Hepatoprotective Effects of Crude Stem Bark Extracts and Solvent Fractions of *Cordia africana* Lam. (Boraginaceae) Against Acetaminophen-induced Liver Injury in Rats

Gudeta Duga (B.Pharm)

A Thesis Submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University, in Partial Fulfillment of the Requirements for the Degree of Master of science in Pharmacology

Addis Ababa University
Addis Ababa, Ethiopia
February, 2021

Addis Ababa University

School of Graduate Studies

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Approved and signed by the examining committee:

External examiner: Dr. Aschalew Nardos (PhD). Signature: _________ Date: ___________

Internal examiner: Dr. Workineh Shibeshi (PhD). Signature: _________ Date: ___________

Advisers: Prof. Eyasu Makonnen (PhD). Signature: _________ Date: ___________

Dr. Shemsu Umer (PhD). Signature: _________ Date: ___________

Dr. Mahlet Arayaselassie (MD). Signature: _________ Date: ___________

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Chair of Department

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Name: ____

Signature: __________________

Place and date of submission: Addis Ababa, Ethiopia, February, 2021

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ABSTRACT

Liver diseases are the major worldwide health problem about 50% of which is drug-induced hepatotoxicity. *Cordia africana* Lam. (Boraginaceae) is widely used in Ethiopian folk medicine for treatment of different types of liver disorders. The aim of this study was to investigate hepatoprotective effects of aqueous (CAAE) and 80% methanol extracts of *C. africana* stem bark (CAME) and the solvent fractions of the CAME against acetaminophen (APAP)-induced liver injury in rats. APAP, 2 g/kg given orally, was used to induce liver injury. Blood samples were taken and serum levels of liver enzymes, and total bilirubin (TB) as well as lipid profiles were determined. Histopathological examination of liver tissues was conducted to confirm the findings of biochemical analysis. Intra-peritoneal (I.P) sodium pentobarbital (SPB)-induced sleep duration was also used to determine the protective effect of the test substances. Oral administration of APAP (2 g/kg) resulted in a significant increase in serum levels of alanine aminotransferase (ALT) (227.80±40.80), aspartate aminotransferase (AST) (417.73±67.10), alkaline phosphatase (ALP) (248.33±34.55), TB (0.19±0.05), low-density lipoprotein (LDL) (13.03±1.70), total cholesterol (TC) (45.95±3.30) and triglycerides (TGs) (114.35±12.14) and decrease in serum high-density lipoprotein (HDL) (19.90±3.44). The levels of all these biomarkers were reversed by the standard drug, silymarin 100 mg/kg. Administration of both crude extracts at different doses and fractions also reversed serum levels of all the parameters tested to ward normal. CAME exerted significant dose-dependent hepatoprotective effects in terms of ALT and AST, while the CAAE showed similar activity in terms of AST, ALP and TGs. The results of histopathological investigations confirmed the hepatoprotective effects shown in the biochemical analysis. *C. africana* stem bark crude extracts and the solvent fractions of the methanol extract showed hepatoprotective effects.

Key words: *Cordia africana*, Ethiopia, Blood biomarkers, Liver enzymes, Lipid profiles, Liver disease, Hepatoprotective effect, Acetaminophen, Silymarin.

ACKNOWLEDGEMENTS

First of all, I would like to thank the Almighty God who gave me health and helped me to successfully complete the thesis. My deepest gratitude goes to my advisers, Professor Eyasu Makonnen, Dr. Shemsu Umer and Dr. Mahlet Arayaselassie for their unreserved encouragement, provision of relevant comments, valuable advice, timely response to my requests and guidance throughout my work. I would like to thank Center for Innovative Drug Development and Therapeutic Trials for Africa (CDT-Africa) for availing chemicals and funding the experimental work. Getahun Alemu, Wogayehu Hailu, Yodit Fikreselasie, Mikias Tesfaye, Hagere Yigazu, Tesfaye Asefa and Bethelhem Fekadu all from CDT Africa are highly acknowledged for their unreserved support in the process of procuring the relevant chemicals and facilitating research staff payment.

I want to acknowledge Ethiopian Public Health Institute (EPHI) for sponsoring the biochemical analysis. Frehiwot Teka, Dr. Getachaw Adis, Yosef Tolcha, Rekik Ashebir and Demirew Bikila are acknowledged for their contribution. Fantu Asefa and Etetu Mamo are also acknowledged for allowing me to use the pharmacology laboratory together with the equipment. I would also like to thank Eshetu Asefa and Besufikad Abebe for all their cooperation in using pharmaceutical chemistry and pharmacognosy laboratory, respectively. Molla Wale has also made appreciable role in this study through providing me specifically required laboratory animals and for his general help, I want to thank him. Moti Sori, a histo-technology professional at the department of pathology, is acknowledged for his professional support in preparing the tissue slides for histopathological investigation.

I want to thank my classmates including; Zawdie Gabrie, Fantawu Girmaw, Dejene Hailu and Hana Saif for their voluntarily help. Mr. Tarekegn Tadesse, a Ph.D. candidate at Andhra University, India, played an appreciable role in providing me the standard drug, silymarin, from India. Without his contribution I would never get the drug silymarin, which is commercially unavailable in Ethiopia and without which the study would have been incomplete. I would like to thank him a lot and wish him all success in his life. My acknowledgement further goes to Addis Ababa University and Ambo University for supporting the research work and sponsoring the whole period of the program, respectively. Lastly, but not the least, I want to thank my families for their encouragement and good will throughout my life.

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LIST OF ABBREVIATIONS AND ACRONYMS

ALD: Alcoholic Liver Disease

ALF: Acute Liver Failure

ALP: Alkaline Phosphatase

ALT: Alanine Amino Transferase

APAP: Acetaminophen (N-acetyl-p-aminophenol)

AST: Aspartate Amino Transferase

BUN: Blood Urea Nitrogen

CAAE: *Cordia africana* stem bark Aqueous Extract

CAAF: Aqueous Fraction of 80% methanol stem bark extract of *Cordia africana*

CABF: Butanol Fraction of 80% methanol stem bark extract of *Cordia africana*

CACF: Chloroform Fraction of 80% methanol stem bark extract of *Cordia africana*

CAM: Complementary and Alternative Medicine

CAME: *Cordia africana* stem bark 80% methanol Extract

CAT: Catalase

CCL_3: Trichloromethyl radical

CCl_4: Carbon Tetra-chloride

CLD: Chronic Liver Disease

CT: Computed Tomography

CYP: Cytochrome p450 enzymes

D-GalN: D-Galactosamine

DNA: Deoxyribonucleic acid

DPX: Dibutylphthalate Polystyrene Xylene

ENAO: Ethiopian National Accreditation Office

GGT: Gamma-Glutamyl Transpeptidase

GSH: Glutathione

HBV: Hepatitis B Virus

HDL: High Density Lipoprotein

HE: Hepatic Encephalopathy

IgG: Immunoglobulin G

IP: Intra-peritoneal

ILAC: International Laboratory Accreditation Cooperation

LD_{50}: Median Lethal Dose

LDH: Lactate Dehydrogenase

LDL: Low Density Lipoprotein

MDME: Microsomal Drug Metabolizing Enzymes

MPT: Mitochondrial Permeability Transition

MRI: Magnetic Resonance Imaging

NAFLD: Nonalcoholic Fatty Liver Disease

NAPQI: N-acetyl-p-benzoquinone imine

NFkB: Nuclear Factor kappa B

NS: Normal Saline

POD: Peroxidase

ROS: Reactive Oxygen Species

SBP: Spontaneous Bacterial Peritonitis

SEM: Standard Error of Mean

SPB: Sodium Pentobarbital

TA: Thioacetamide

TB: Total Bilirubin

TC: Total Cholesterol

TGs: Triglycerides

VLDL: Very Low Density Lipoprotein

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1. INTRODUCTION

Liver is the largest internal organ with an extraordinary spectrum of functions. It weighs approximately 1,500 g and sits in the right upper abdominal cavity beneath the diaphragm protected by the rib cage. It is separated into lobes covered on their external surfaces by a thin connective tissue capsule. The liver is composed of several cell types adapted to perform specific functions. The principal cell type is the hepatic parenchymal cell, generally referred to as hepatocytes. They account for 60% of the total cell population and 80% of the volume of the organ. Other cell types are non-parenchymal and include; Kupffer cells, sinusoidal endothelial cells and stellate cells. Together with sinusoidal space, the non-parenchymal cells comprises the majority of the remaining liver volume (Cagle, 2020).

Most of the functions generally associated with the liver are executed by hepatocytes. Extracting and processing nutrients and other materials from the gut via portal circulation is the primary role. Toxic metabolite generated as by-product of red blood cell destruction, bilirubin, is excreted through hepatocytes in conjugated and soluble form. Except for immunoglobulins, which are synthesized by plasma cells, all of the major plasma proteins for both hepatic and non-hepatic use are secreted by hepatocytes. Hepatocytes play a great role in maintaining steady blood glucose levels. In response to pancreatic islet hormones, hepatocytes either synthesize glycogen from glucose (glycogenesis) or metabolize glycogen to produce glucose (glycogenolysis). Hepatocytes can also manufacture glucose from other carbohydrates (e.g., fructose) and from amino acids (gluconeogenesis) (Boyer *et al.*, 2016).

Phagocytosis of blood-borne toxicants and particulates such as bacteria from the circulation, secretion of inflammatory mediators, forming a leaky barrier between the parenchymal cells and the blood flowing in sinusoids, and storing fat-soluble vitamins are activities done by non-parenchymal liver cells. Lipid metabolism is another important role of liver. The most commonly stored and circulating forms of energy are fatty acids, among which triacylglycerols are the most common non-toxic form. Levels of fatty acids/triacylglycerols originated from different sources are regulated by the activity of cellular molecules that facilitate hepatic fatty acid uptake, fatty acid synthesis, and esterification. Bile acid synthesized by hepatocytes from cholesterol is used as fat emulsification agents in the small intestine (Nguyen *et al.*, 2008).

1.1. Liver Disorders

Multidimensional functions and its strategic location make the liver prone to many diseases. Liver diseases are largely neglected health issue in developing countries which carry the highest burden but receive little attention (Cainelli, 2012). Based on its duration, liver disease might be acute or chronic. While acute liver disease is characterized by a rapid loss of liver function in days or weeks, while chronic liver disease is a disordered liver function lasting for six or more months. It results from a process of progressive destruction and regeneration of the liver parenchyma and encompasses a wide range of liver pathologies including chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Chronic liver disease (CLD) is a major cause of morbidity and mortality, and was responsible for an estimated 1.3 million deaths worldwide in 2015 (Wang *et al*., 2016).

In addition to genetic cause, there are multiple known forms of liver disease caused by a variety of factors. The most common liver diseases include viral hepatitis (A, B, C, D and E), alcoholic hepatitis, non-alcoholic fatty liver disease, cirrhosis, and liver cancer. Hepatitis viruses are the most common infectious causes of liver diseases. They are strongly associated with chronic liver disease (Abdelmenan *et al*., 2019). The immune system also might go wrong and attack some parts of the liver causing autoimmune liver disease. Cancer from other parts of the body like; lung, breast, colon, can spread to and cause liver disease (Liberal & Grant, 2016).

Many drugs, toxins and herbal medicines have been reported to cause liver injury and drugs account for 20-40% of all instances of hepatic failure. Consequently, many approved drugs are withdrawn due to drug-induced hepatic injury. Acetaminophen (N-acetyl-p-aminophenol, APAP), also called paracetamol, is a frequently used analgesic-antipyretic drug which causes hepatotoxicity at overdoses (Gibson *et al*., 1996).

1.2. Pathophysiology of Liver Diseases

Above a certain quantity, alcohol consumption can elicit a spectrum of liver lesions among which steatosis is presented in nearly all drinkers who consume more than 40 g/day regularly. Alcoholic liver disease (ALD) is also a leading cause of cirrhosis, liver cancer, and acute and chronic liver failure leading to significant morbidity and mortality. Direct toxic effects of alcohol and its main intermediate metabolite, acetaldehyde, plays a critical role in the pathophysiology of ALD. Recently, novel putative mechanisms have been identified in systematic scans covering the entire human genome and raise new hypotheses on previously

unknown pathways. The latter also identify host genetic risk factors for significant liver injury, which may help design prognostic risk scores (Stickel *et al.*, 2017).

Chronic viral hepatitis is a chronic inflammatory reaction of the liver for at least six months duration. Several infectious agents have been identified among which the hepatitis B virus (HBV) infection is the common one. T-lymphocyte-mediated cytotoxicity probably directed against HBc antigen on the hepatocellular membrane has a leading role. A loss of tolerance to autologous liver tissue is regarded as the major pathogenic mechanism in autoimmune chronic hepatitis. Tissue destruction is probably mediated by infiltrating T-lymphocytes, although their antigen specificity is not yet known. Patients with autoimmune hepatitis have an increased expression of the histocompatibility complex antigens (Schölmericn & Holstege, 1986).

Over the last four decades, considerable progress has been made in rodent models toward understanding the mechanisms of APAP induced hepatotoxicity. The majority of a therapeutic dose of APAP is glucuronidated or sulfated and then excreted. A small percentage is metabolized by cytochrome p450 enzymes (CYP) to the reactive intermediate NAPQI (N-acetyl-p-Benzoquinone Imine), which is readily detoxified by conjugation with GSH. Formation of excess NAPQI results from higher doses of APAP and leads to saturation of the glucuronidation and sulfation pathways. The additional reactive metabolite depletes liver GSH and binds to proteins. Ultra-structural and biochemical studies demonstrated that toxic doses of APAP could cause changes in the morphology and function of liver mitochondria (Yan *et al.*, 2018).

1.3. Complications of Liver Diseases

A variety of complications are associated with acute and chronic liver diseases. Cirrhosis is the result of chronic inflammation and development of fibrosis that leads to various complications of CLD. It results in around 29,000 deaths annually in the United States (Mohammad *et al.*, 2010). One of the complications of CLD is portal hypertension which results from the replacement of normal hepatic parenchyma with fibrotic tissue leading to resistance to blood flow through the liver. It can lead to other complications of CLD, including the development of varices and variceal bleeding, ascites, and spontaneous bacterial peritonitis (SBP). In addition, the loss of hepatocytes and intra-hepatic shunting of blood caused by portal hypertension diminishes the liver's metabolic and synthetic function. This may result in a reduction in ammonia metabolism, further leading to hepatic encephalopathy (HE) (Schölmericn & Holstege, 1986).

HE is a complex neuropsychiatric syndrome marked by personality changes, intellectual impairment, and an altered level of consciousness. It is associated with hepatocyte loss and dysfunction, and portosystemic shunting, which allow nitrogenous substances derived from the gut to adversely affect brain function. HE is a common and distressing complication, developing in 30% to 45% of patients with decompensated cirrhosis. Ammonia continues to be the toxin most often implicated in the pathogenesis of HE. However, an elevated serum ammonia level is not required to make a diagnosis of HE and does not aid in staging or prognosticating the disease (Perri & Khosravani, 2016).

Anemia is a frequently observed manifestation during the clinical course of CLD and occurs in up to 75% of patients. The frequent occurrence of anemia in CLD is due to the structural relationship between the liver and the hematopoietic system, the role of the enterohepatic cycle in supplying the elements and vitamins necessary for hematopoiesis, and the effect of hepatocyte function on red blood cell production and survival (Sobhonslidsuk *et al.*, 2006). Increased portal pressure causes a common complication of cirrhosis, esophageal varices. The presence of esophageal varices usually correlates with the severity of liver disease. Approximately half of patients with cirrhosis develop esophageal varices, and of these, one-third will develop a variceal bleed. With an episode of active bleeding, there is a 30% chance of mortality and a 70% risk of hemorrhage recurrence within; all these might account for the occurrence of anemia (Schölmericn & Holstege, 1986; Perri & Khosravani, 2016).

The general causes, pathophysiology and complication of liver diseases are summarized in figure1 (Nallagangula *et al.*, 2017).

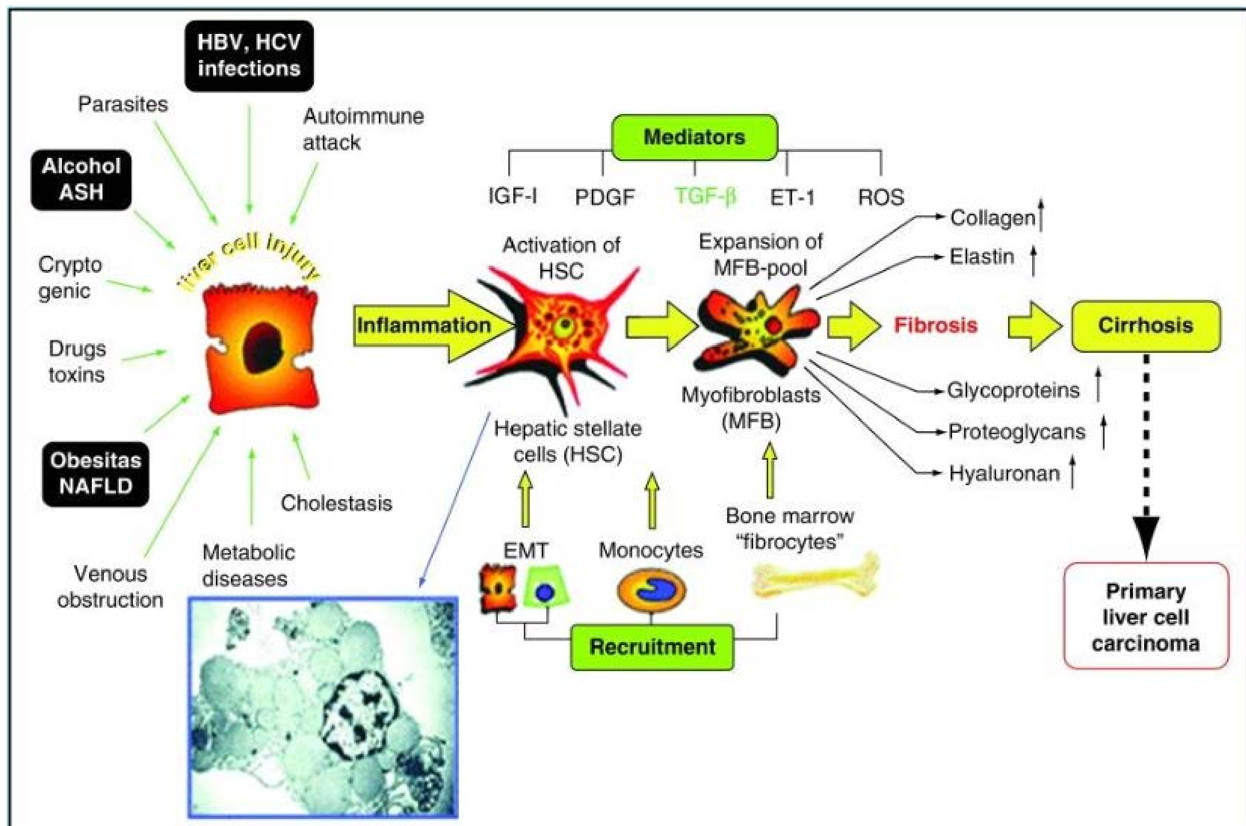


Figure 1. 1: Causes, pathophysiology and complications of liver fibrosis.

After chronic liver injury, necrotic or apoptotic cells will be replaced by regenerated parenchymal cells. Inflammation-connected activation of hepatic stellate cells takes place and trans-differentiation into myofibroblast-like cells which attain contractile, proinflammatory, and fibrogenic property. ASH: Alcoholic steatohepatitis; EMT: Epithelial-mesenchymal transition; ET-1: Endothelin-1; HSC: Hepatic stellate cell; IGF-I: Insulin-like growth factor-I; NAFLD: Nonalcoholic fatty liver disease; PDGF: Platelet derived growth factor; ROS: Reactive oxygen species; TGF β: Tumor growth factor beta.

1.4. Clinical Features and Diagnosis of Liver Disease

Clinical features of the liver can be both hepatic- and extra-hepatic manifestations. In the early stage of cirrhosis, there are usually no symptoms. However, the early signs of cirrhosis, in B-ultrasonography include inhomogeneity of the hepatic tissue, irregularity of the hepatic surface, or enlargement of the caudate lobe. Portal hypertension leads to splenomegaly. In advanced liver disease approaching the stage of cirrhosis, thrombocytopenia is seen, along with impaired hepatic biosynthesis (as shown by low concentration of albumin and cholinesterase, elevation

of the international normalized ratio (INR), and impairment of the detoxifying function of the liver (as shown by elevated bilirubin concentration) (Wiegand & Berg, 2013).

Extra-hepatic manifestations, although uncommon, represent clinically important aspects of hepatitis B and C. Dermatological presentations include porphyria cutanea tarda (PCT), lichen planus and vasculitic rashes associated with cryoglobulinemia. Arthropathy, Sjogren's syndrome and polyarteritis nodosa are rheumatological manifestations of the disease. A high serum globulin level may indicate the presence of cryoglobulinemia, which may be associated with systemic complications such as glomerulonephritis and vasculitis. Other hematological abnormalities are thrombocytopenia and leucopenia. Thrombocytopenia may be the result of hypersplenism or drug therapy, or it may be immune-mediated. Neurological complications may be related to cryoglobulinemia and present with mononeuritis of cranial or peripheral nerves (Sim *et al.*, 2008).

Diagnosis of liver disorders can be made through serological test, histological test and radio techniques. Serological test provides values of aspartate aminotransferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), bilirubin, prothrombin time (PT), Gamma-glutamyl transpeptidase (GGT), albumin, immunoglobulins mainly immunoglobulin G (IgG), creatinine level and sodium level. These commonly used noninvasive clinical biomarkers of liver disease can be grouped in to two, viz. markers of liver injury and markers of liver function. The aminotransferases ALT and AST are the best-known examples of liver injury biomarkers. The presence of these two biomarkers in serum, particularly ALT, is generally thought to indicate loss of hepatocyte membrane integrity. Serum bilirubin and various measures of coagulation time are biomarkers of liver function (Mcgill *et al.*, 2016).

Since liver plays a critical role on lipid metabolism, lipid profiles are other biomarkers whose levels are affected by liver injury. Accordingly, in chronic liver disease, lower levels of lipid biomarkers such as total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol could be observed due to decreased biochemical synthetic capacity of the liver (Subhan *et al.*, 2012). On the other hand, there could be pathological accumulation of triglycerides in liver cells resulting from damage in homeostasis of hepatic lipid metabolism. This could be due to compensatory up regulation of triglyceride synthesis, impaired decomposition of lipid droplet, and decreased secretion of triglyceride and

very low density lipoprotein (VLDL), all these will ultimately result in fatty liver (Ding *et al.*, 2018).

However, the reverse is true when liver is intoxicated by potent environmental toxicants such as carbon tetra chloride (CCL_4) and hepatotoxic drugs such as APAP. Levels of liver biomarkers such as ALT, AST, total bilirubin (TB), ALP, TC, TG, LDL, VLDL, blood urea nitrogen (BUN) and globulin have been significantly increased by acute administration of the widely used hepatotoxic agents such as CCL_4 and APAP. Experimental studies used CCL_4-induced liver damages (Mekky *et al.*, 2016; Shahwan & Zain Al Abdin, 2018) have shown significant increase in the levels of the mentioned biomarkers. Intoxication of animals by APAP resulted in significant increase of the parameters (Oyagbemi & Odetola, 2010; Kumar *et al.*, 2012; Asadollahi *et al.*, 2014; Al-harbi, 2015) in experimental models. On the other hand, the levels of HDL, total protein and albumin were significantly decreased by both CCL_4 and APAP. The same results were obtained from D-Galactosamine (D-GalN), other hepatotoxin agent used in models of liver injury (Mondal *et al.*, 2020).

Low serum sodium indicates severe liver disease due to excessive diuretic therapy or defective free water clearance. Albumin level decreases, serum creatinine concentration increases and the prothrombin time prolong. Liver biopsy is considered the gold standard histological test for diagnosis and sequential histological grading of fibrosis. It also serves to confirm the type and severity of liver disease. Stains are required for copper and iron measurement to confirm the diagnosis of Wilson's disease or iron overload. Immunocytochemical stains detect viruses, bile ducts and angiogenic structures. Ultrasound examination, endoscopy, Magnetic Resonance Imaging (MRI) and Computerized Tomography (CT) scan are radio techniques used to diagnose liver disorders (Suva, 2018).

1.5. Management of Liver Disease

Many patients complain of anorexia, which may be due to direct compression of ascites on the gastrointestinal tract. Patients should receive adequate calories and protein in diets. Multiple feedings per day, regular exercises including walking and swimming to prevent inactivity and muscle wasting are required. Supervised formal exercise program also benefit debilitated patients. Specific therapies are needed in liver diseases for treatment or to prevent the development of cirrhosis (Suva, 2018). Hepatitis A and B immunization status should be documented and immunizations performed, if indicated. Patients with cirrhosis should be

screened for hepatocellular carcinoma every six to twelve months using imaging. Even though they are only 0 to 75 percent sensitive for hepatocellular carcinoma, CT or right upper quadrant ultrasonography can be used for imaging. Improved modalities may include gadoxetate disodium-enhanced MRI, which has a reported sensitivity of 80 percent (Starr & Raines, 2011).

Salt restriction and diuretics are used to treat ascites. Combination of spironolactone and a loop diuretic are widely used with great caution on serum level of sodium level. Diagnostic paracentesis should be performed for patients with new-onset ascites and it consists cell count, total protein test, albumin level, and bacterial culture and sensitivity. SBP is a common complication of uncontrolled ascites and is diagnosed by ascitic fluid polymorphonuclear cell count greater than 250 cells per mm^3 or positive Gram stain. Patients who have SBP should receive antibiotics within six hours if hospitalized; in those who are ambulatory, antibiotics should be started within 24 hours (Kanwal *et al.*, 2010).

In patients with active encephalopathy, reversible factors should be sought and managed, including constipation, noncompliance with medical therapy, infection (i.e., SBP), electrolyte imbalances, gastrointestinal bleeding, and use of benzodiazepines. Paracentesis should be performed to rule out peritonitis as a cause of encephalopathy. If encephalopathy persists, after paracentesis, then the patient should be treated with disaccharides or rifaximin (Xifaxan). Lactulose is a non-absorbable disaccharide believed to induce absorption of nitrogen into bacteria of fecal flora, making it less available to generate absorbable ammonia (Weber, 1996).

Pharmacological therapy has been associated with a wide variety of alterations in the structure and functions of the liver and biliary system. The majority of these alterations present acutely, identified by elevations of liver enzymes with or without non-specific symptoms, with the development of jaundice or occasionally acute liver failure (ALF) with coagulopathy and encephalopathy in the presence of jaundice. Medications have also been associated with CLD with more distinct imaging and histopathological features such as fatty liver disease, fibrosis, granulomatous hepatitis and nodular regenerative hyperplasia. Each of these forms is identified using the same characteristic features as those used to define the primary condition unrelated to drug etiology (Andrade *et al.*, 2019).

1.6. Experimental Models of Liver Injury

There are numerous causes and incidence of human liver diseases globally. With high prevalence in developing countries, liver diseases such as fibrosis are major worldwide health problem. NAFLD and nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma have become a worldwide health concern of all ages and ethnicities. Due to the scarcity of effective pharmacological treatment, it is necessary to investigate appropriate treatment using appropriate model systems (Torres *et al.*, 2012).

The availability of different model systems for hepatic injury plays a crucial role for a successful development of therapy for the liver diseases. Antihepatotoxic activity of any substance can be screened through several models and these models can be *in vivo*, *in vitro*, or *ex-vixo*. In these systems, hepatic toxicity is induced by different hepatotoxins in cellular cultures, organs or in experimental animals (rats, mice, etc.) and the ability of the drug to prevent or cure will be measured. Because of limitations of the outcome of any model, it is important to recognize the benefits of combining different methods for confirmation of the findings (Delgado-montemayor *et al.*, 2016; Farghali *et al.*, 2016).

1.6.1. *In vitro* Experimental Models

In this type of model, the hepatoprotective effect is measured using fresh hepatocytes, primary hepatocyte cultures and immortalized cell line. The possibility of establishing mechanisms of action at a cellular and molecular level makes this model the best option. Even though it is not possible to maintain primary hepatocyte cultures for a long time, normal metabolic liver properties can be maintained. However biochemical and metabolic aspects of immortalized or carcinogenic lines may differ from normal cells. Parameters used in this model to evaluate protection include, transaminase liberation, cell multiplication, morphology, macromolecular synthesis, oxygen consumption, etc. (Delgado-montemayor *et al.*, 2016).

In vitro experimental models have its own advantage and disadvantage. The tests are affordable and quick in that only two to three days are required, little variability, reproducibility, small amounts of the test substances are required, different samples may be analyzed in the same test. Additionally, primary cultures or fresh hepatocytes require few experimental animals compared to *in vivo* models. On the other hand, *in vitro* data should be verified with *in vivo* systems. The reason is that in an organism, cells do not function independently, but they form close and complicated nets with each other and with the extracellular matrix. In this model, however, it is

little to no cell-to-cell interaction. Therefore, this should be taken into consideration during the interpretation of *in vitro* data. Due to the absence of natural environment, which would control it, there is an elevated cell differentiation rate in isolated cells as well as cell lines (Vodovotz *et al.*, 2005).

1.6.2. *Ex vivo* Experimental Model

As described in the above section, the *in vitro* models in liver research cannot mimic the complex cellular interactions that occur *in vivo*. Due to loss of the natural environment, including cues from the extracellular matrix and neighboring or migratory cells, differentiation rapidly occurs. On the other hand, the utilization of *in vivo* animal experiments itself has other shortcomings. It raises both ethical and financial issues because it requires large numbers of animals for extended time periods. It suffers from inter-individual variation and also has limited implications for human disease due to relevant species differences in molecular pathogenesis (Robin *et al.*, 2012).

Therefore, *ex vivo* models, which: resemble the *in vivo* environment, are reproducible, are low cost and reduce the requirement of live animals, and permit the testing in complex human systems, are needed. Precision-cut tissue slices (PCLS) represent an *ex vivo* tissue culture technique that imitates multi-cellular characteristics of *in vivo* organs, with the possibility of performing morphological studies. Metabolizing enzymes and biliary canaliculus are also maintained in liver slices. PCLS has proven to be a valid *ex vivo* system to study metabolism and liver damage and function as a bridge between *in vivo* systems and cell cultures (Olinga & Schuppan, 2013).

1.6.3. *In vivo* Experimental Models

In vivo models have been widely used. The protection mechanism can be determined through these models. Different biochemical and metabolic markers, as well as histopathological examinations, are used to determine the damage produced and the magnitude of the damage and/or protection due to known dosage administration of different hepatotoxins. *In vivo* models have advantages of the highest degree of correlation with what occurs in humans and all biochemical and histopathological parameters can be measured. The possible effects of the immune and central nervous systems in the development of hepatic diseases can be taken into account. However *in vivo* models increase ethical and financial aspects because it requires a

large number of animals, and the study usually lasts long (Van de Bovenkamp *et al.*, 2007; Delgado-montemayor *et al.*, 2016).

1.7. Mechanisms of Induction of Hepatotoxicity

Hepatotoxins are molecules responsible for liver damage, and the process of hepatotoxicity can be classified as intrinsic or idiosyncratic. If the agent has predictable behavior such as constant latency between development of liver damage and time of exposure, or if it causes dose-dependent injury the process is classified as intrinsic. Hepatotoxins such as; CCl_4, thioacetamide (TA), acetaminophen and ethanol have such behavior. Halothane, sulfonamides and isoniazid are hepatotoxins with idiosyncratic behavior in that they are not predictable but produce toxicity just in a small portion of exposed population, toxicity is not dose-related, occurs after a variable latent period and it is not reproducible in experimental models. Moreover, CCL_4 and APAP are the most widely used models of liver injury (Roth & Ganey, 2010).

CCl_4 toxicity depends on dose and duration of exposure. Effects like loss of Ca_2^+ homeostasis, lipid peroxidation, release of cytokines, apoptotic events and cellular regeneration are produced at low doses. The effects are more severe and the patient may develop fibrosis, cirrhosis, or even cancer at high doses, or if there is a longer exposure. It is metabolized by the cytochrome p450 dependent of monooxygenases, mainly through the CYP2E1 isoform in the endoplasmic reticulum and mitochondria. The metabolite trichloromethyl radical (CCl_3), which is highly reactive, is responsible for hepatotoxicity. The organism's antioxidant defense system is saturated by these radicals and it also reacts with proteins, attack unsaturated fatty acids, generating lipid peroxidation and reduce the amount of CYP450 (Qadrie *et al.*, 2015).

All these lead to a functional failure which results in lowering of protein and accumulation of triglycerides (fatty liver), and alter water and electrolyte equilibrium with increased hepatic enzymes in plasma (Delgado-Montemayor *et al.*, 2015). Level of liver biomarkers such as ALT, AST, TB, ALP, TC, TG, LDL, VLDL, BUN and globulin increased in CCL_4-induced animal models of liver injury. However, levels of HDL, GSH, total protein and albumin were significantly lowered compared to normal control group (Jyothi *et al.*, 2008; Mekky *et al.*, 2016; Shahwan & Zain Al Abdin, 2018).

APAP is an analgesic antipyretic drug which induces acute liver damage by causing necrosis of the hepatocytes at high doses. N-acetyl cysteine, a known scavenger of ROS, is recommended as an effective clinical antidote against APAP-induced acute liver injury even though its narrow

therapeutic range limits its use. Because APAP-induced liver injury is among drug-induced liver damage, it is a widely used experimental model of clinical importance. While APAP is mainly metabolized to non-toxic sulfate and glucuronide conjugates, some fraction of the drug is converted to an electrophilic reactive product, NAPQI, which is detoxified by GSH (Chavan & Kuvalekar, 2019).

Moreover, mitochondrial damage and nuclear deoxyribonucleic acid (DNA) fragmentation has been identified in APAP-induced liver damage in both human and mice (Mcgill *et al.*, 2012). When the GSH in cytoplasm and mitochondria is depleted it covalently binds to protein-thiols. Mitochondria and endoplasmic reticulum are the key sites of dysfunction and an oxidative stress is induced which ultimately leads to mitochondrial permeability transition (MPT)-mediated necrosis. Signal transduction pathways in which the initial oxidative stress activates kinases leading to JNK activation plays a critical role in the development of MPT. Activated JNK binds to its target, SH3BP5 (Sab) on the cytoplasmic face of the mitochondria outer membrane. This leads to a self-sustaining mechanism which further impairs mitochondria function and amplifies ROS production which has two consequences: sustaining JNK activation and promoting MPT (Win *et al.*, 2011a; Win *et al.*, 2014b).

Even though the cellular or intracellular sources and the nature of the ROS in its mechanism remain debatable, APAP-induced liver injury has served as the most popular, mechanistically well studied and clinically relevant model for testing of phytotherapeutics and other hepato-protective interventions (Jaeschke *et al.*, 2013). It has been witnessed by different studies that APAP toxicity caused significant elevation in the levels of ALT, AST, TB, ALP, TC, TG, LDL, VLDL, BUN and globulin while levels of HDL, total protein and albumin were significantly decreased (Oyagbemi & Odetola, 2010; Asadollahi *et al*., 2014).

This model has many advantages over other models. As described earlier APAP is the most accessible and widely used analgesic drug worldwide making it the usual cause of liver injury. Therefore this makes the model best to represent drug-induced liver damage and it highly correlates experimental tests with clinical studies. Additionally, this model has a preliminary screening test, APAP-induced lethality test, used to estimate the hepatoprotective activity before conducting the main studies (Gilani *et al.*, 1997a; Gilani *et al.*, 2005b & Anusha *et al.*, 2011).

While studying hepatoprotective effects of a given product, a standard drug available on the market with proved hepatoprotective effect should be used as a reference. Silymarin is a well-

known hepatoprotective agent widely used as standard in hepatoprotective studies (Fraschini *et al*., 2002). The data in the literature indicate that silymarin and one of its structural congeners, silibinin, act via four mechanisms. They can act as antioxidants, scavengers of free radicals and regulators of the intracellular content of glutathione. They can also stabilize and regulate cell membrane permeability that prevents hepatotoxic agents from entering hepatocytes. It has also been observed that silymarin and silybinin can act through promoting ribosomal ribonucleic acid (RNA) synthesis and stimulating liver regeneration (Papackova *et al*., 2018).

Moreover, transformation of stellate hepatocytes into myofibroblasts can be inhibited by them and the deposition of collagen fibers leading to cirrhosis can be prevented. They also have documented anti-inflammatory and anticarcinogenic properties. Among all mechanisms free radical scavenging activity appears to be the key mechanism that ensures hepatoprotection. Therefore hepatotoxicity of several agents, including, ethanol, acetaminophen and CCL_4 used in animal models can be neutralized by silymarin (Fraschini *et al*., 2002). While acetyl N-Cysteine is a well-known antidote for APAP overdose and which could be used in such experiments as a reference drug, using silymarin has advantage over it. Silymarin itself is a polyphenolic component isolated from the fruits and seeds of the milk thistle plant *Silybum marianum,* Asteraceae family (Papackova *et al*., 2018).

Therefore, it is rational to use silymarin when the products to be tested are plant-derived as this narrows the characteristic differences between test products and the standard reference. Other drugs having confirmed hepatoprotective activity includes L-carnitine, SAMe (S-Adenosylmethionine), Ursodiol, Zinc, and vitamins like; vitamin C and E (Chavan & Kuvalekar, 2019).

1.8. Hepatoprotective Roles of Medicinal Plants

Natural products, such as plants, animals, microorganisms, and marine organisms, have been used by humans to alleviate and treat diseases. The use of these natural products as medicines, however, was through a tremendous challenge to early humans. They suffered from vomiting, diarrhea, coma, or other toxic reactions—perhaps even death. Probably it is when seeking food, they often consumed poisonous plants and this helps them be able to develop knowledge about edible materials and natural medicines. However, in this way, early humans were able to make use of Traditional Medicines (TM) and are of great importance. In their various forms, they may have certain defects, but they are still a valuable repository of human knowledge (Yuan *et al.*,

2016). Medicinal plants widely used in the treatment of liver diseases include *Justicia schimperiana* (Hochst. ex Nees) (Acanthaceae), *Verbascum sinaiticum* Benth. (Scrophulariaceae), *Coptis chinensis* Franch (berberine), *Glycyrrhiza uralensis* Fisch (glycyrrhizin), *Silybum marianum (L.)* Gaertn. (silymarine and silybinin), *Bupleurum chinensis* DC (saikosaponins), *Salvia miltiorrhiza* Bunge (salvianolic acid), *Cordia africana* Lam. (Boraginaceae) and *Scutellaria baicalensis* Georgi (baiclin, wogonin) (Hong *et al.*, 2015).

Ethiopia is one of the most important biodiversity hot spots in the world. Around seven thousands plant species, one thousand of which are endemic to Ethiopia, are documented in the Flora of Ethiopia (2003). However, many of the species are in danger of extinction because of rapid conversion of forest to agricultural land and over-grazing. Due to the cultural acceptability, relatively low cost of TM and limited access to modern health facilities, about 80% of populations in Ethiopia relied on TM for their primary health care (Bekele-tesemma & Tengnäs, 2007). Herbal medicine has become a major contributor to the treatment of liver diseases. As modern drugs may induce liver damage, relying on natural products seems promising to avoid such problems. There are many medicinal plants whose hepatoprotective activities have been confirmed experimentally. Plant-derived natural products and herb medicines have gained significant considerations in recent years due to their various pharmacological properties such as anti-oxidant and anti-inflammatory for hepatoprotective effect (Qadir & Ahmad, 2017).

The results of a comparative study done to demonstrate hepatoprotective and antioxidative activity of *Phyllanthus niruri*, *Maytenus emarginata*, *Eclipta alba*, *Aloe vera*, *Solanum indicum* and *Aegle marmelos* indicate hepatoprotective and antioxidative activity of all studied plants extract against APAP-induced liver toxicity (Parmar, 2010). Similarly, the ethanolic extract of *Ficus carica* leaves was screened for hepatoprotective and antioxidant activity in CCl_4-induced liver damage. The ethanolic extract (50mg/kg, 100mg/kg and 200mg/kg) exhibited significant hepatoprotection against CCl_4-induced liver injury in a dose-dependent manner comparable with the standard drug, silymarin, 10mg/kg, intra-peritoneal (I.P) (Mujeeb *et al.*, 2011). *In vivo* hepatoprotective activities of two Ethiopian medicinal plants, namely, *Justicia schimperiana* (Hochst. ex Nees) (Acanthaceae) and *Verbascum sinaiticum* Benth. (Scrophulariaceae) were evaluated against CCl_4-induced hepatotoxicity in Swiss albino mice. The plasma levels of AST and ALT were significantly suppressed in mice pretreated with the hydro-alcoholic extracts of both plants compared with the CCl_4-intoxicated controls (Umer *et al.*, 2010).

The study done to evaluate the hepatoprotective properties of the stem ethanol extract of *Homalium letestui* Pellegr (Flacourtiaceae) revealed a significant dose-dependent reduction in levels of ALT, AST, ALP, TC, direct and TB whose levels had been increased by APAP, while serum levels of total protein, albumin and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione Peroxidase (GPx) and GSH have been elevated. Marked hepatoprotective effect was observed by consistency of the chemical pathological changes with histopathological observations (Okokon *et al.*, 2017). Hepatoprotective activity of ethanolic extract of *Salix subserrata* Willd flower was evaluated against CCl_4-induced chronic hepatotoxicity in rats. Administration of the extract showed hepatic protection at an oral dose of 150 mg/kg. The extract significantly reduced the elevated serum levels of intracellular liver enzymes and the expression levels of tumor necrosis factor alpha (TNF-α) and nuclear factor kappa B (NFk B) proteins in comparison to CCl_4-intoxicated group. The findings were confirmed by histopathological observations (Wahid *et al.*, 2016).

Moreover, methanol leaf extract of *Diaphananthe bidens* has shown hepatoprotective effect by shortening SPB-induced sleeping time and by reducing levels of biomarkers in liver injury caused by APAP in rats (Aba *et al*., 2019). Similarly, the phenobarbitone-induced increase in sleeping time was reduced by polyherbal liquid formulations both in CCl_4 and APAP-induced liver toxicity in mice. The shortened sleeping time, along with amelioration of liver enzymes increased by CCL_4 and APAP, has accounted for the hepatoprotective effect of the formulations (Girish *et al*., 2009a; Girish *et al*., 2009b).

Most liver injuries need long-term treatment because they are chronic disease. When developing novel hepatoprotective agents, therefore, reducing side-effects of the therapy is critical. Use of herbal medicines in developing nations has risen in recent times. It is estimated that more than 75% of the population in sub-Saharan Africa depends on traditional herbal remedies for primary health care. Although most of the patients believe that medicinal herbs and phytochemicals are natural and safe to be administered without significant side effects, all medicinal agents including herbal medicines potentially have toxicity and side effects. For safe use of medicinal herbs and phytochemicals, the potential side effects and toxicity of these hepatoprotective herbal medicines should be seriously taken into consideration (Xiong & Guan, 2017; Amadi, 2018).

1.8.1. The Genus *Cordia* (Boraginaceae)

Cordia (generic name after a German botanist, Valeris Cordus) is a pantropical genus of flowering plants that belong to the family of Boraginaceae. About 100 genera are found within the family and it comprises more than 2000 species (Orwa *et al.*, 2009). The botanical characteristics of the genus *Cordia* include alternate petiolate leaves with the entire or dentate margin. The flowers are white, yellow or orange in color with cyme, spike or head inflorescences. Many species of the genus *Cordia* have long since been used to treat several ailments in the various traditional systems of medicine. Plants such as *Cordia dichotoma*, *Cordia latifolia*, *Cordia macleodii*, *Cordia myxa*, *Cordia rothii* and *Cordia obliqua* are used in Ayurveda, Unani and Siddha systems of medicine. The phytochemistry and pharmacology of the various species of the genus *Cordia* have been reported since many years. The major reported pharmacological activities of extracts and isolated compounds include anti-inflammatory, antioxidant, larvicidal, hepatoprotective, analgesic, antimicrobial and antidiabetic (Oza & Kulkarni, 2017).

Besides numerous ethnobotanical reports on the traditional use of genus *Cordia* for liver disorders, there are many experimental studies that have confirmed hepatoprotective effects of species in the genus. *C. dichotoma*, *C. myxa*, *C. obliqua*, *C. sebestena* and *C. macleoddi* are among species of the genus *Cordia* which confirmed hepatoprotective effect in animal studies. Additionally, due to their prominent anti-oxidant activities, other species of genus *Cordia*, including *C. africana*, are considered potentially hepatoprotective plants (Lawal *et al.*, 2016). Hepatoprotective activity of aqueous – methanol extract of *C. dichotoma* fruit against APAP-induced hepatic damage in rabbit has been done. The extract at 300 mg/kg exhibited a significant reduction in biochemical parameters such as random blood sugar (RBS), ALT, AST, total serum bilirubin (TSB), total serum protein (TSP), BUN and Creatinine whose levels were elevated by APAP and it was confirmed by histopathological studies (AL-Zuhairi *et al.*, 2020).

Anti-oxidant activity and protective role of *C. myxa* L. (CM) against liver fibrosis induced by CCL_4 or TA were assessed in terms of AST, ALT and ALP. The level of these enzymes significantly improved in rats after administration of CM (Afzal & Khan, 2014). The ethanol extract of *C. obliqua* (EECO) Willd leaves were evaluated for hepatoprotective activity in Wistar rats in APAP-induced hepatic damage. Significant decrease in ALT, AST, ALP, and TB levels was seen in rats treated with EECO at 200 and 400 mg/kg. Similarly a significant elevation of

TSP, albumin, and globulin has been seen in rats treated with the same dose of EECO when compared with APAP-treated rats (Sivagnanam & Devarasu, 2019).

C. macleodii is another species of genus *Cordia* which has shown antioxidant and hepatoprotective activity. The ethanolic extract of the plant's leaves demonstrated a significant dose-dependent antioxidant activity which is comparable with ascorbic acid. Also it has shown hepatoprotective activity through CCl_4-induced liver damage in rats. The increase in levels of ALT, AST, ALP and TB was inhibited after treating the rats with 100, 200 and 400 mg/kg PO of the extract and the inhibition was comparable with 100 mg/kg PO silymarin (Qureshi & Haleem, 2009). The ethanol extract of the whole plant of *C. sebestena* possessed a significant hepatoprotective effect. Administration of ethanolic extract of *C. sebestena* resulted in reduction of serum levels of ALT, AST, ALP, TB, total protein (TP) and cholesterol (Pradeep & Balavardhan, 2015; Sunaryo *et al*., 2019).

1.8.2. Plant Secondary Metabolites with Hepatoprotective Activity

Plant secondary metabolites have different pharmacological activities. Many of them possess antioxidant effect, which is a promising activity of plants to be hepatoprotective. The major secondary metabolites isolated from the genus *Cordia* include terpenoid hydroquinone, triterpenoids, prenylated hydroquinone, meroterpenoid naphthoquinone, polysaccharides, fatty acids, sesquiterpenes, flavonol glycosides, oleanane- and ursane-type triterpenes, arylnaphthalene-type lignin, dammarane-type triterpenes, alkaloids, phenols, tannins and hydrocarbons. There are similar secondary metabolites from other plants that have shown hepatoprotective activity (Ganesan & Banu, 2015). Alkaloid fractions from *Solanum pseudocapsicum* (Vijayan *et al.*, 2008), *Hygrophila auriculata* (Raj *et al*., 2010) *Murraya koenigii* (Sangale & Patil, 2017) and *Tiliacora racemosa* (Darvin *et al.*, 2018) have experimentally confirmed hepatoprotective activity.

Moreover, polyphenols from leaves extract of *Liquidambar styraciflua* L. (Eid *et al.*, 2015), fraction of *Folium Microcos* (Wu *et al*., 2017) and polyphenols isolated from virgin coconut oil (Famurewa *et al*., 2019) have shown hepatoprotective activities in different animal models. Flavonoids are among polyphenolic secondary metabolites whose hepatoprotective activities have been studied. Flavonoids from *Laggera alata* (Wu *et al*., 2006), *Rosa laevigata* Michx fruit (Liu *et al*., 2011), *Cichorium glandulosum* (Tong *et al*., 2015) and *Cleome viscosa* L. (Nguyen *et al*., 2017) have shown hepatoprotective activities against APAP or CCL_4-induced liver injury.

Hepatoprotective activities of saponins also have been shown in different studies. Total saponins isolated from *Taraphochlamys affinis* (Huang *et al*., 2012), *Actinidia valvata* Dunn (Qu *et al*., 2012), *Rhizoma panacis* Majoris (Zhang *et al*., 2014) and *Metapanax delavayi* (Wei *et al*., 2020) have shown hepatoprotective activities in CCL_4 or alcohol-induced liver injury. Studies have shown potential hepatoprotective activities of different types of terpenoids from *Scoparia dulcis* L (Krishnamurthy *et al*., 2010), *Juniperus procera* (Alqasoumi & Abdel-kader, 2014) and *Juniperus sabina* L (Abdel-kader *et al.*, 2019) in CCL_4-induced liver injury models.

1.8.3. *Cordia africana* Lam. (Boraginaceae)

Cordia africana (specific name '*africana*', formerly *abyssinica*, to refer the tree's natural homeland) is one of the common forest tree species in the family. It has different synonymous scientific names; *Cordia abyssinica* R. Br. Ex A. Rich., *Cordia holstii* Gürke ex Engl., *Cordia unyorensis* Stapf., *Gerascanthus africanas* (Lam.) Borhidi, *Gerascanthus holstii* (Gürke ex Engl.) M. Kuhlm. & Mattos. Its English common names are East African Cordia, large-leafed Cordia and Sudan teak (Orwa *et al.*, 2009; Wondafrash *et al.*, 2019). *Cordia africana* is native to such countries as Angola, the Democratic Republic of Congo, Djibouti, Eritrea, Ethiopia, Ghana, Guinea, Kenya, Malawi, Mozambique, South Africa, Sudan, Tanzania, Uganda, Zimbabwe, Saudi Arabia and Yemen. Generally, the species grows in areas with altitudes between 550 and 2600 m above sea level and annual rainfall of 700 to 2000 mm (Alemayehu *et al.*, 2016).

C. africana is a medium sized tree which, on average, attains a height between 14 and 21 m and a diameter between 0.60 and 0.90 cm, with great morphological variation. Leaves are dark green above, paler green and velvety below, with prominent parallel tertiary net-nerves and alternate ovate to sub-circular, 7.5-17.5 (max. 30) cm long and 3.5-10.2 (max. 30) cm broad. Apex broadly tapering or rounded; base rounded to shallowly lobed; margin entire; petiole slender, 2.5-7.6 cm long. The fruits are smooth, spherical, oval tipped, fleshy which is 1.3-1.5 cm long. Its color is green when young, yellow to orange when mature; with a sweet, mucilaginous pulp and contains 2-4 seeds, which lack endosperm (Derero, 2017).

In Ethiopia, it is known by different vernacular names; 'Wanza' in Amharic, 'Waddeessa' in Afan Oromo, and 'Ahwi' in Tigrigna. The plant has different uses. Its characteristic branching habit and broad leaves make *C. africana* useful as a shade tree for various shade-loving plants, particularly coffee trees. But this attribute is often season dependent, as the tree sheds its leaves quite heavily during some of Ethiopia's hot and dry seasons (Negash, 2010). The showy pure

white flowers, together with their fragrance and abundant nectar, are attractive to honeybees. Consequently, *C. africana* is an excellent honey tree. The wood is used for timber, farm equipment, high-quality furniture, doors, windows, cabinet making, drums, joinery, interior construction, paneling, fuel and veneering. In cities such as Addis Ababa, the finished and sized plank is used for lining external edges of roofs. *C. africana*'s fruit is widely eaten in most parts of Ethiopia and other countries in Africa. It is used both fresh and dried, and for both food and medicinal purposes (Negash, 2010; Isa *et al.*, 2016).

Different ethno-botanical studies have reported that several parts of the plant have many traditional medicinal uses in Ethiopia. The medicinal uses of *C. africana* have been reported by different authors for example, it helps cure acute febrile, wound, cough, toothache, stomach ache, fire burn, Michi, diarrhea, tonsillitis, eye problems, anthrax of cattle, epilepsy, urination at night, malaria, chest pain, skin rash, smell of foot, scabies, tonsillitis, abdominal pain, evil eye, gastritis, sore, spider poison, fever and influenza, and liver diseases (Alemayehu *et al.*, 2016). Additionally, *C. africana* is one of Ethiopian traditional medicinal plants whose traditional uses for treatment of various diseases have been validated in several experimental studies. Ethanol extracts of the plant exhibited *in vivo* anti-inflammatory (Tijjani *et al*., 2015), antioxidant (Alhadi *et al*., 2015; Tijjani *et al*., 2015) and antinociceptive activities (Ashmawy *et al*., 2020).

Another study on the leaf and stem bark extracts of *C. africana* also showed that the plant possesses anti-inflammatory, antibacterial, and antioxidant activities (Alhadi *et al*., 2015; Isa *et al*., 2016; Zemichael *et al*., 2019). The leaf extract of the plant showed *in vivo* anti-malarial activity (Wondafrash *et al*., 2019). Leaf, bark and roots of the plant had activity of controlling termite attack (Abdelrahim *et al*., 2016). Moreover it has already confirmed that both aqueous (Ganesan & Banu, 2015) and methanol (Alhadi *et al*., 2015; Isa *et al*., 2016) stem bark extract contains major secondary metabolites such as alkaloids, flavonoids, phenols and tannins, whose hepatoprotective effects have been shown in various studies (Table 1). Moreover, it is one of African plants having antioxidant activities and, potentially, hepatoprotective effect (Lawal *et al.*, 2016).

It has also been widely reported that different parts of the plant are used for liver disorders. Aqueous decoction of the dried powdered stem bark is drunk (Yineger *et al*., 2008) and boiled leaves are inhaled for treatment of hepatitis. Moreover root of the plant is dried, grinded, mixed with water and taken orally for liver disorders. It is also reported that the plant is used together

with other plants used traditionally for liver disorders. The dried powdered stem bark is boiled with powdered *Croton macrostachyus* and given orally for the treatment of jaundice (Giday *et al*., 2007; Gebeyehu *et al*., 2014; Mekuanent *et al*., 2015; Taha & Shimekit, 2020).

Although, there are effective treatments such as colchicine, corticosteroid, interferon and penicillamine but the incidence of the adverse effects is severe (Qadir & Ahmad, 2017). Therefore, for the management of hepatic diseases, there is a need to innovate alternative pharmaceuticals having more effectiveness and less toxicity. Traditional plants are one of the sources for development of such drugs. Despite a frequent ethno-botanical report on the traditional use of *C. africana* for treatment of liver disorders, there is no experimental study done yet validating the claim. Additionally, many other studies are now in consensus on the plant's antioxidant activity. On other hand, most chemicals share similar liver-damaging mechanism in common; oxidative liver damage. Plants having anti oxidant activity possibly can be used for liver disorders caused by those causative agents damaging the liver through this mechanism. Therefore the aim of the study was to evaluate the hepatoprotective effects of *C. africana* extracts and the solvent fractions of the methanol extract in rat model.

Table 1. 1: Preliminary phytochemical screening of aqueous (Ganesan & Banu, 2015) and methanol (Alhaddi *et al*., 2015) stem bark extracts of *Cordia africana* Lam. (Boraginaceae).

Secondary metabolites	**Aqueous extract**	**80% methanol extract**
Alkaloids	+++	-
Protein and amino acids	-	
Anthraquinones	-	-
Flavonoides	++	+++
Glycosides	-	-
Saponins	-	+
Steroids	-	-
Total phenols and Tannins	++	++
Triterpenoides	-	++
Coumarins		+

(-): Absent (+): Low concentration (++): Moderate concentration (+++): High concentration

Figure 1. 2: Picture of *Cordia africana* Lam. (Boraginaceae) tree from Ambo area, December, 2019.

1.9. Hypothesis

Some Ethiopian medicinal plants have hepatoprotective effects. *Cordia africana* Lam. (Boraginaceae), a plant which is traditionally used for liver diseases and there is no scientific evidence for its use, could be used for the management of liver disorders.

2. OBJECTIVES

2.1. General Objective

To evaluate hepatoprotective effects of crude extracts of *Cordia africana* Lam. (Boraginaceae) stem bark and the solvent fractions of the methanol extract in APAP-induced liver injury in rats

2.2. Specific Objectives

- To conduct acute toxicity test
- To conduct acetaminophen (APAP)-induced lethality test
- To evaluate hepatoprotective effects of 80% methanol and aqueous extracts of *Cordia africana* Lam. (Boraginaceae) stem bark.
- To evaluate hepatoprotective effects of aqueous, chloroform and n-butanol fractions of 80% methanol extract of *Cordia africana* Lam. (Boraginaceae) stem bark.
- To evaluate hepatoprotective effects of both crude extracts and solvent fractions in terms of acetaminophen (APAP)-prolonged sodium pentobarbital (SPB)-induced sleeping time in rats.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Instruments

Mechanical grinder, filter paper (Whatman number one), gauze (Nylon clothes), plastic sample holder, lyophilizer (OPERON, Made in Korea), deep freezer, rotary evaporator (BÜCHI Rotavapor R-200), heating bath (BÜCHI Heating Bath B- 490), volumetric flasks, funnel, aluminum foil, mortar and pestle, separator funnel, spoon, beakers, measuring cylinders, spatula, serum separator tubes (SST) (Vacuum Blood Collection Tube Gel & Clot Activators, 5ml, Henso Medical (Hangzhou) Co., Ltd., China), centrifuge machine (Eppendorf Centrifuge 5804^R), fully automated serum analyzer (Cobas^R 6000), oral gavages, frost-ended slides, syringe, desiccators, heater, refrigerator, sensitive digital weighing balance, surgical blades, surgical glove, disposable glove, scissors, forceps, tissue cassettes, tissue processor (Tissue Tek II Rotary Sekura Tissue Processor), autostainer, cool plate, water bath, labeler, markers, rotary microtome, Leica DM750 microscope with ICC50 HD camera, mice cages and rat cages were used.

3.1.2. Chemicals and Reagents

Methanol (99.8% For HPLC-Gold-Ultra gradient, CARLO ERBA Reagent), chloroform (99.8% For HPLC-Gold-Ultra gradient, CARLO ERBA Reagent), n-Butanol (99%, LOBA CHEMIE Pvt.Ltd.), Tween-80, distilled water, silymarin tablets (Liverubin^R Cure Quick Herbals Alchem international Pvt. Ltd.), APAP tablets (Para-Denk 500 Denk Pharma), normal saline (0.9% sodium chloride solution), 10% buffered neutral formalin solution (Formaldehyde 35%; SIGMA-ALDRICH Co.), paraffin, xylene, hemotaxylin solution, 2% eosin solution, Closed reagent cassettes of Roche Company for all ALT, AST, ALP TB, HDL, LDL, TC, and TGs (Roche Diagnostic, Indiapolis, made in Germany), bought from Germany were used.

3.2. Methods

3.2.1. Collection and Preparation of Plant Material

The stem bark of *C. africana* was collected from Ambo, West Shoa Zone, Oromia, about 114 kilometers West of the capital city, Addis Ababa, Ethiopia. The plant was identified and authenticated by Dr. Getachaw Adis, a botanical taxonomist at the Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia. The specimen was given voucher number (GD-001) and kept in the herbarium of EPHI Traditional and Modern Medicine Research Directorate for future reference.

3.2.1.1. Preparation of Crude Extracts

The stem barks of *C. africana* was dried under shed for two weeks after washing it with tap water to remove any dirty materials, chopped and grinded using mechanical grinder in to course powder. A hydro-alcoholic extract of *C. africana* stem bark was prepared by macerating 600gram of the powder in 80% methanol in the ratio of 1: 7 (one gram of the powder in 7ml of 80% methanol) for 72 hrs three times with occasional shaking and the extracts were combined. To prepare aqueous decoction 500gram of the powder was weighed, boiled with distilled water for 30 minutes and allowed to be cooled.

Both extracts were separately filtered with gauze and then filter paper using filter-aider vacuum. The filtrate of 80% methanol extract was concentrated using a rotary evaporator under reduced pressure at a temperature not exceeding 40°C. Aqueous filtrate was kept in a refrigerator to freeze the water, which was removed by freeze drier, lyophilizer. Finally, solid crystals were obtained and percent yields were calculated from the dried mass using the following formula:

$$\text{Yield \%} = \frac{\text{Weight of extract obtained}}{\text{Weight of plant sample}} \times 100$$

It was then powdered, packed into a glass vial, properly labeled and stored in a desiccator until use.

Figure 3. 1: Photos of collected stem bark sample of *C. africana*, powdered sample, methanol and aqueous extracts, filtering of extracts, concentrating extracts and their yields.

3.2.1.2. Preparation of Solvent Fractions

As the 80% methanol extract of *C. africana* was found to have better hepatoprotective effect at the medium dose, it was subjected to solvent-solvent fractionation using three solvents of different polarity: chloroform, n-butanol and water using a separator funnel. Optimum amount of 80% methanol extract was taken and dissolved in distilled water; equal volume of chloroform was added to it and the mixture was shaken. The mixture was allowed to form visibly different layers. The chloroform layer was separately taken and equal volume of n-butanol was added to the water residue and layers were separately taken. The organic solvents were removed under reduced pressure using a rotary evaporator. The aqueous fraction was freeze-dried following same procedure used for the aqueous crude extract using lyophilizer. The resulting semisolid masses of fractions were placed in a vacuum oven at 30°C for about 48 h to remove any residual solvent and they were stored in a desiccator under the same condition as that of crude extracts until use.

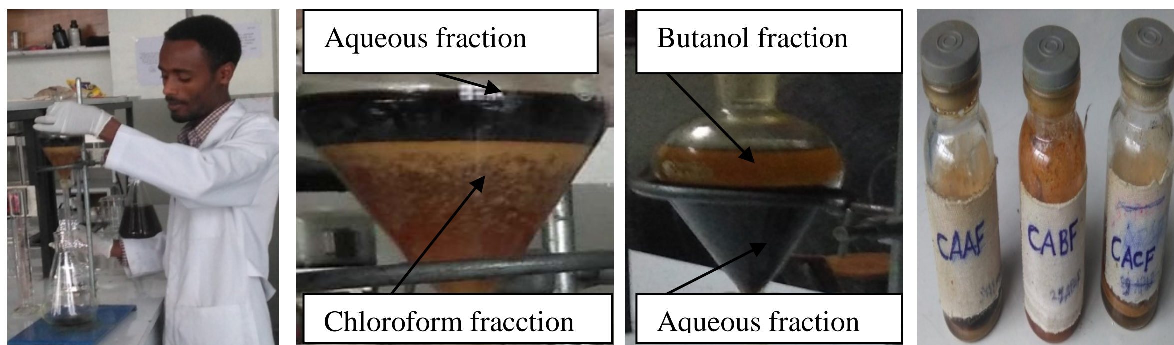


Figure 3. 2: Solvent fractionation of the 80% methanol extract of *C. africana* stem bark.

3.2.2. Experimental Animals

Rats and mice of either sexes weighing 150-215g and 30-35g respectively, were obtained from Addis Ababa University, Pharmacology Laboratory. All experimental procedures were conducted in accordance with the standards set forth in the guidelines for the care and use of experimental animals by Committee for Purpose and Control of Supervision of Experiments on Animals (Albus, 2012). Animals were acclimatized to animal house conditions for a period of seven days before experimentation to minimize effects of environmental change and stress associated with transportation induced physiological changes such as cardiovascular, immunological, neural and endocrine changes. Then, they were randomized in to various groups and housed in groups of five to ten animals in polypropylene cages, lined with soft wood flakes

as bedding (renewed every 24 h), with artificial 12 h light and 12h dark time and at room temperature. They were allowed free access to standard pellets, except during fasting period, and tap water regularly.

3.2.3. Ethical Considerations

The proposal got approval from department of pharmacology and clinical pharmacy scientific and ethics review committee, College of Health Sciences, Addis Ababa University. All animals were handled according to OECD guideline on experiment animals.

3.2.4. Administration of Extracts and Fractions to Animals

Animals received their corresponding doses of extracts and fractions. The doses were calculated per kilogram of their body weights. Animals were given code mark using permanent marker in order to separate them as per their body weight. The standard drug, plant extracts, fractions and vehicles were administered using oral gavages of appropriate size. All administrations were recorded on the prepared checklist to prevent missing or double administration.

3.2.5. Acute Oral Toxicity Test

Acute toxicity test on the stem bark extracts of *C. africana* was carried out based on the limit test recommendations of Organization for Economic Co-operation and Development guideline-425 (OECD, 2008), on mice of either sex. Two groups of animals containing five mice each were used, i.e., group I for CAME and group II for CAAE. Mice were fasted for four hours prior to the experiment and were allowed for access to free water *ad libitum* during fasting. A single dose of 2000 mg/kg of CAME or CAAE dissolved in 2% Tween-80 in distilled water was administered to each animal orally using oral gavages. After administration, mice were fasted for two hours and given free access to food and water. Animals were then observed for physical and behavioral changes within twelve hours; with special attention during the first four hours. The major signs of toxicities observed were difficulty in breathing, loss of appetite, general weakness, irritability, writhing, loss of motor coordination, muscle relaxation, sedation and deep sleep. They were further observed every day for fourteen days to see any mortality and the test was repeated with 3000 mg/kg of extracts.

3.2.6. Acetaminophen-Induced Lethality Test

Acetaminophen (APAP)-induced lethality test in both male and female mice was conducted according to the method described by Gilani *et al* (2005). Briefly, the animals were grouped into three with 10 per group and fasted for twelve hours with free access to water prior to extract

administration and for one hour after administration of APAP. Group I served as a control and received the vehicle, 2% of Tween-80 dissolved in distilled water; Groups II and III received 400 mg/kg of CAME and CAAE, respectively, dissolved in 2% Tween-80. One hour later, all animals received lethal dose of APAP (1 g/kg). Number of deaths within 24 hours was recorded and percent protection was calculated using the following formula:

$$\text{Percent protection (\%)} = \frac{a}{b} \times 100,$$

where *a* is number of surviving animals 24 hours after administration of lethal dose of APAP, 1 g/kg, and *b* is number of animals in the group at the beginning.

3.2.7. Acetaminophen Dose Selection Test

The test was aimed to determine the optimum dose of APAP used for induction of liver injury. Three frequently used doses of APAP; 640 mg/kg (Gilani & Janbaz, 1995), 1 g/kg (Janbaz *et al*., 2002) and 2 g/kg (Azarmehr *et al*., 2019) were taken from three scientific papers. Four groups of animals containing five female rats each were used for the test. Group I served as a control and received normal saline (NS), 10ml/kg. Groups II-IV received 640mg/kg, 1g/kg, and 2g/kg of APAP, respectively. All animals were anaesthetized with chloroform 48 h after APAP administration, and 3-4 ml blood samples were taken through cardiac puncture. Blood samples were put in to serum separator tube (SST) containing clot activator and allowed to clot at room temperature for 1 h. Then, it was centrifuged at 3000 revolutions per minute for 15 minutes at 4°C, and serum was taken to determine levels of ALT and AST. The dose inducing significant biochemical change without causing any physical damage was selected to be used for the main test. Accordingly, experimental liver damage was induced by administering single dose (2g/kg) of APAP (Afeefa *et al*., 2016).

3.2.8. Hepatoprotective Activity Studies

3.2.8.1. Grouping and Dosing of Animals

Study animals were randomly classified in to different groups. There were nine (I-IX) groups of animals containing five male rats each for hepatoprotective test of the crude extracts (CAME and CAAE). It includes: normal control group (received vehicle, 2% Tween-80), standard drug group (received silymarin 100 mg/kg in 2% Tween-80), CAME test groups received 100, 200 or 400 mg/kg, CAAE test groups received 100, 200 or 400 mg/kg and negative control group received 2% Tween-80), respectively. All extracts were freshly prepared by dissolving in 2% Tween-80 and given daily for ten days. On day ten, all animals in group II-IX were given 2g/kg APAP in

NS one hour after administration of last doses; while group I animals received the vehicle, NS, instead of APAP. Dosing was according to the one used for acute toxicity test, i.e., 2000mg, and previous studies (Pradeep & Balavardhan, 2015). One-tenth of dose used for acute toxicity test was taken as a medium dose, twice of which was taken as maximum dose, and its half was taken as a minimum dose.

After identifying the extract with better activity, fractionation of CAME was done using different solvents. Thus, the fractions were also investigated for their hepatoprotective effect using similar procedure mentioned above. Accordingly, there were three groups, containing five animals each, which received 400 mg/kg CAAF, 400 mg/kg CABF and 400 mg/kg CACF, respectively, for ten days. On the tenth day, all groups received 2 g/kg APAP.

3.2.8.2. Biochemical Analysis

Animals were fasted for twelve hours and anesthetized with chloroform. Blood samples were collected through cardiac puncture forty eight hours after administrating toxic dose of APAP, 2 g/kg. The levels of liver enzymes, ALT, AST, ALP and GGT, and level of total bilirubin were determined. Lipid profiles including TC, TGs, HDL and LDL were also evaluated. All the biomarkers were analyzed using a fully automated machine, cobas^{R} 6000. Cobas^{R} is a fully automated biomarker analyzing machine, at EPHI clinical chemistry laboratory. It is composed of three parts analyzing electrolytes, chemistry and hormones. It has common sample reception and different analyzing part for all three samples. Test principles of all sample is based on spectrophotometry. There are independent reagent cassettes for each biomarker in a closed system. All reagents used in the analysis of the biomarker are contained in the readymade closed system reagent cassettes.

The hepatoprotective activity, expressed as percent protection, of the test substances in terms of biochemical parameters were calculated using the following formula:

$$\% \text{ Protection} = [(a - b) \div (a - c)] \times 100,$$

where, a is the mean value of the biomarker produced by hepatotoxin (APAP); b is the mean value of the marker produced by toxin plus test material; and c is the mean value produced by the vehicle (Umer *et al.*, 2010). Test principles for each biomarker, as obtained from the leaflet of the system, was according to Maqsood *et al* (2017). Percent protection in terms of SPB-induced sleeping time was also determined in the same manner.

3.2.8.3. Histopathological Analysis

Three gross sections of liver samples were taken from left lateral lobe, right medial lobe and caudate lobe. Sections were kept in the tissue cassette with respective identity of the liver and remain fixed in 10% BNFS until processing. They were then dehydrated using different increasing concentrations of ethanol. Accordingly, cassettes were immersed in 70%, 85%, 95% and finally into absolute, 100%, ethanol. Then, tissues were cleared of any residual ethanol using xylene in two containers. The whole process was carried out over night using auto processing machine. Molten paraffin was put into moulds to fix samples on tissue cassette externally and it was kept on cool plate to help the molten paraffin in the mould solidify. The mould was separated from the tissue embedded on the external surface of the cassette and the tissue blocks were trimmed to remove excess paraffin.

Sections of 4µm thickness were taken from each tissue block and put into warm water bath to prevent contraction. Sections were then picked up from the water bath onto frosted microscope slides to prepare for staining. The staining was done using an auto stainer, where the slides were deparaffinized by xylene in absolute alcohol and rehydrated. The slides were then immersed in graded alcohols (95%, 85%, 60%), and tap water in a step wise manner. Slides were then stained in hematoxylin for 10 minutes and counter stained in 2% eosin solution for 20 seconds. The stained slides were taken out of the machine and cover-slipped using DPX (Dibutylphthalate Polystyrene Xylene) mounting medium. Histomorphologic abnormalities were scored based on type and severity of morphologic changes according to Yahya *et al* (2013). Selected images of the sections were captured under magnification of 10x by Leica DM750 microscope using ICC50 HD camera.

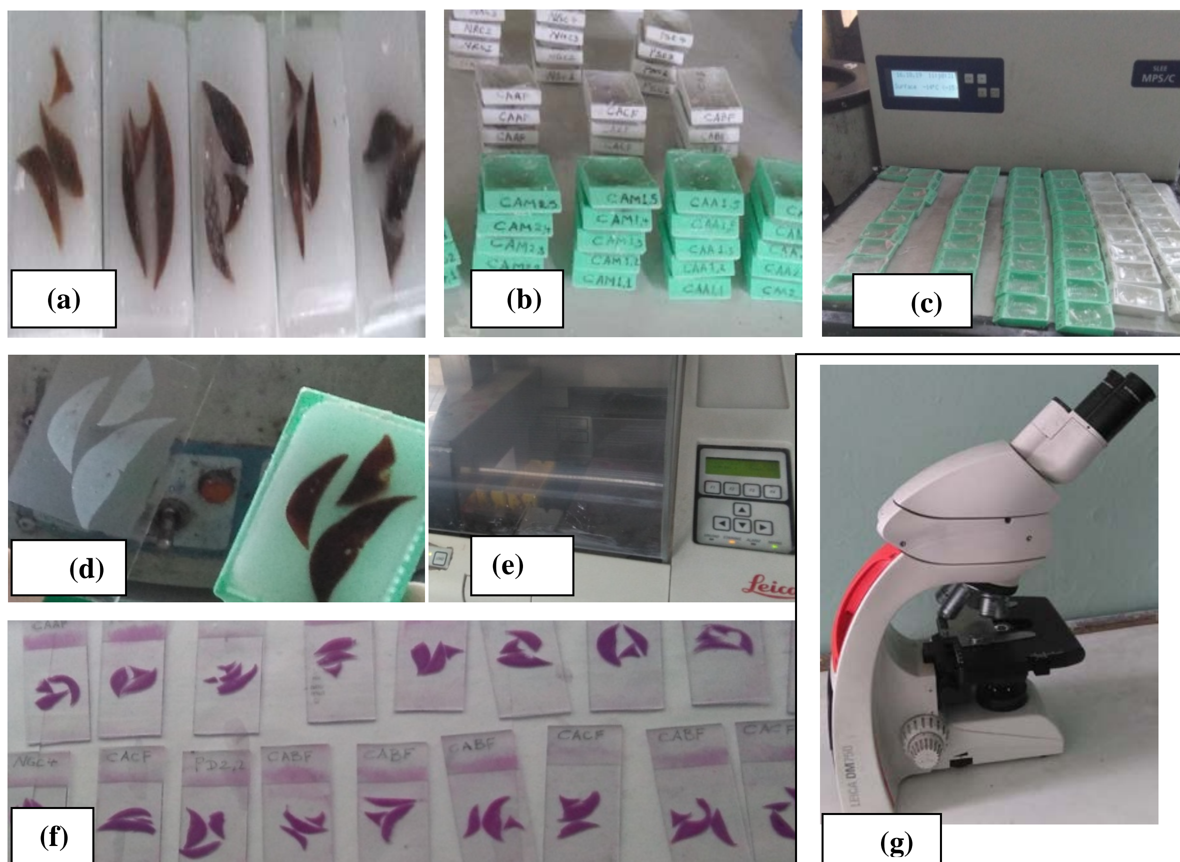


Figure 3. 3: Tissue blocks **(a)**, Trimmed blocks **(b)**, Cool plate **(c)**, Tissue sections **(d)**, Auto-staining machine **(e)** stained and mounted liver tissue section **(f)**, Leica DM750 Microscope **(g)**.

3.2.8.4. Sodium Pentobarbital-Induced Sleeping Time

The test was done according to the method described by Gilani & Janbaz (1994) with some modification on ten groups of animals, containing six male rats each. Liver injury was induced by APAP instead of CCL_4 and 150 mg/kg sodium pentobarbiatal (SPB) was used. Groups I-III served as normal, negative and positive controls, respectively. Groups I and II received the vehicle, 2% Tween-80, and group III received the standard drug, silymarin 100 mg/kg. Groups IV and V were used to see effects of the extracts alone on the sleeping time and received 400 mg/kg CAME and CAAE, respectively. Groups VI to X received CAME, CAAE, CAAF, CABF and CACF at a dose of 400 mg/kg, respectively. All animals received four doses of their corresponding vehicle, standard drug or test substances at twelve hour interval. Animals in all groups, except groups I, IV and V, respectively, received 2 g/kg APAP one hour after last dose of test substances or vehicle. Twenty-four hour after APAP administration, animals in all groups were given 150 mg/kg of SPB I.P. Data recording formats were prepared for all animals and each

animal was recorded individually. Recorded data include: time of injection, time of sleep onset (loss of writhing reflex), time of awakening (gain of reflex) and duration of sleep (time between loss and gain of reflex) in minutes.

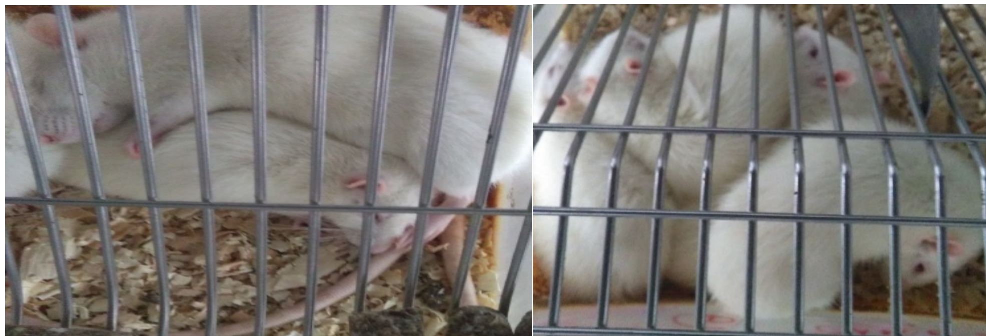


Figure 3. 4: Animals in deep sleep after I.P injection of SPB.

3.2.9. Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM). SPSS (Statistical Package for Social Sciences) version 20 software was used for statistical analysis. Differences between means were determined using analysis of variance (ANOVA) and Tukey post-hoc test with multiple comparisons was used to determine significant differences. $P < 0.05$ was considered statistically significant.

3.2.10. Quality Assurance

To assure quality of the data all necessary preconditions were set during all time of the study. There were activities done before analyses to assure quality of data which include skill developing on laboratory animals handling and data recording techniques. For proper handling of laboratory animals, brief training was offered by the department before starting the study. Additionally, chemicals, reagents and drugs used were of analytical grade, except APAP, silymarin and NS which were therapeutic grade. All animals were given identification code in their respective groups and data were registered on previously prepared data recording format. In order to obtain data of high quality, all biochemical analyses were done in clinical chemistry laboratory of EPHI. The laboratory is accredited by Ethiopian National Accreditation Office (ENAO), which is member of International Laboratory Accreditation Cooperation (ILAC). Accordingly, before analysis of sample, calibration has been done by calibrator and samples were analyzed against control to assure quality of data. Moreover, histopathological examination was blindly done and scored by experienced pathologist.

4. RESULTS

4.1. Percent Yields of Extraction

The percentage yields obtained from aqueous and 80% methanol extractions were calculated to be 6.97 and 7.90 %, respectively.

4.2. Acute Oral Toxicity Test

No mortality was observed following administration of both aqueous and 80% methanol extracts of *C. africana* stem bark at doses of 2000 and 3000 mg/kg within 14 days. Moreover, no signs of toxicity such as difficulty in breathing, loss of appetite, general weakness, irritability, writhing, loss of motor coordination, muscle relaxation, sedation and deep sleep were observed during the observation period. Thus, oral LD_{50s} (median lethal dose) of the extracts are assumed to be greater than 3000 mg/kg.

4.3. Acetaminophen-Induced Lethality Test

Among the animals that received 400 mg/kg CAME or CAAE, only one and two animals died, respectively, within 24 hrs of APAP (1 g/kg) administration showing the percent protections to be 90% and 80%, respectively (Figure 4.1). Moreover, these observations were maintained for one-week duration after which no more observations were made.

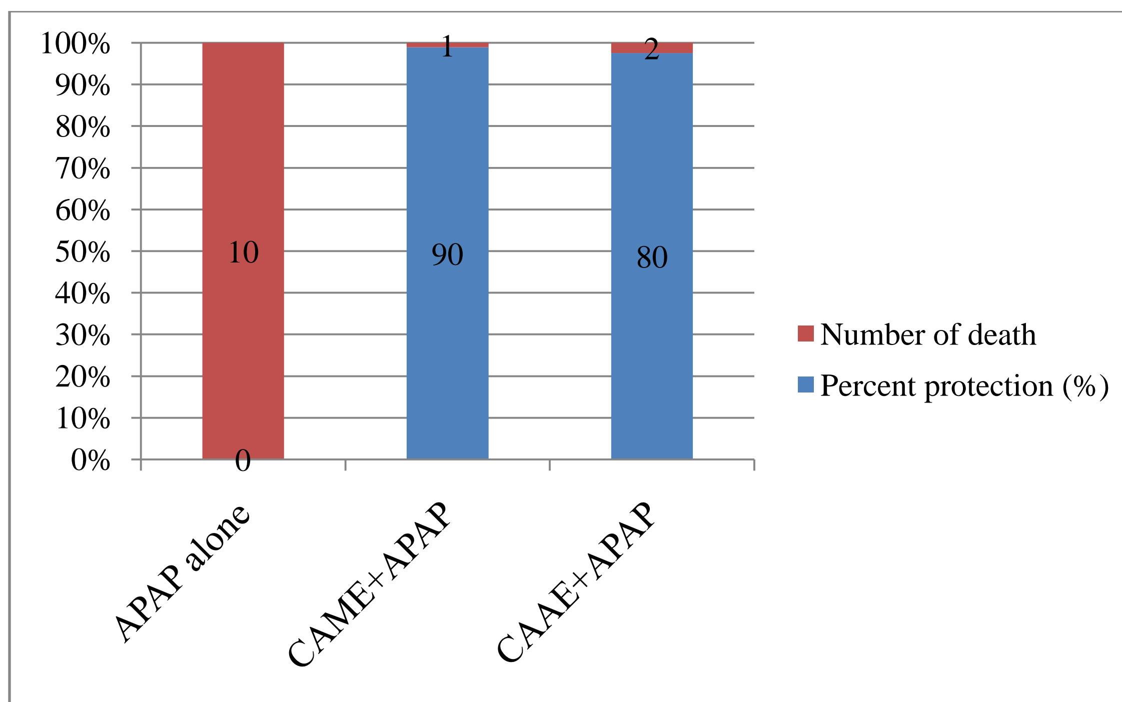


Figure 4. 1: Effects of 80% methanol and aqueous extracts of *C. africana* stem bark on APAP-induced mortality.

4.4. Acetaminophen Dose Selection Test

The results of APAP dose selection study are presented in Table 1. As can be seen from Table 1, the mean levels of liver enzymes (ALT and AST) were not significantly increased in animals that received 640 mg/kg of APAP compared to control. 2000 mg/kg of APAP caused a significant ($p < 0.05$) increase in the serum levels of both ALT and AST and hence, this dose was chosen for induction of liver injury.

Table 4. 1: Effects of different dose of APAP on serum level of liver enzymes (ALT and AST), (Mean ± SEM).

Group	**ALT (U/L)**	**AST (U/L)**
NS, 10 ml/kg	58.75 ± 23.1	134.20 ± 32.03
APAP, 640 mg/kg	58.00 ± 19.4^{cd}	134.65 ± 35.02^{cd}
APAP, 1000 mg/kg	118.50 ± 30.9^{abd}	333.50 ± 25.03^{abd}
APAP, 2000 mg/kg	151.95 ± 33.9^{abc}	390.05 ± 30.03^{abc}

Compared to: ^{a}10 ml/kg, NS, ^{b}640mg/kg APAP, ^{c}1000mg/kg APAP, ^{d}2000mg/kg APAP, $p<0.05$

4.5. Hepatoprotective Activity Studies

4.5.1. Effects of aqueous and 80% methanol extracts of *C. africana* stem bark on liver enzymes and total bilirubin

The results of the effects of crude extracts (CAAE and CAME) of *C. africana* stem bark on liver enzymes ALT, AST, ALP and total bilirubin (TB) in APAP induced liver damage is presented in Table 4.2. Administration of 2g/kg APAP caused significant ($p < 0.05$) increase in serum level of all these biomarkers compared to normal control group. On the other hand, administration of different doses of extracts decreased the increased level of those biomarkers. Serum levels of ALT and AST were decreased dose-dependently by CAME and that of AST and ALP were decreased by CAAE in the same manner.

Table 4. 2: Effects of aqueous and 80% methanol extracts of *C. africana* stem bark on serum levels of liver enzymes and total bilirubin in rats orally administered 2 g/kg APAP, (Mean ± SEM), n=5.

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	TB (mg/dL)
Normal control	101.57±45.88	218.30±73.18	118.00±42.31	0.09±0.02
Negative control	227.80±40.80^{a}	417.73±67.10^{a}	248.33±34.55^{a}	0.19±0.05^{a}
100 mg/kg, CAME	110.47±35.18^{bdef} (92.9)	347.53±70.28^{bde} (35.2)	201.00±44.65^{bdef} (36.3)	0.15±0.01^{b} (40)
200 mg/kg, CAME	108.62±40.72^{bceg} (94.4)	292.23±63.38^{bce} (62.9)	225.50±42.31^{bceg} (17.5)	0.11±0.01^{bg} (80)
400 mg/kg, CAME	102.75±39.74^{bcdh} (99.1)	254.33±53.20^{bcd} (81.9)	228.50±32.30^{bcd} (15.2)	0.14±0.01^{b} (50)
100 mg/kg, CAAE	121.96±35.54^{b} (83.8)	324.77±73.18^{bgh} (46.6)	248.00±34.55^{bgh} (0.3)	0.11±0.01^{b} (80)
200 mg/kg, CAAE	178.95±56.19^{b} (38.7)	261.45±80.68^{bfh} (78.4)	245.00±40.33^{bfh} (2.6)	0.15±0.01^{b} (40)
400 mg/kg, CAAE	153.15±39.74^{b} (59.1)	254.93±71.53^{bfg} (81.6)	219.67±44.50^{bfg} (22)	0.13±0.02^{b} (60)
100 mg/kg, Silymarin	98.50 ±45.88 ^{bcdefgh} (102.4)	241.75±89.62^{bcdefgh} (88.2)	177.67±34.55^{bcdefgh} (54.2)	0.12±0.01 ^{bcdefgh} (70)

Compared to: [a]normal control, [b]negative control, [c]CAME100, [d]CAME200, [e]CAME400, [f]CAAE100, [g]CAAE200, [h]CAAE400, P<0.05, values in bracket indicate percent (%) protection

4.5.2. Effects of aqueous and 80% methanol extracts of *C. africana* stem bark on lipid profiles

Administration of APAP caused a significant increase in serum levels of LDL, TC and TGs and a significant decrease in HDL levels (Table 4.3). However, administration of both crude extracts at different doses resulted in decrement of those values that have been increased by APAP. CAAE showed dose-dependent effect on serum level of TGs.

Table 4. 3: Effects of aqueous and 80% methanol extracts of *C. africana* stem bark on lipid profiles of rats orally administered 2 g/kg APAP, (Mean ± SEM), n=5.

Group	**HDL (mg/dL)**	**LDL (mg/dL)**	**TC (mg/dL)**	**TGs (mg/dL)**
Normal control	23.13±1.65	8.40±2.05	38.60±4.02	61.55±14.02
Negative control	19.90±3.44[a]	13.03±1.70[a]	45.95±3.30[a]	114.35±12.14[a]
100 mg/kg, CAME	23.10±2.20[bf] (99.1)	10.20±1.34[b] (61.1)	42.85±2.66[b] (42.2)	81.78±15.48[bde] (61.7)
200 mg/kg, CAME	21.24±1.70[bg] (41.5)	11.80±1.30[b] (26.6)	42.40±3.58[bg] (48.3)	82.40±14.00[bce] (60.5)
400 mg/kg, CAME	22.92±1.90[bh] (93.5)	10.40±2.00[b] (56.8)	42.83±2.48[b] (42.4)	87.50±11.76[bcd] (50.8)
100 mg/kg, CAAE	22.58±1.56[b] (83)	9.50±2.05[b] (76.2)	42.43±2.05[b] (47.9)	78.35±10.86[bgh] (68.2)
200 mg/kg, CAAE	21.10±2.25[b] (37.2)	11.10±2.14[b] (41.7)	42.63±2.00[b] (45.2)	75.78±7.94[bfh] (73.0)
400 mg/kg, CAAE	21.80±2.20[b] (58.8)	9.00±1.44[b] (87.0)	41.48±2.61[b] (60.8)	74.52±9.20[bfg] (75.4)
100 mg/kg, Silymarin	23.12±1.85[bcdefgh] (99.70)	9.43±1.51[bcdefgh] (77.8)	39.85±4.02[bcdefgh] (82.9)	61.63±8.11[bcdefgh] (99.8)

Compared to: [a]normal control, [b]negative control, [c]CAME100, [d]CAME200, [e]CAME400, [f]CAAE100, [g]CAAE200, [h]CAAE400, P<0.05

4.5.3. Effects of solvent fractions of 80% methanol extract of *C. africana* stem bark on liver enzymes and total bilirubin

Administration of all aqueous, n-butanol and chloroform solvent fractions of *C. africana* stem bark 80% methanol extract at the dose of 400 mg/kg resulted in a significant reduction of serum levels of liver enzymes and total bilirubin, whose levels were increased by APAP, Table 4.4. Chloroform fraction of 80% methanol extract showed highest percent protection in terms of ALT, AST and ALP among all fractions. Serum level of total bilirubin was highly reduced by n-butanol fraction than others.

Table 4. 4: Effect of aqueous, butanol and chloroform fractions of *C. africana* stem bark 80% methanol extract on liver enzymes and total bilirubin (TB) of rats orally administered 2 g/kg APAP, (Mean ± SEM), n=5.

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	TB (U/L)
Normal control	101.57±45.88	218.30±73.18	118.00 ±42.31	0.09±0.02
Negative control	227.80± 40.80^{a}	417.73 ± 67.10^{a}	248.33 ±34.55^{a}	0.19±0.05^{a}
400 mg/kg, CAAF	113.50±56.20^{bj} (90.5)	263.38±63.38^{bj} (77.4)	248.00±37.64^{b} (0.3)	0.13±0.01^{b} (60)
400 mg/kg, CABF	116.80±45.88^{b} (87.9)	305.58±54.30^{b} (56.2)	193.67±33.13^{bi} (41.9)	0.11±0.02^{bik} (80)
400 mg/kg, CACF	103.10±61.44^{bij} (98.8)	242.00±71.27^{bij} (88.1)	128.50±50.10^{bij} (91.9)	0.13±0.02^{bj} (60)
100 mg/kg, Silymarin	98.50±45.88 (102.4)	241.75±89.62 (88.2)	177.67±34.55 (54.2)	0.12±0.01 (70)

Compared to: ^{a}normal control, ^{b}negative control, ^{i}CAAF, ^{j}CABF, ^{k}CACF, P<0.05

4.5.4. Effects of solvent fractions of 80% methanol extract of *C. africana* stem bark on lipid profiles

As it is shown in Table 4.5, 400 mg/kg of all aqueous, n-butanol and chloroform solvent fractions of *C. africana* stem bark 80% methanol extract decreased serum levels of LDL, TC and TGs and increased serum level of HDL compared to negative control. Serum levels of all lipid profiles were highly reduced by chloroform fraction than others.

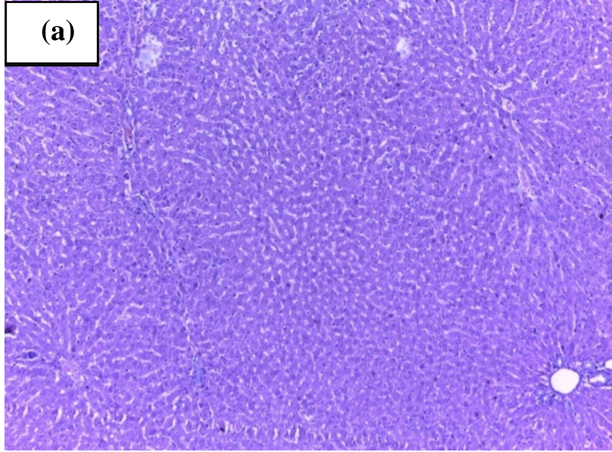
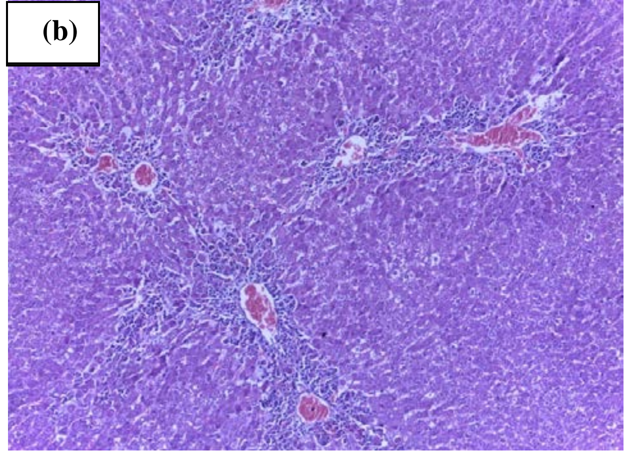
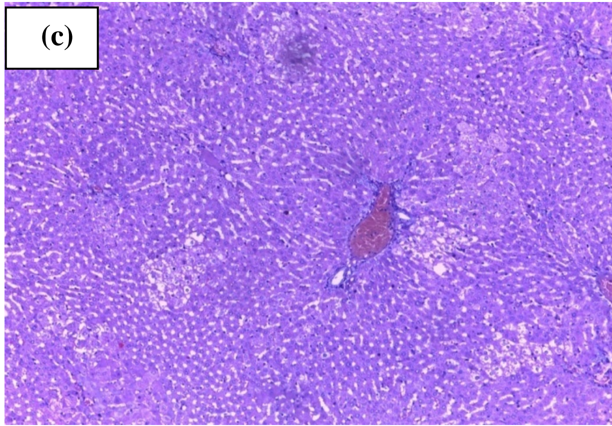
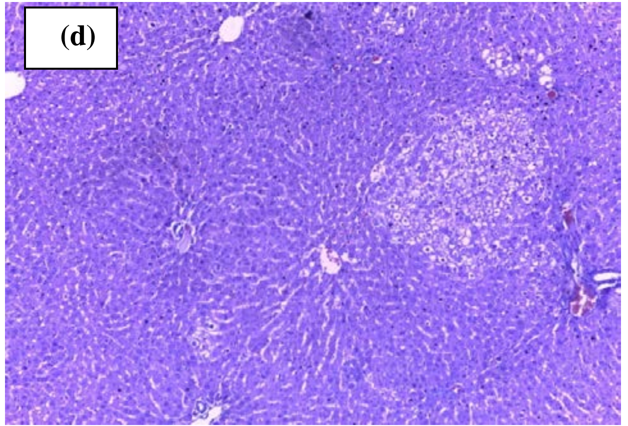
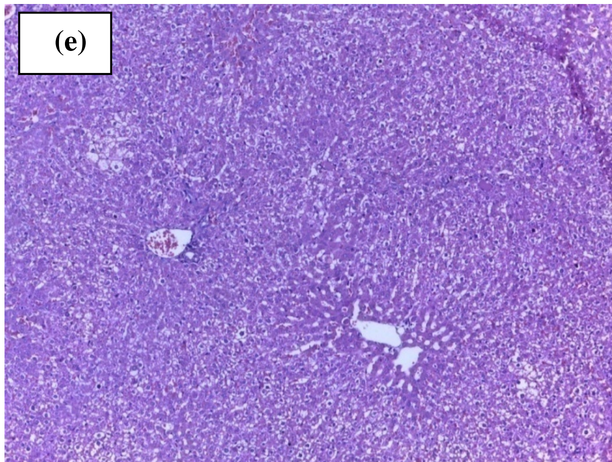
Table 4. 5: Effects of aqueous, n-butanol and chloroform fractions of *C. africana* stem bark 80% methanol extract on lipid profiles of rats orally administered 2 g/kg APAP, (Mean ± SEM), n=5.

Group	HDL (mg/dL)	LDL (mg/dL)	TC (mg/dL)	TGs (mg/dL)
Normal control	23.13±1.65	8.40±2.05	38.60±4.02	61.55±14.02
Negative control	19.90±3.44[a]	13.03±1.70[a]	45.95±3.30[a]	114.35±12.1[a]
400 mg/kg, CAAF	22.80±1.90[bj] (89.8)	12.50±1.60[b] (11.4)	45.30±3.11[b] (8.8)	77.40±30.39[bj] (70.0)
400 mg/kg, CABF	22.28±2.12[b] (73.7)	10.10±2.70[bi] (63.3)	43.30±4.32[bi] (36.1)	81.45±21.38[b] (62.3)
400 mg/kg, CACF	23.03±2.20[bij] (96.9)	9.38±2.92[bij] (78.8)	40.05±4.02[bij] (80.3)	69.50±6.98[bij] (84.9)
100 mg/kg, Silymarin	23.12±1.85[bijk] (99.70)	9.43±1.51 [bijk] (77.8)	39.85±4.02 [bijk] (82.9)	61.63±8.11 [bijk] (99.8)

Compared to: [a]normal control, [b]negative control, [i]CAAF, [j]CABF, [k]CACF, P<0.05

4.5.5. Effects of crude extracts and solvent fractions of 80% methanol extract of *C. africana* stem bark on histopathology of APAP-induced liver injury

The effects of aqueous and 80% methanol extracts and the solvent fractions of the methanol extract on histopathology were investigated. As shown in figure 4.2, normal architecture of the liver was altered in negative control group which received 2 g/kg APAP compared to the normal control. However, pre administration of standard treatment, extracts and fractions of *C. africana* stem bark resulted in protection of the tissue to varying extent as in Table 4.6.


(a)

(b)

(c)

(d)

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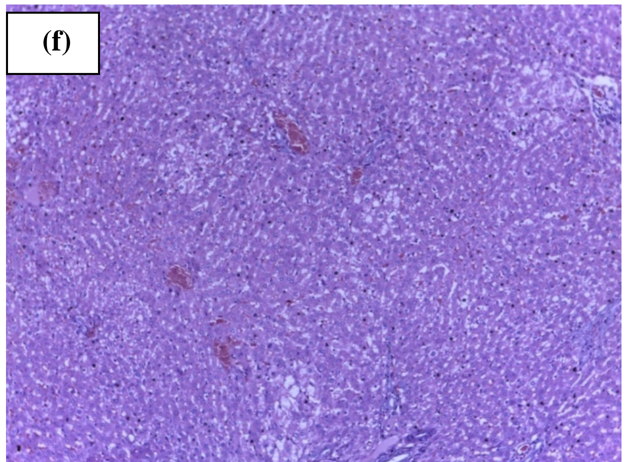
(f)

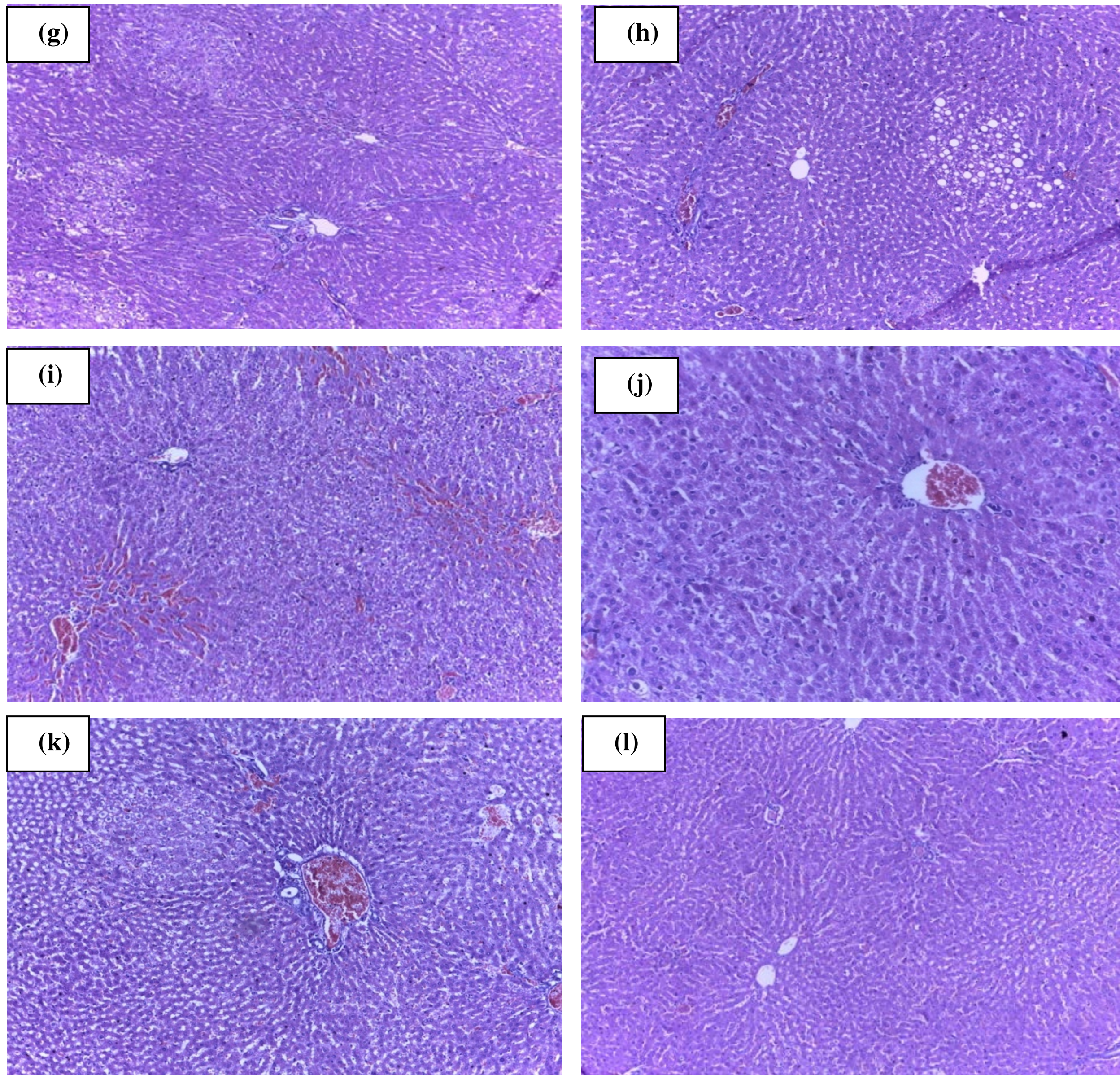

Figure 4. 2: Microscopic pictures of liver sections from different groups.

(a) Normal control groups: rats treated with 2% Tween-80 followed by normal saline, **(b)** Negative control groups: rats received APAP alone, **(c)** Rats received 100 mg/kg CAME, **(d)** Rats received 200 mg/kg CAME, **(e)** Rats received 400 mg/kg CAME, **(f)** Rats received 100 mg/kg CAAE, **(g)** Rats received 200 mg/kg CAAE, **(h)** Rats received 400 mg/kg CAAE, **(i)** Rats received 400 mg/kg CAAF, **(j)** Rats received 400 mg/kg CABF, **(k)** Rats received 400 mg/kg CACF, **(l)** Positive control group: rats received 100 mg/kg silymarin.

Table 4. 6: Effects of different doses of crude extracts and solvent fractions of *C. africana* stem bark on APAP-induced liver injury.

Group	Necrosis	Vacuolar degeneration
Normal control, NS	-	-
Negative control,	+++	++
100 mg/kg, CAME	+	++
200 mg/kg, CAME	-	+
400 mg/kg, CAME	+	+
100 mg/kg, CAAE	-	+
200 mg/kg, CAAE	+	++
400 mg/kg, CAAE	-	+
400 mg/kg, CAAF	-	+
400 mg/kg, CABF	+	+
400 mg/kg, CACF	-	+
100 mg/kg, Silymarin	-	-

- normal, + mild effect, ++ moderate effect, +++ severe effect

4.5.6. Effects of extracts and solvent fractions of the 80% methanol extract of *C. africana* stem bark on sodium pentobarbital-induced sleeping time

Administration of 150 mg/kg SPB prolonged sleep duration in animals treated with 2 g/kg APAP from 75.70 ± 6.60 to 161.33 ± 9.70. However, administering extracts without APAP resulted in no change in SPB-induced sleep duration compared to normal control. Pretreatment of animals with silymarin, crude extracts and solvent fractions restored the normal sleep duration, Table 4.7. CAME showed percent protection comparable to standard treatment, silymarin. Moreover, CAAF showed highest reduction in sleep duration.

Table 4. 7: Effects of high doses (400 mg/kg) of crude extracts and solvent fractions of the methanol extract of *C. africana* stem bark on sodium pentobarbital-induced sleeping time in rats orally administered 2 g/kg APAP, (Mean ± SEM), n=6.

Group	Sleep duration (in minutes)
Normal control, Vehicles + SPB	75.70±6.60
Negative control, APAP + SPB	161.33±9.70[a]
CAME + SPB	80.25±11.44
CAAE + SPB	75.80±12.00
CAME + APAP + SPB	91.60±4.73[bdef] (81.4)
CAAE + APAP + SPB	129.30±3.06[b] (37.4)
CAAF + APAP + SPB	102.80±5.70[bfg] (68.4)
CABF + APAP + SPB	128.50±7.94[b] (38.3)
CACF + APAP + SPB	132.80±5.44[b] (33.3)
Silymarin, 100 mg/kg+APAP+SPB	85.25±5.70[bdefg] (88.8)

Compared to: [a]normal control, [b]negative control, [c]CAME, [d]CAAE, [e]CAAF, [f]CABF, [g]CACF
SPB: Sodium pentobarbital

5. DISCUSSION

Natural products remain the trusted solution of diseases for which no effective treatment has yet been obtained. Diseases whose interventions are with threatening adverse effects or unknown safety profiles are also benefited. Even though studies show that the majority of the populations of the country rely on TM, scientific evidences for their safety and efficacy still remain scarce. Furthermore, studies done so far usually focus on some common areas, while others remain unnoticed. Liver is a critical organ which is important for normal functioning of all other organs and organ systems of the body. On the other hand, it seems clear that it is one of the areas at which experimental studies aimed toward investigating safe and effective new drug remain relatively deficient.

All activities intended to the discovery of new therapeutic entity should first consider the liver, the usual house of metabolism and the target for the toxic effects of that new agent. Unless otherwise, it seems just trying to treat one case and, on the other side, inducing other problem. The aim of this study was to test hepatoprotective activity of one of the Ethiopian medicinal plants, *Cordia africana* Lam. (Boraginaceae). The broad medicinal uses (Alemayehu *et al*., 2016) of the plant *C. africana* by different ethnic groups in Ethiopia are witnessed by the consistency in reports of different published ethno-medicinal studies (Yineger *et al*., 2008; Gebeyehu *et al*, 2014; Mekuanent *et al*., 2015; Taha & Shimekit, 2020) and made the plant candidate to be screened for hepatoprotective effect in this study.

Animal model of APAP-induced liver injury was selected for this experiment. While other chemicals could be used instead, APAP was selected to induce liver injury to assess hepatoprotective effect in our study for two reasons. First, the model has its own prescreening test called APAP-induced lethality test which serves as a pre-study test. Second, while other chemicals induce liver toxicity indirectly through environmental exposure, APAP induces hepatotoxicity by its direct effect on liver because it is the most widely used drug with its hepatotoxic effects (Ishitsuka *et al*., 2020). As drug-induced liver injury has been investigated to be responsible for 5% of all hospital admissions and 50% of all acute liver failures (Pandit *et al*., 2012; Rotundo & Pyrsopoulos, 2020), APAP-induced lethality model has been widely used in other studies (Gilani *et al*., 2005) for the same purpose. Therefore, this model has advantage of inducing hepatotoxicity which resembles liver injury in humans.

SPB-induced sleeping time was used to assess the metabolizing activity of the livers in different animals group. SPB was chosen for the reason that it is metabolized solely by the liver so that its metabolism cannot be interfered by other organ. It is metabolized by hepatic microsomal drug metabolizing enzymes (MDME), cytochrome p-450, to inactive metabolites. In liver injury, the metabolizing activities of the liver become defected and the plasma level of the drug remains higher which results in prolongation of sleep duration. The duration of SPB-induced sleep in animal is considered to be a reliable index for the activity of hepatic MDME. The damage caused by APAP to hepatocytes as well as hepatic MDME causes loss of drug metabolizing capacity of the liver, resulting in prolongation of SPB-induced sleeping time (Gilani & Janbaz, 1994).

In this study, aqueous and methanol extracts of the plant's stem bark were prepared through decoction and maceration, respectively. Both extraction methods were used according to the traditional use. The percent yield of methanol 80% extract of *C. africana* stem bark was higher than aqueous extract (7.90% vs. 6.97%); this might be due to the relatively higher amount of sample powder taken for maceration than for decoction which may account for the difference. Moreover, the relative polarity difference between the two solvents which might have affected concentration of constituents to be extracted could also be the reason (Muhamad *et al*., 2017). Both extracts showed no mortality and sign of toxicity which might justify the edibility of the pulp of mature fruit of the plant by human being in Ethiopia (Gebreegziabher, 2014).

APAP-induced lethality was used as a prescreening test to estimate hepatoprotective effects of the plant. APAP-induced deaths were reduced by 90% and 80% after oral administration of 400 mg/kg CAME and CAAE, respectively. Reduction in deaths induced by lethal dose of APAP, which is in agreement with that of a previous study (Gilani *et al*., 1997), could be attributed to the hepatoprotective activities of the extracts. This might be associated with the inflammation and oxidative stress modulating activities (Hohmann *et al*., 2015), i.e., anti-inflammatory and antioxidant activities of the plant (Isa *et al*., 2016). The decreased lethality effect of the extracts observed in the present study led to subsequent investigations carried out to confirm further the hepatoprotective activities.

The optimum dose of APAP which induced liver damage was determined to be 2g/kg through selection of three doses from the previous studies which was similar procedure used in other studies (Salwe *et al*., 2017; Azarmehr *et al*., 2019). APAP 2 g/kg is among the highest doses that have been used so far in previous studies which might have accounted for optimal induction of

liver injury in the present study. In the present study, administration of 2 g/kg APAP resulted in significant elevation of serum levels of liver enzymes; ALT, AST, ALP; TB, and lipid profiles; LDL, TC and TGs; and, decreased serum level of HDL which was in agreement with the previous study (Asadollahi *et al.*, 2014).

These findings are also similar to those of other studies (Oyagbemi & Odetola, 2010; Okokon *et al.*, 2017) in which serum levels of all above biomarkers were elevated after oral administration of both less and greater than 2 g/kg dose of APAP. This dose variation might be due to the differences in susceptibility of animals to the drug resulting from age and inter-species variations. Moreover, it has been reported that, the aforementioned biomarkers for liver damage are all increased after administration of other inducers of liver injury like CCL_4, which share common mechanism of cellular injury with APAP (Anusha *et al.*, 2011; Nasir *et al.,* 2013).

The increase in serum level of liver enzymes is due to the damage of hepatocytes by the APAP metabolite (NABQI) which cause rupture of the cell membrane resulting in leakage of the enzymes from hepatocytes to the serum, where the level of these enzymes is normally lower, and their serum levels become abnormally increased (Du *et al.*, 2016; Yan *et al.*, 2018). Lipid metabolizing incapability of the liver caused by APAP intoxication results in abnormally high levels of LDL, TG, TC and low level of HDL which would otherwise be metabolized to bile acids, transported into and stored in other body parts. After liver injury, bilirubin cannot appropriately conjugate and excreted which results in higher serum level of total bilirubin (Ozougwu, 2017).

Pre administration of standard drug, silymarin 100 mg/kg, significantly ($p < 0.5$) prevented HDL level from getting decreased and other biomarkers from getting increased by APAP administration. Percent protection of silymarin in terms of ALT was found to be beyond normal groups, i.e. serum level of ALT was lowered below normal controls, however the difference was found to be non significant ($p>0.05$) from multiple comparison. Silymarin is well known for its hepatoprotective activity which has been widely used as a standard drug in several similar studies. It had revealed protective activities in many previous studies which used APAP (Asadollahi *et al.*, 2014) or other liver damaging chemicals (Mondal *et al.*, 2020). It has been confirmed by Papackova *et al* (2018) that free radicals scavenging properties of silymarin accounts for its hepatoprotective activity.

Similarly, administration of both crude extracts at different doses and high dose of the solvent fractions of the CAME significantly reversed the levels of biomarkers compared to negative control. Methanol extract at dose of 200 mg/kg showed better activities than aqueous extract in terms of ALT, ALP, TB, TC and HDL levels. Additionally, almost similar results were obtained with both 100 mg/kg and 400 mg/kg extract doses with better activities in terms of ALT, ALP, HDL and ALT, AST, HDL, respectively, than aqueous extract at same doses which showed better activities in more than half of total biomarkers than methanol extract. This might be attributed to polarity difference between the two solvents in which more hepatoprotective components might have been extracted by methanol than water (Muhamad *et al*., 2017, Felhi *et al*., 2017).

CAME significantly reduced serum levels of ALT and AST dose-dependently, while CAAE did the same for AST, ALP and TGs which is similar to the stem extract of *Homalium letestui* Pellegr which reduced the above biomarkers dose-dependently in APAP-induced liver injury (Okokon *et al.*, 2017). This might be due to the increment in concentrations of responsible constituents with dose and/or to the strong role the three enzymes play for biomarker activity. However, CAME has shown dose-dependent decrease in percent protection in terms of ALP and TGs which could be due to some toxic phytoconstituents in the extract whose concentration increased with dose. Percent protections of 200 mg/kg CAME, 100 mg/kg CAAE and 400 mg/kg CABF in terms of serum TB level were found to be more than that of the standard treatment, 100 mg/kg silymarin. However the mean difference in serum TB level between the test substances and silymarin was not significant.

The same was true for serum level of ALP and LDL which seems to be more reduced by 400 mg/kg CACF than silymarin. As CAME showed better activity at medium dose, it was further fractionated with three solvents among which 400 mg/kg CACF resulted in highest percent protection in almost all biomarkers; ALT, AST, ALP, HDL, LDL, TC and TG. On the other hand CAAF has shown better activities in percent protection of HDL, ALT, AST and TGs than CABF. This is similar to Anyasor *et al* (2020) in which aqueous fraction of methanol extract of *Costus afer* leaves showed highest hepatoprotective effect at all three different doses in diethyl nitrosamine-induced liver carcinoma in rats.

However, CABF has shown highest percent protection in terms of TB than others and better activity than CAAF in terms of ALP, LDL and TC. This is similar to Sabbani *et al* (2016) in

which methanol extract and its n-butanol fraction of whole plant of *Gardenia gummifera* has shown a potent hepatoprotective activity against APAP induced liver injury. All these variations could be due to the differences in composition among the fractions in which CACF contained more responsible constituents and offered it highest hepatoprotective effects than others which might attribute to the polarity differences among the solvents. Histopathological examination was done to confirm findings of biochemical analysis.

In addition to liberation of liver enzymes, as APAP also induces oxidative stress followed by necrosis, cellular damage can also occur (Kaplowitz *et al*., 2015). In present study, necrosis and vacuolar degeneration have been observed through histopathological examination. Degrees of damage in terms of necrosis and vacuolar degeneration, as shown in Table 5.7, were higher in negative control groups which received only APAP compared with normal control groups. However, the damages were found to be lower in groups that received silymarin and test products. This is similar with previous studies (Moshaie-nezhad *et al*., 2018; Yan *et al*., 2018) in which necrosis, vacuolar degeneration and other cellular processes have been identified to be involved in the pathogenesis of APAP-induced liver injury and the damages were ameliorated by the extracts. All the findings of histopathological investigations were in agreement with those of biochemical findings, thereby confirming the hepatoprotective effect of the extracts and fractions.

In present study the sleeping time was prolonged from 75.70 ± 6.60 minutes to 161.33 ± 9.70 minutes by APAP, however both extracts and the solvent fractions of the CAME shortened SPB-induced sleeping time which could further confirm the hepatoprotective effects of the plant. This is similar to other study (Sabbani *et al.,* 2016) in which the APAP-prolonged SPB sleeping time was shortened by methanol extracts and solvent fractions *Gardenia gummifera* Linn. Moreover CAME has reduced the sleeping time comparable to the standard treatment, silymarin 100 mg/kg. This is also similar to the previous studies in which methanol extracts of *Olax viridis* root (Nwaigwe *et al*., 2012) and *Diaphananthe bidens* leaf (Aba *et al*., 2019) have shown hepatoprotective effect comparable to the standard drug, silymarin, by shortening the SPB-induced sleeping time in 2 g/kg APAP-induced liver injury in rats.

Sleeping time of animals which received CAME and CAAE without APAP was almost similar to that of the control groups which received sodium SPB alone confirming that the plant has no enzyme inhibitory activity and the sleeping time prolongation was caused merely by APAP. This

is similar with Gilani & Janbaz (1994) in which the plant *Artemisia scoparia* showed hepatoprotective activity with no effect on pentobarbital sleeping time. Among solvent fractions, CAAF showed the highest percent protection perhaps due to the variability in concentration and activity of hepatoprotective secondary metabolites in the fractions which accounted for the differences in hepatoprotective effects. The shortened SPB-induced sleeping time could explain that the extracts and solvent fractions might have protected the liver from getting damaged by APAP so that SPB was metabolized by xenobiotics-metabolizing enzymes resulting in reduction of its plasma concentration.

Findings of the present study are in agreement with the previous studies done on other species of the genus *Cordia*. *Cordia macleodii* (Qureshi & Haleem, 2009), *Cordia myxa* L (Afzal & Khan, 2014), *Cordia sebestena* (Pradeep & Balavardhan, 2015; Sunaryo *et al*., 2019) and *Cordia obliqua* (Sivagnanam & Devarasu, 2019) all have shown hepatoprotective effects. Being under the same genus might have accounted for the hepatoprotective effect shown by *C. africana* in the present study. Moreover, APAP induces liver injury through its free radical metabolite, NABQI. Plants having anti oxidant activity could possibly reverse all oxidative stresses caused by the APAP. Inflammatory reaction is also activated by these free radicals and it contributes in the cause of liver injury.

C. africana is a plant whose antioxidant (Tijjani *et al*., 2015) and anti-inflammatory (Isa *et al*., 2016) activities of its stem bark extracts had been confirmed experimentally. Furthermore, secondary metabolites which have shown anti oxidant and hepatoprotective activities in other studies were found in the phytochemical screening of *C. africana*. These secondary metabolites, which include alkaloids, flavonoids, phenols and tannins, were found in both aqueous (Ganesan & Banu, 2015) and methanol (Alhadi, *et al*., 2015; Isa *et al*., 2016) stem bark extracts of *C. africana.* Therefore, the hepatoprotective effects of *C. africana* in terms of biochemical, histological and pharmacological parameters in the present study could be associated with the anti-inflammatory and/or anti oxidant activities of the constituents present in the extracts and fractions.

6. LIMITATIONS

The following were the limitations of the present study:

- It did not investigate the reversal effect of the test substances on already injured liver, only the protective activity was assessed
- Only single dose of APAP was used to induce liver injury, so it did not address what would happen with repeated doses of APAP
- Only the stem bark of plant was evaluated for the hepatoprotective effect, i.e., it did not evaluate the other parts of the plant

7. CONCLUSION AND RECOMMENDATIONS

7.1. Conclusion

The result of present study revealed hepatoprotective activity of crude stem bark extracts and solvent fractions of *Cordia africana* lam. (boraginaceae) against acetaminophen-induced liver injury in rats. The present findings might provide scientific justification for the traditional claim in the use of *Cordia africana* for different forms of liver disease.

7.2. Recommendations

As the present study focused on some aspects of hepatoprotective effects of *Cordia africana*, further detailed studies including the following are recommended.

- Subchronic and chronic toxicity studies
- Hepatoprotective effects against various models of liver injury
- Reversal effect of the test substances on already injured liver
- Mechanism for hepatoprotective activity
- Isolation, purification and structural elucidation of phytochemicals responsible for bioactivity

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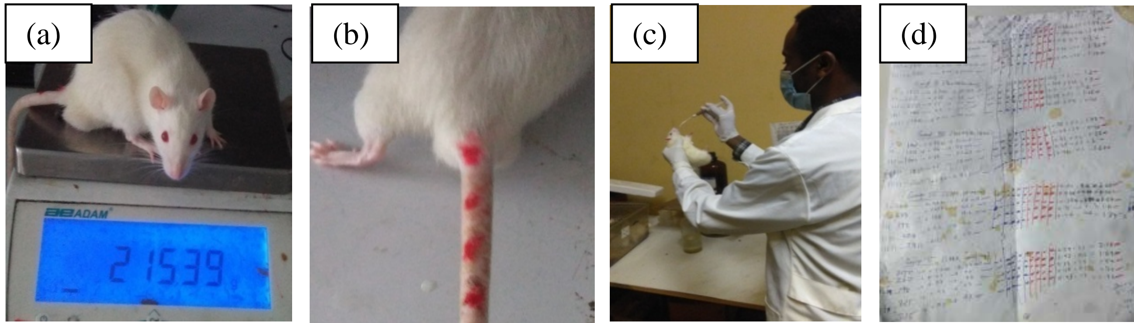
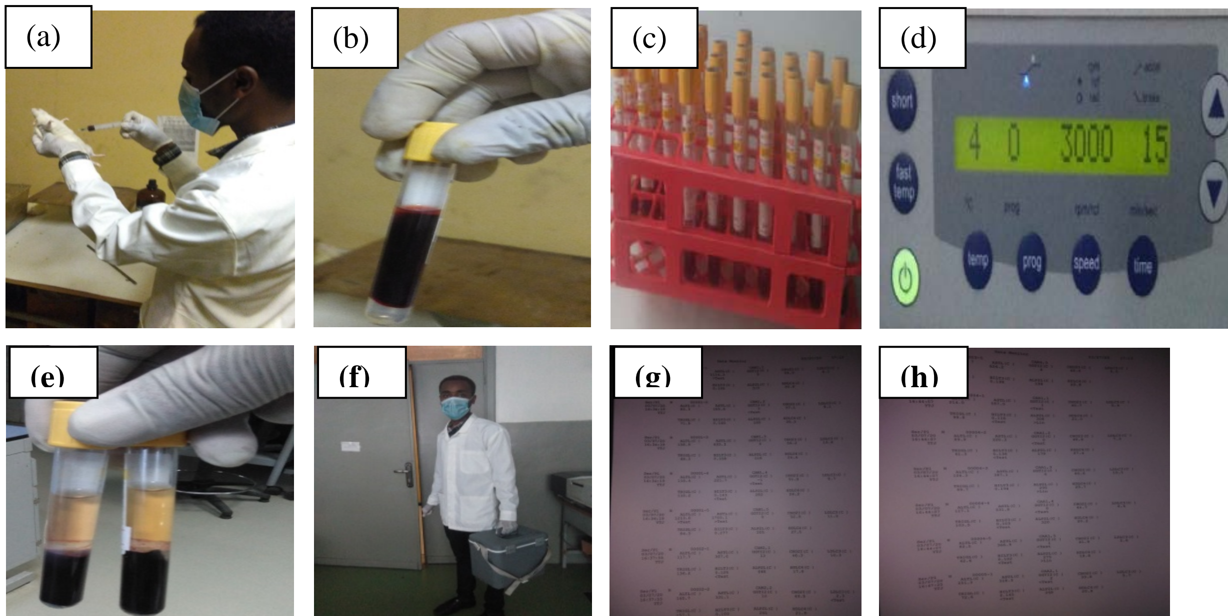
9. ANNEXES

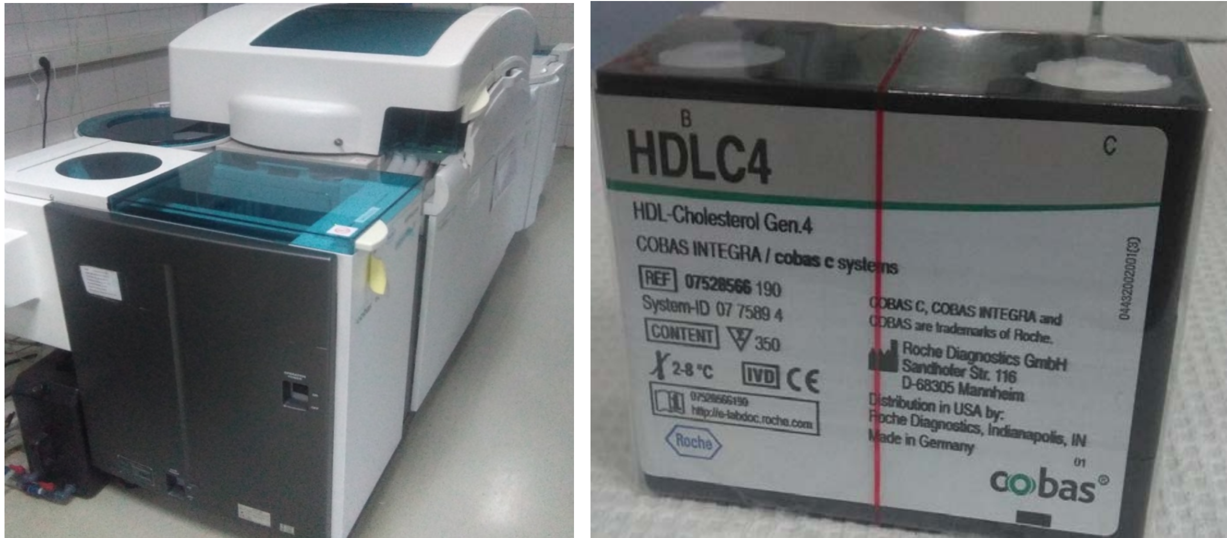
9.1. Photos of Weighing (a), Marking (b), Administering (c) and Recordings (d) of rats



9.2. Photos of Blood Sample Taking (a-c), Centrifugation (d-e), Transport to EPHI (f) and Received Results (g-h).



9.3. Photos of Comprehensive Bio-Analyzing System (Cobas^{R} 6000) and HDL reagent cassette at EPHI



9.4. Photos of Liver *in situ* before harvest (a), formalin-fixed livers from one group (b) and liver sections ready for processing (c).

9.5. Photos of Accreditation certificate of EPHI clinical chemistry laboratory by ENAO (a) and chemicals used as a calibrator and as control to assure quality of the result (b)

(a)

ETHIOPIAN NATIONAL ACCREDITATION OFFICE

ACCREDITATION CERTIFICATE

Ethiopian Public Health institute
National Clinical Chemistry Reference Laboratory
Addis Ababa
Ethiopia
Facility Accreditation No: **M0025**

Is accredited by the Ethiopian National Accreditation Office (ENAO) to perform tests in accordance with the attached Scope of Accreditation in the field of

Medical testing

The facility is accredited in accordance with the requirements of ES ISO 15189:2013, General requirements for the competence of Medical laboratories. The accreditation demonstrates technical competence for a defined scope and the operation of a laboratory quality management system. While this certificate remains valid, the Accredited Facility named above is authorized to use the relevant ENAO accreditation symbol to issue test reports.

Effective Date: **May 30, 2019**
Certificate Expires: **November 25, 2021**

Mr.Araya Fesseha
Director General
Ethiopian National Accreditation Office

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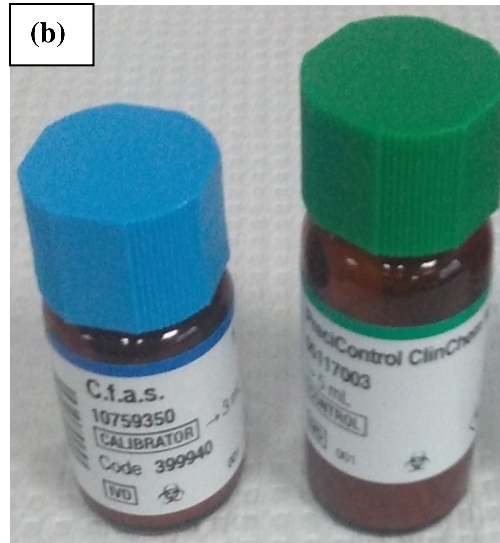
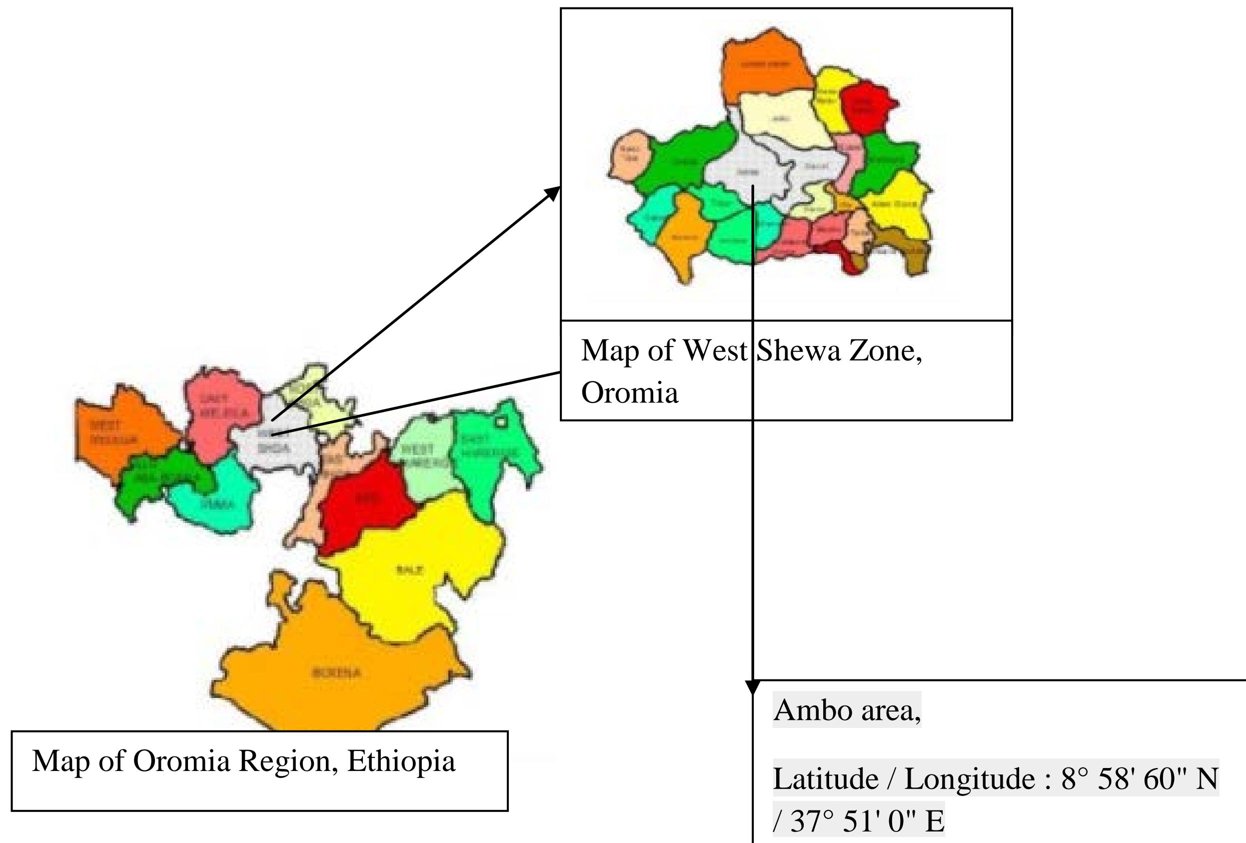


9.6. Map showing *C. africana* collection area.



Images were taken from Geleta & Grausgruber (2013).