 2007. Africa, seeds biofuel. *Journal of Multidisciplinary Reseach*, **1**:1-6.

Kedija, H., Azage, T., Mohammed, Y. and Berhanu, G. 2008. Traditional cow and camel milk production and marketing in agro-pastoral and mixed crop-livestock systems: The case of Mieso

district, Oromia regional state, Ethiopia. Improving productivity and market success of Ethiopian farmer, Project working paper, 13. *International Livestock Research Institute*, **56**:1-3.

Krause, W.J. 2005. Krause's essential human histology for medical students, 3rd ed. Columbia, Missouri:67-87.

Kumar, G., Banu, G.S., Pappa, P.V., Sundararajan, M. and Pandian, M.R. 2004. Hepato protective activity of *Trianthema portulacastrum* L. against paracetamol and thioacetamide intoxication in albino rats. *Journal of Ethnopharmacology*, **92(1)**:37-40.

Kumar, V., Cotran, R. and Robbins, S. 2002. Robbins Basic Pathology, 7th edition, Elsevier Saunders, Philadelphia: 592-593.

Kuppast, I.J., Nayak, P.V., Ravi, M.C. and Biradar, S.S. 2009. Studies on the hematological effect of the extracts of *Cordia dichotoma* forst. f. fruits. *Research Journal of Pharmacology and Pharmacodynamics*, **1(3)**:117-119.

Lee, B.H., Annis, P.C. and Choi, W.S. 2004. Fumigant toxicity of essential oils from the *Myrtaceae* family and 1, 8-cineole against 3 major stored-grain insects. *Journal of Stored Products Research*, **40(5)**:553-564.

Lee, S.E., Choi, W.S., Lee, H.S. and Park, B.S. 2000. Cross-resistance of a chlorpyrifos-methyl resistant strain of *Oryzaephilus surinamensis* (Coleoptera: Cucujidae) to fumigant toxicity of essential oil extracted from *Eucalyptus globulus* and its major monoterpene, 1, 8-cineole. *Journal of Stored Products Research,* **36(4)**:383-389.

Ley, K., Laudanna, C., Cybulsky, M.I. and Nourshargh, S. 2007. Getting to the site of inflammation: The leukocyte adhesion cascade updated. *Nature Reviews Immunology,* **7(9)**:678-689.

Longmuir, K.J., Robertson, R.T., Haynes, S.M., Baratta, J.L. and Waring, A.J. 2006. Effective targeting of liposomes to liver and hepatocytes *in vivo* by incorporation of a plasmodium amino acid sequence. *Pharmaceutical Research*, **23(4)**:759-769.

Lucia, A., Juan, L.W., Zerba, E.N., Harrand, L., Marcó, M. and Masuh, H.M. 2012. Validation of models to estimate the fumigant and larvicidal activity of Eucalyptus essential oils against *Aedes aegypti* (Diptera: Culicidae). *Parasitology Research*, **110(5)**:1675-1686.

Mansi, K. and Lahham, J. 2008. Effects of *Artemisia sieberi* Besser (A. herbaalba) on heart rate and some hematological values in normal and alloxan-induced diabetic rats. *Journal of Basic and Applied Sci*ences, **4(2)**:57-62.

Marone, G., Galli, S.J. and Kitamura, Y. 2002. Probing the roles of mast cells and basophils in natural and acquired immunity, physiology and disease. *Trends in Immunology*, **23(9)**:425-427.

Mdhluli, M. 2003. Toxicological and antifertility investigations of oleanolic acid in male vervet monkeys. PhD Thesis, Discipline of physiological sciences, University of the Western Cape. Bellville:67-74.

Mengel,F., Mark, B., Schwiebert, L. and Peter, P. 2005. Family medicine: Ambulatory care & prevention. McGraw-Hill Professional: 268– ISBN 978-0-07-142322-9. Retrieved 5 August 2016.

Mesfine, T. and Lemma, M. 2001. The role of traditional veterinary herbal medicine and its constraints in the animal health care system in Ethiopia. In: Conservation and sustainable use of medicinal plants in Ethiopia. Medhin Zewdu and Abebe Demissie (Eds); Institute of Biodiversity Conservation and Research, Addis Ababa, Ethiopia:22-28.

Michalopoulos, G.r. and Defrances.1997. Liver regeneration. *Science*, **276**:60-66.

Mohandas, N. and Gallagher, P.G. 2008. Red cell membrane: Past, present, and future. *Blood*, **112(10)**:3939-3948.

Moore, K. and Dalley, A. 2006. Clinically Oriented Anatomy, 5th ed. Lippincott Williams and Wilkins:289-311.

Musyimi, D.M. and Ogur, J.A. 2008. Comparative assessment of antifungal activity of extracts from *Eucalyptus globules* and *Eucalyptus citriodora*. *Research Journal of Phytochemistry*, **2(1)**: 35-43.

Mwale, M., Bhebhe, E., Chimonyo, M., and Halimani, T.E. 2005. Use of herbal plants in poultry health management in the Mushagashe small-scale commercial farming area in Zimbabwe. *International Journal of Applied Research in Veterinary Medicine*, **3**:163–170.

Naito, M., Hasegawa, G. and Takahashi, K. 1997. Development, differentiation, and maturation of Kupffer cells. *Microscopy Research and Technique*, **39(4)**:350-364.

Negese, T. 2011. *In vivo* Acaricidal efficacy of *Eucalyptus globulus* and *Cympogon citratus* against *Sacroptes scaieis* var caprea of goats in Kemise, Oromia zone of Amhara regional state. DVM thesis, Addis Ababa University, College of Agricultural Science and Veterinary Medicine, School of Veterinary Medicine, Department of Veterinary Parasitology.

Nematbakhsh, M., Ebrahimian, S., Tooyserkani, M., Eshraghi-Jazi, F., Talebi. A. and Ashrafi, F. 2013. Gender difference in cisplatin-induced nephrotoxicity in a rat model: Greater intensity of damage in male than female. *Nephrology Urology*, **5(3)**:818-821.

Njoroge, G.N. and Bussmann, R.W. 2006. Herbal usage and informant consensus in Ethnoveterinary management of cattle diseases among the Kikuyus (Central Kenya). *Journal of Ethnopharmacology*, **108**: 332-339.

Nortier,G., Martinez, K., and Schmeiser. R. 2000. Urothelial carcinoma associated with the use of a Chinese herb (*Aristolochia fangchi*). *New England Journal of Medicine*, **342**:1686-1692.

Nwafor, S.V. 2004. Investigation of the antiulcer properties of the methanolic leaf fraction of *Cissampelos mucronata*. *African Journal of Science and Technology*, **5**:109-114.

Nyengaard, J.R. and Bendtsen, T.F. 1992. Glomerular number and size in relation to age, kidney weight, and body surface in normal man. *The Anatomical Record*, **232(2)**:194-201.

OECD Guidelines for the testing of chemicals. 2008. Acute Oral Toxicity – Up-and-Down-Procedure (UDP):1-8.

OECD, 2001. The Organization of Economic Co-operation and Development guideline for testing of chemicals: 420 Acute Oral Toxicity. France:1-14.

Okpuzor, J., Ogbunugafor, H.A. and Kareem, G.K. 2009. Hepatic and hematologic effects of fractions of *Globimetula braunii* in normal albino rats. *Excli Journal*, **8**:182-189.

Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., Lilly, P., Sanders, J., Sipes, G., Bracken, W. and Dorato, M. 2000. Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regulatory Toxicology and Pharmacology*, **32(1)**:56-67.

Panda, N.C. 1989. Kidney. In: Talwar GP, Srivastava LM, Moudgil KD, editors. Textbook of biochemistry and human biology. 2nd ed. India: Prentice-Hall:276-292.

Papachristos, D.P and Stamopoulos, D.C. 2004. Fumigant toxicity of three essential oils on the eggs of *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae). *Journal of Stored Products Research*, **40**:517-525.

Parawira, W., 2010. Biodiesel production from *Jatropha curcas*: A review. *Scientific Research and Essays*, *5*(14), pp.1796-1808.

Phillipson, M. and Kubes, P. 2011. The neutrophil in vascular inflammation. *Nature Medicine*, **17(11)**:1381-1390.

Pillai, P., Suresha, P., Mishrab, G. and Annapurnaa, M. 2011. Evaluation of the acute and sub-acute toxicity of the methanolic leaf extract of *Plectranthus amboinicus* (Lour) Spreng in Balb C mice. *European Journal of Experimental Biology*, **1**:236-245.

Pillai, P.G. and Suresh, P. 2011. Evaluation of acute and subacute toxicity of methanolic extract of *Caesalpinia bonducella* (L) Fleming. *European Journal of Scientific Research*, **53(3)**:462-469.

Piper, M., Treuting, D. and Suzanne, M. 2012. Comparative Anatomy and Histology: First edition:198-206.

Piper, M.J. and Suzamme, M.D. 2012. Comparative Anatomy and Histology : A mouse and human atlas first edition:193-199.

Ponte, F.L.R., Silva, A.A.R. and Maia, M.B.S. 2008. Bee-honey, propolis and *Eucalyptus globulus* extract: Preclinical toxicity study in rodents. *Pharmacognosy Magazine*, **4(16)**:278.

Quaggin, S.E. and Kreidberg, J.A. 2008. Development of the renal glomerulus: Good neighbors and good fences. *Development*, **135(4)**:609-620.

Radostits, O.M., Gay, C., Hinchcliff, K.W. and Constable, P.D. 2007. A textbook of the diseases of cattle, sheep, goats, pigs and horses. Veterinary medicine 10th edition Bailliere, Tindall, London, UK:1576-1580.

Randhawa, M.A. 2009. Calculation of LD50 values from the method of Miller and Tainter, 1944. *Journal of Ayub Medical College, Abbottabad*, **21(3)**:184-185.

Rassaeifar, M., Hosseini, N., Asl, N.H.H., Zandi, P. and Aghdam, A.M. 2013. Allelopathic effect of *Eucalyptus globulus* essential oil on seed germination and seedling establishment of *Amaranthus blitoides* and *Cyndon dactylon*. *Trakia Journal of Sciences*. **11(1)**:73-81.

Raza, M., Al-Shabanah, O., El-Hadiyah, T. and Al-Majed, A. 2002. Effect of prolonged vigabatrin treatment on haematological and biochemical parameters in plasma, liver and kidney of Swiss albino mice. *Journal of Pharmaceutical Sciences*, **70 (2)**:135-145.

Rechav, Y. and Nuttall, P.A. 2000. The effect of male ticks on the feeding performance of immature stages of *Rhipicephalus sanguineus* and *Amblyomma americanum* (Acari: Ixodidae). *Experimental and Applied Acarology*, **24(7)**:569-578.

Robertson, R.T., Baratta, J.L., Haynes, S.M. and Longmuir, K.J. 2008. Liposomes incorporating a Plasmodium amino acid sequence target heparan sulfate binding sites in liver. *Journal of Pharmaceutical Sciences*, **97(8)**:3257-3273.

Rockey, D., Don C., Caldwell, S.H., Goodman, Z.D., Nelson, R.C. and Smith, A.D. 2009. Liver biopsy. *Hepatology*, **49**:1017–1044.

Ross, H. and Pawlina. W. 2011. Histology: A Text and Atlas: With cell correlation and Molecular Biology. 6th edn, Lippincott Williams and Wilkins, pp. 268-299; 628-643; 699-720.

Rothenberg, M.E. and Hogan, S.P. 2006. The eosinophil. *Annual Review of Immunology*, **24**:147-174.

Rowin, J. and Lewis, S.L. 1996. Spontaneous bilateral subdural hematomas associated with chronic *Ginkgo biloba* ingestion. *Neurology*, **46**:1775-1776.

Ryu, J., Ohtsubo, K., Izumiyama, N., Nakamura, K,. Tanaka, T., Yamaura, H. and Ueno, Y. 1988. The acute and chronic toxicities of nivalenol in mice. *Fundamental and Applied Toxicology*, **11**:38-47.

Saengkhae, C., Arunnopparat, W. and Sungkhajorn, P. 2008. Antioxidant activity of *Nelumbo nucifera Gaertn* on oxidative stress-induced erythrocyte hemolysis in hypertensive and normotensive rats. *Journal of Physiological Sciences,* **20**:70-78.

Sakai, S., Toida, T., Kawahara, N. and Goda, Y. 2005. Purification and characterization of major components in" Eucalyptus leaf extract" as a natural food additive. *Japanese Journal of Food Chemistry*, **12**:135-139.

Sarin, R., Sharma, M., Sinharay, S. and Malhotra, R.K. 2007. Jatropha- palm biodiesel blends: An optimum mix for Asia. *Fuel*, **86(10)**:1365-1371.

Schneck, K., Washington, M., Holder, D., Lodge, K. and Motzel, S. 2000. Hematologic and serum biochemical reference values in nontransgenic FVB mice. *Canadian Journal of Comparative Medicine,* **50(1)**:32-35.

Schnitzler, P., Schön, K. and Reichling, J. 2001. Antiviral activity of Australian tea tree oil and eucalyptus oil against herpes simplex virus in cell culture. *Die Pharmazie*, **56(4)**: 343-347.

Schuppan, D., Schmid, M., Somasundaram, R., Ackermann, R., Ruehl, M., Nakamura, T. and Riecken, E.O. 1998. Collagens in the liver extracellular matrix bind hepatocyte growth factor. *Gastroenterology,* **114(1)**:139-152.

Sigal, S.H., Rajvanshi, P., Gorla, G.R., Sokhi, R.P., Saxena, R., Gebhard, D.R., Reid, L.M. and Gupta, S. 1999. Partial hepatectomy-induced polyploidy attenuates hepatocyte replication and activates cell aging events. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, **276(5)**:1260-1272.

Sireatawong, S., Lertprasertsuke, N., Srisawat, U., Thuppia, A., Ngaamjariyawat, A., Suwannlihio, N. and Jaijoy, K. 2008. Acute and sub-chronic toxicity study of the water extract from *Tiliacora triandra*. *Songklanakarin Journal of Science and Technology*, **30**:729-737.

Sköld, C.M., Liu, X.D., Umino, T., Zhu, Y.K., Ertl, R.F., Romberger, D.J. and Rennard, S.I. 2000. Blood monocytes attenuate lung fibroblast contraction of three-dimensional collagen gels in coculture. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, **279(4)**: 667-674.

Smith, G.L., Shlipak, M.G., Havranek, E.P., Foody, J.M., Masoudi, F.A., Rathore, S.S. and Krumholz, H.M. 2006. Serum urea nitrogen, creatinine, and estimators of renal function: Mortality in older patients with cardiovascular disease. *Archives of Internal Medicine*, **166(10)**:1134-1142.

Steenkamp, P.A., Harding, N.M., Van Heerden, F.R. and Van Wyk, B.E. 2006. Identification of atractyloside by LC–ESI–MS in alleged herbal poisonings. *Forensic Science International*, **163(1)**:81-92.

Stockert, R.J. and Wolkoff, A.W. 2001. Cellular and molecular biology of the liver. *Gastroentrol*, **17**:205-210.

Stöhr, G., Deimann, W. and Fahimi, H.D. 1978. Peroxidase-positive endothelial cells in sinusoids of the mouse liver. *Journal of Histochemistry and Cytochemistry*, **26(5)**:409-411.

Suriawinata, A.A. and Thung, S.N. 2007. Liver Histology for Pathologists. 3rd ed. Philadelphia, PA: Lippincott Williams & Wilkins:685-703.

Thapa, B. and Walia, A. 2007. Liver function test and their interpretation. *Indian Journal of Pediatrics*, **74**:67-75.

Thoolen, B., Maronpot, R.R., Harada, T., Nyska, A., Rousseaux, C., Nolte, T., Malarkey, D.E., Kaufmann, W., Küttler, K., Deschl, U. and Nakae, D. 2010. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicologic pathology*, **38(7)**:5-81.

Tietz N.W. 2000. Fundamentals of Clinical Chemistry. 6th ed. Philadelphia, PA: Saunders:744-745, 789-790.

Tortora, G. and Derrickson, B. 2009. Principles of anatomy and physiology, 12th ed, John Wiley & Sons: 114-132, 234-260.

Toyin, Y.M., Adewumi, A.M. and Temidayo, O.A. 2008. Alterations in serum lipid profile of male rats by oral administration of aqueous extract of *Fadogia agrestis* stem. *Research Journal of Medicinal Plant*, **2(2)**:66-73.

Uhrmacher, S., Erdfelder, F. and Kreuzer, K.A. 2010. Flow cytometry and polymerase chain reaction-based analyses of minimal residual disease in chronic lymphocytic leukemia. *Advances in Hematology*, **1**:1-11.

Vahalia, M.K., Thakur, K.S., Nadkarni, S. and Sangle, V.D. 2011. Chronic toxicity study for Tamra bhasma (A generic Ayurvedic mineral formulation) in laboratory animals. *Recent Research in Science and Technology*, **3(11)**:76-79.

Vainio, S. and Lin, Y. 2002. Coordinating early kidney development: Lessons from gene targeting. *Nature Reviews Genetics*, **3(7)**:533-543.

Vankar, P.S., Tiwari, V. and Srivastava, J. 2006. Extracts of steam bark of *Eucalyptus globules* as food dye with high antioxidant properties. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, **5(6)**:1664-1669.

Viegi, L., Pieroni, A., Guarrera, P.M. and Vangelisti. R. 2003. A review of plants used in folk veterinary medicine in Italy as basis for a databank. *Journal of Ethnopharmacology*, **89(2)**:221-244.

Whitman, B.W. and Ghazizadeh, H. 1994. Eucalyptus oil: Therapeutic and toxic aspects of pharmacology in humans and animals. *Journal of Paediatrics and Child Health*, **30(2)**:190.

WHO. 2001. Legal status of traditional medicine and complementary/alternative medicine: A World Wide Review. Geneva:5-12.

WHO. 2003. SARS: Clinical trials on treatment using a combination of traditional Chinese medicine and western medicine. Geneva:53-61.

Witthawaskul, P., Panthong, A., Kanjanapothi, D., Taesothikul, T. and Lertprasertsuke, N. 2003. Acute and subacute toxicities of the saponin mixture isolated from *Schefflera leucantha* Viguier. *Journal of Ethnopharmacology*, **89(1)**:115-121.

Wondimu, W. 2010. Major ectoparasites of small ruminants in Arsi zone of Oromia region, prevalence, species composition and acaricidal efficacy of medicinal plants against lice of sheep. MSc thesis, Addis Ababa University, School of Graduate Studies, Faculty of Veterinary Medicine, Department of Veterinary Parasitology.

Yacob, A. and Catley, A. 2010. Livestock exports from pastoralist areas: An analysis of benefits by wealth group and policy implications. *IGAD Livestock Policy Initiative*, **1(10)**:52.

Yamada, M., Naito, M. and Takahashi, K. 1990. Kupffer cell proliferation and glucan-induced granuloma formation in mice depleted of blood monocytes by strontium-89. *Journal of Leukocyte Biology*, **47(3)**:195-205.

Yasine, A. 2010. Study on Mange mites of small ruminants in Oromia zone of Amhara regional state: *In vitro* and *in vivo* acaricidal efficacy of selected medicinal plants against *Sacroptes scabiei* var. caprae. MSc thesis, Addis Ababa University, School of Graduate Studies, Faculty of Veterinary Medicine, Department of Veterinary Parasitology.

Yirdaw, E. and Luukkanen, O. 2003. Indigenous woody species diversity in *Eucalyptus globulus* ssp. Globulus plantations in the Ethiopian highlands. *Biodiversity and Conservation*, **12**:567-582.

Young, B. 2006. Wheater's Functional Histology: A text and colour atlas., 5th edn. Churchill Livingstone Elsevier:256-281.

9. Appendices

Appendix I: Preparation of working solutions

10% Neutral Buffered Formalin	
40% formaldehyde	100 ml
Distilled water	900 ml
Sodium dihydrogen phosphate monohydrate	4 gm
Disodium hydrogen phosphate anhydrous	6.5 gm
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
1% Alcoholic Eosin (E)	
Eosin Y, water soluble (CI 45380)	1 gm
95% Ethanol	100 ml
Glacial acetic acid	0.5 ml
1% Acidic alcohol	
70% alcohol	500 ml
Hydochloric acid, concentrated	5 ml
Bluing solution	
Sodium bicarbonate	2.5 gm
Distilled water	1000 ml

Appendix II: Tissue processing procedures

Fixation	
10% Neutral Buffered Formalin	24 hrs
Washing	
Tap water	several changes
Dehydration	
70% Ethanol	2 hrs
90% Ethanol	2 hrs
Absolute alcohol I	11/2 hrs
Absolute alcohol II	11/2 hrs
Absolute alcohol III	11/2 hrs
Absolute alcohol IV	overnight
Clearing	
Xylene I	11/2 hrs
Xylene II	21/2 hrs
Infiltration	
Paraffin wax I	11/2 hrs
Paraffin wax II	21/2 hrs
Paraffin wax III	overnight

Appendix III: Hematoxylin and Eosin (H & E) Staining Protocol

Deparaffinization	
Xylene I	5 min
Xylene II	5 min
Rehydration	
Absolute alcohol I	3 min
Absolute alcohol II	3 min
95% Ethanol	3 min
70% Ethanol	3 min
Rinse in distilled water	5 min
Stain in Hematoxylin	15 min
Rinse in running tap water	5 min
Decolorize in acid alcohol	1-3 sec
Rinse in running tap water	5 min
Immerse in Sodium bicarbonate solution	1 min
Rinse in running tap water	5 min
Counterstain in Eosin	1 min
Dehydration	
70% Ethanol	3 min
95% Ethanol	3 min
Absolute alcohol II	3 min
Absolute alcohol I	3 min
Clearing	
Xylene II	5 min
Xylene I	5 min

Appendix IV: Dilution of stock and preparation of volume for administration

Dilution of stock solution

$$C1V1=C2V2$$

C1= Concentration of stock solution

V1=? Volume required

C2= Concentration to be prepared

V2= Volume of solution to be prepared

C1=20%

V1=?

C2=1.25%

V2=10ml

20% × V1 = 1.25 × 10ml

0.6 ml of stock solution + 9.4 ml of distilled water

- The volume for administration is calculated 1ml/kg body weight and multiplied by 10 factorial.

Declaration

This is to certify that the thesis prepared by Shewit Gebre, entitled: Toxicity study of anti-ectoparasitic formulation comprising *Eucalyptus globulus* and *Jatropha curcas* oils blended using industry based emulsifier on the histopathology of liver, kidneys and some blood parameters in mice and submitted in partial fulfillment of the requirements for the degree of Master of Science in Anatomy complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

The Thesis has passed with----------remark.

Shewit Gebre ---------- ----------

Candidate Signature Date

Signed by the examining committee:

Dr. Girmai Gebru ---------- ----------

Examiner Signature Date

Dr. Mekbeb Afework ---------- ----------

Principal Advisor Signature Date

Dr. Asfaw Debela ---------- ----------

Co-Advisor Signature Date

Dr. Wondwosen Ergete ---------- ----------

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Prof. Eyasu Makonnen ---------- ----------

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