Hepatic histomorphological changes following highly active antiretroviral therapy and the intervention of *Hypoxis hemerocallidea* in an experimental animal model

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Preface

The study described in this dissertation was carried out in the Discipline of Clinical Anatomy, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal Durban, South Africa from February 2014 to December 2015, under the supervision of Dr. O.O Azu and Dr. E.C.S Naidu.

Declaration

I, Mr. Salem Kharwa declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. This thesis does not contain other person's writing, data, pictures, or other information, unless specifically acknowledged as being sourced from other persons or researchers. Where other written sources have been quoted then:
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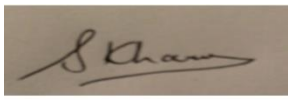
3. Signed  Date…05-04-2016……

Dedication

My sincere pride, joy and dedications to my daughters Tasnim and Yasmin Kharwa for making me proud dad with all their academic success and being the best daughters a dad could have.

I also would like to dedicate my Master's degree to my parents Hasim and Sarah Kharwa

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To my dear friend Mr Dan Naidu I would like to thank you for being beside me through my years in Anatomy and wish you all the best in your future retirement .

Table of contents

List of figures

Page

List of tables

Page

Tables from manuscript

List of plates from manuscript

Page

Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ANOVA	Analysis of variance
ART	Antiretroviral Therapy
BIL-T	Total bilirubin
BPH	Benign prostrate hyperplasia
cART	Combination Antiretroviral Therapy
CD4	Cluster of differentiation 4
CDC	Centre for Disease Control
COX	Cyclooxygenase
CYP3A4	Cytochrome P450 3A4
DPX	Permanent mount glue
FDA	Food and Drug Administration
GGT	Gama glutamyl transferase
HAART	Highly active Antiretroviral Therapy
HDL	High density lipoprotein
HIV	Human Immunodeficiency Virus
LDL	Low density lipoproteins
LFT	Liver function test
NASCOP	National AIDS and STI Control Program
NNRTIs	Non-nucleoside Reverse Transcriptase Inhibitors

NRTIs	Nucleoside Reverse Transcriptase Inhibitors
PIs	Protease Inhibitors
PLWHAs	People living with HIV and AIDS
PROT	Total protein
SADC	Southern African Development Community
T- Lymphocytes cells	Type of white blood cells
TNF-α	Tumor necrosis factor- alpha
UNAIDS	Joint United Nations Program on AIDS
WHO	World Health Organization

Abstract

Introduction

Hepatotoxicity has remained a serious complication limiting the efficacy of highly active antiretroviral therapy (HAART) regimen. While this challenge continues to exist, finding possible solutions continues to attract scientific solutions.

Materials and Method:

Sixty- three adult male Sprague-Dawley rats were used for the study and were divided into 9 groups (A-I). Group A received HAART cocktail (Lamivudine, Stavudine & Nevirapine), Group B received HAART and *H. hemerocallidea* extract (100 mg/kgbw), Group C received HAART and *H. hemerocallidea* extract (200 mg/kgbw), Group D received HAART and vitamin C, Group E received HAART and vitamin E, Group F received HAART, vitamin C and vitamin E, Group G received *H. hemerocallidea* extract (100 mg/kgbw), Group H received *H. hemerocallidea* extract (200 mg/kgbw), and Group I received water as placebo. The experiment lasted for 56 days after which, the animals were sacrificed, the liver were harvested and prepared for histological examination and blood samples were collected through cardiac puncture and centrifuged to get the serum for biochemical assessment.

Results

While no mortality was reported, animals treated with adjuvant HAART and AP recorded least %body weight gain. Significant derangements in serum lipid profiles were exacerbated by treatment of with AP as LDL (increased $p<0.03$), TG (increased $p<0.03$) with no change in total cholesterol levels. Adjuvant AP with HAART recorded reduced LDL ($p<0.05$ and 0.03), increased HDL ($p<0.05$) and TG ($p<0.05$ and 0.001). Markers of liver injury assayed showed significant increase ($p<0.003$, 0.001) in AST in AP alone as well as HAART+ vitamins C and E groups respectively. Adjuvant HAART and AP and vitamins C and E also caused significant declines in ALT and ALP levels. Serum GGT were not markedly altered. Histopathological derangements ranged from severe hepatocellular distortions, necrosis and massive fibrosis following co-treatment of HAART with vitamins C and E as well as HAART alone.

Conclusion

The results warrant caution on the adjuvant use of H. hemerocallidea with HAART by PLWHAs as implications for hepatocellular injuries are suspect with untoward cardio metabolic changes. More vigilant monitoring of patients at risk of antiretroviral toxicity is necessary and may prove helpful.

Keywords: *Liver, histoarchitecture, H. hemerocallidea,* HAART.

CHAPTER ONE

INTRODUCTION

1.1 Background

The Centers for Disease Control and Prevention first described human immunodeficiency syndrome and acquired immune deficiency syndrome (HIV/AIDS) in 1981 (Fenton and Silverman, 2008). Since then, HIV/AIDS have reached epidemic proportions affecting millions of people globally. HIV is of retrovirus family that can lead to AIDS, a condition in humans in which the immune system begins to fail leading to life threatening opportunistic infections such as tuberculosis, pneumonia, diarrhea, meningitis and tumors (Brooks *et al*., 2010). HIV is spread from human to human through direct sexual contact with sexual fluids and blood (Schreibman and Friedland, 2003). Infants can also be exposed to the virus through pregnancy and lactation (Schreibman and Friedland, 2003). The virus targets the immune system, in particular the CD4 lymphocytes also referred to as T-helper lymphocyte cells which are involved in protecting the body against infection (Fenton and Silverman, 2008).

According to the UNAIDS Report on the global AIDS epidemic (2008) there were an estimated 33 million people living with HIV in 2007 (UNAIDS, 2010). Sub-Saharan Africa, the geographical region most heavily affected by this epidemic accounts for 67% of all people living with HIV (UNAIDS, 2010). Famine, droughts, floods, poverty, food insecurity, war and political insecurities are common factors that affect the lives of people living in this region (Spencer *et al*., 2007). Reports have shown that during 2007 72% of AIDS deaths occurred in Sub-Saharan Africa (UNAIDS, 2010).

The introduction of highly active antiretroviral therapy (HAART) has led to a significant reduction in AIDS-related morbidity and mortality. HAART usually consists of a combination of at least three antiretroviral agents used with the intent to suppress viral replication and progression of HIV disease (Montessori *et al*., 2004). Due to HAART, HIV has become a manageable chronic condition, which results in an increase in life expectancy.

Despite substantial benefits of HAART, a variety of adverse effects have been associated with their use which reduces adherence and efficacy levels of the medication (d'Arminio *et al*., 2000). Metabolic complications including insulin resistance, glucose intolerance, lactic acidosis, liver

enzyme abnormalities, anemia, osteopenia and fat abnormalities (lipodystropy and dyslipidemia) have been associated with long-term usage of antiretroviral medications and occur in approximately half of all HAART-treated patients, (Carr, 2003; Montessori *et al*., 2004). Apart from underlying metabolic conditions, patients often have difficulties eating due to side effects like nausea, vomiting and loss of appetite and coupled with increased incidence of diarrhea these patients are at a high risk of becoming malnourished (Spencer *et al*., 2007). Among other toxicities associated with HAART, hepatotoxicity has also been another complication bedeviling the use of most antiretroviral drugs like zidovudine, stavudine or didanosine (Walker *et al*., 2004).

Sulkowski *et al*., (2000) observed that 18 out of 31 drugs causing hepatotoxicity in humans showed toxicity in liver enzymes in animals. The liver functions in the digestion of food, the formation of excretory products, the degradation of complex cellular materials (e.g., hemoglobin), the detoxification of unnatural compounds, the synthesis of plasma proteins, and the maintenance of energy and vitamin stores. Because of this functional complexity, and the dependence of liver function on its structure, any changes in liver anatomy become important considerations in the study of diseased or abnormal organisms (Champetier *et al*., 1985).

The human liver is fairly similar to that of mammals such as the presence of lobes and ligaments in its external surface and also functionally heterogeneous with the presence of lobules. The rat liver shares some common similarities with the human liver.

1.1.1 Gross anatomy of the rat liver

The rat liver is multilobulated as in other mammals. In rats, the liver mass represents approximately 5% of the total body weight, while in adult humans it represents 2.5% (Kongure *et al*., 1999). It has three surfaces: superior, inferior and posterior. The superior surface comprises a part of the left lateral lobes and medial lobes, and as a whole, is convex, and fits under the vault of the diaphragm. It is completely covered by the peritoneum, except along the line of attachment of the falciform ligament. The inferior surface is concave and is in relation to the stomach, duodenum, right colic flexure, the superior part of the pancreas, the right kidney and suprarenal gland (Kongure *et al*., 1999).

Although the rat liver inferior surface does not have the fossae in the shape of the letter H as in humans. This surface is almost completely invested by the peritoneum. The posterior surface is not covered by the peritoneum over some part of its extent, and is in direct contact with the diaphragm. It extends obliquely between the caudate lobe and the bare area of the liver (Kongure *et al*., 1999).

However, similar to the human liver, the rat liver is connected to the under surface of the diaphragm and to the anterior wall of the abdomen by five ligaments: the falciform, the coronary, and the two laterals are peritoneal folds; the fifth, the round ligament, is a fibrous cord, the obliterated umbilical vein. The liver is also attached to the lesser curvature of the stomach by the hepatogastric ligament and to the duodenum by the hepatoduodenal ligament (Lorente *et al*., 1995).

The rat liver lobes, like the human liver, are named after the portal branches that supply them. The medial lobe (ML) is the largest, accounting for approximately 38% of the liver weight. It has a trapezoidal shape and is fixed in the diaphragm and abdominal wall by the falciform ligament. It is subdivided by a vertical fissure into a large right medial lobe and a smaller left medial lobe (Bismuth, 1982).

The right lobe (RL) is located on the right of the vena cava and posteriorly in the right hypochondrium and is almost completely covered by the medial lobe. It comprises about 22% of the liver weight and is divided by a horizontal fissure into two pyramidal-shaped lobules called superior right lobe and inferior right lobe. The left lateral lobe (LLL) has a rhomboid shape, is flattened and situated in the epigastric and left hypochondriac regions over the anterior aspect of the stomach. Its medial portion is covered by the left part of the medial lobe. Its upper surface is slightly convex and is molded on the diaphragm. It has no fissures. The caudate lobe (CL) is situated behind the LLL and on the left of the vena porta and inferior cava vein. It comprises 8-10% of the liver weight. The origin and course of the major vessels are similar to those of humans (Bismuth, 1982).

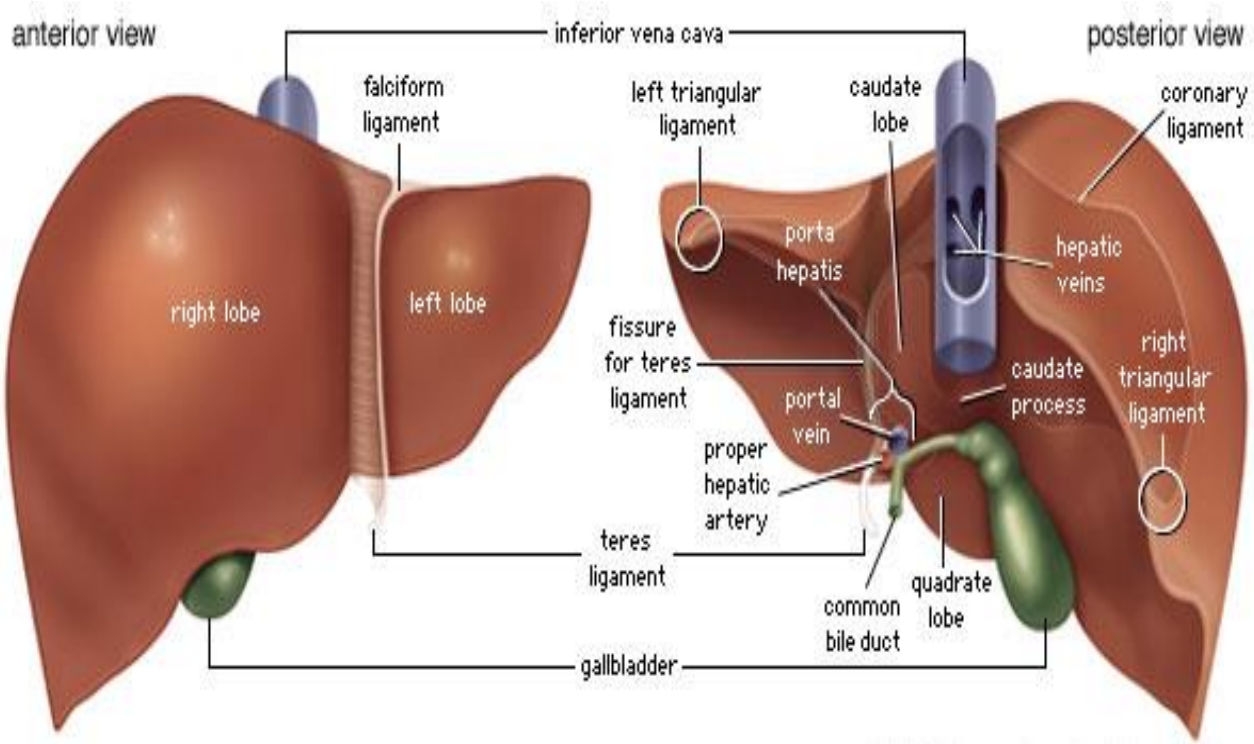

Figure 1.1: Schematic illustration of the gross anatomy of the rat liver (Adapted *from www. Ivyroses.com / Digestive system_ Liver Diagram*). Assessed on June 22, 2015.

1.1.2 Histology of the liver

The basic functional unit of the liver is the liver lobule. A single lobule is about the size of a sesame seed and is roughly hexagonal in shape (David *et al*., 2005). The primary structures in a lobule include:

a. Plates of hepatocytes form the bulk of the lobule
b. Portal triads at each corner of hexagon
c. Central vein
d. Liver sinusoids that run from the central vein to the portal triads
e. Hepatic macrophages (Kupffer cells)
f. Bile canaliculi ("little canals") – formed between walls of adjacent hepatocytes
g. Space of Disse – a small space between the sinusoids and the hepatocytes.

The portal triads consist of three vessels: a hepatic portal arteriole, a hepatic portal venule, and a bile duct. The blood from the arteriole and the venule both flow in the same direction, through the sinusoids toward the central vein, which eventually leads to the hepatic vein and the inferior vena cava. Secreted bile flows in the opposite direction, through the bile canaliculi away from the central vein, toward the portal triad, and exiting via the bile duct. As blood flows through the sinusoids and the space of Disse toward the central vein, nutrients are processed and stored by the hepatocytes, and worn out blood cells and bacteria are engulfed by the Kupffer cells (David *et al*., 2005).

The liver has 5 cell types: hepatocytes, Kupffer cells, sinusoidal endothelial cells, bile duct epithelial cells and Ito cells.
Hepatocytes represent 60% of the liver‟s cells, and about 80% of the liver‟s total cell mass. Most of the liver‟s synthetic and metabolic capabilities are functions of the hepatocytes. Hepatocytes are arranged in plates only a single cell thick. Blood flowing toward the hepatic vein within the space of Disse passes both exposed surface areas of the hepatocyte plates, and toxins and nutrients within the blood are extracted by the hepatocytes (Saxena *et al*., 1999).

Kupffer cells are macrophages that reside in the sinusoids. These cells help clear out old red blood cells and bacteria. They also break down heme (the iron-containing pigment in hemoglobin) into bilirubin, which then becomes one of the chief pigments of bile. A later by-product of bilirubin gives feces its characteristic brown color (Saxena *et al*., 1999).

Sinusoidal endothelial cells are fenestrated meaning they have large pores that allow most proteins to pass freely through the sinusoidal endothelium into the space of Disse, where they can make direct contact with hepatocytes. The pores are also bi-directional, meaning that proteins created by the liver and other substances stored or processed by the liver can also be passed back into the blood (Wisse *et al*., 1996).

Bile duct epithelial cells line the interlobular bile ducts within the portal triads (Strazzabosco and Fabris, 2008).

Ito cells are found in the space of Disse. They are important because when the liver is injured, the Ito cells transform into cells that produce collagen, which leads to liver fibrosis. If this occurs on a large scale, it can lead to cirrhosis of the liver (David *et al*., 2005).

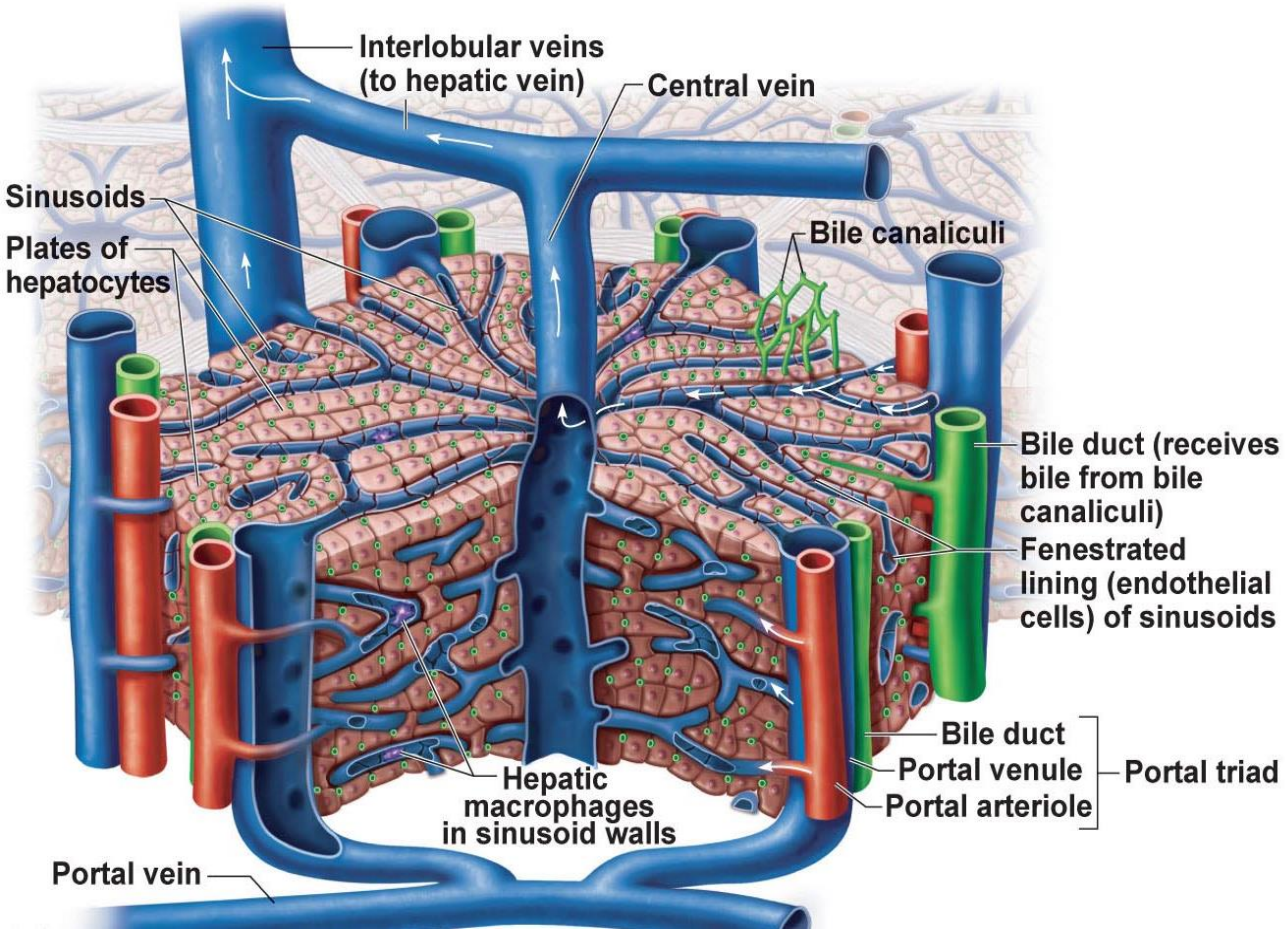

Figure 1.2: Schematic illustration of the structure of the liver lobule (Adapted *from www. Ivyroses.com/Human Body/Digestive_system_ Liver lobule Diagram*). Assessed on June 22, 2015.

1.1.3 Liver functions

The liver has 4 essential functions:

1. Synthesis of many proteins that circulate in the blood. These include albumin, coagulation factors, alpha1-antitrypsin, very low density lipoprotein, and many others (David *et al*., 2005).
2. Stores nutrients for later use. The liver balances the supply of nutrients with demand. For example, the liver stores glucose as glycogen, and converts it back to glucose as needed. If the supply of glycogen is depleted, the liver can also synthesize glucose from amino acids, lactate, and glycerol, although this is less efficient than breaking down glycogen into glucose. Additionally, the liver metabolizes fatty acids, cholesterol, and amino acids. When there is a surplus of glucose in the bloodstream, the liver can convert excess glucose and amino acids into fatty acids for storage. The liver both synthesizes cholesterol and removes it from circulation. Finally, the liver can synthesize non-essential amino acids when needed by the body (David *et al*., 2005).
3. Detoxification and elimination of toxic substances. Toxins are detoxified by the liver‟s ability to metabolize lipophilic compounds. These compounds (bound to albumin) enter the liver sinusoids and then the space of Disse. Enzymes in the hepatocytes (cytochrome P-450 enzymes) are involved in the metabolism of the lipophilic compounds, which include toxins and many drugs (David *et al*., 2005).
4. Production of bile. Bile acts as a detergent, and breaks fats down into smaller components so they can be digested in the small intestine. Bile also provides a way for the liver to remove wastes, including bilirubin, cholesterol, and toxins. Bile is formed in the biliary canaliculi, which drain into the interlobular bile ducts. These ducts then merge with other ducts, forming larger intermediate ducts, which eventually merge into the right and left hepatic ducts, which themselves merge into the common hepatic duct, which merges with the cystic duct from the gallbladder, finally forming the common bile duct, which empties into the small intestine (David *et al*., 2005).

Although, animal models cannot always predict human toxicities, however the frequency of drug toxicities described in clinical trials is not so thoroughly monitored and drug-induced toxicity is often detected long after a drug enters the market. The study was therefore carried out to mimic the therapeutic conditions of patients under antiretroviral therapy.

1.2 Pathophysiology and etiology of HIV/AIDS

Human immunodeficiency virus replicates in CD4+ T cells and results in the depletion of cells which in turn is responsible for gradual decline in the T cell immunity. HIV viral replication results in the production of viral particles which are responsible for the development of chronic inflammation that contributes hugely to the disease pathogenesis and progression towards AIDS and death (Deeks, 2009). There are two distinct serotypes of HIV virus: type 1 and type 2. The HIV-1 is the primary cause of acquired immunodeficiency syndrome (AIDS) worldwide while, HIV-2 is found predominantly in West Africa and its vertical transmission develops more slowly and milder compared to HIV-1 (Sanders *et al*., 2007).

Acquired immune deficiency (AIDS), is the late stage of HIV infection, a condition characterized by destruction of CD4+ T cells which help the body fight diseases (NASCOP, 2002). The syndrome was first identified in 1981 among homosexual men and intravenous drug users in New York and California and after its detection evidence of AIDS epidemics grew shortly after among heterosexual men, women, and children in sub-Saharan Africa (CDC, 2009). Although initial infection with HIV can result in flu-like symptoms, infected persons typically can show no symptoms for many years but as HIV replicate in the body, infected persons begin to show signs and symptoms of e.g., shingles, tuberculosis, oral or vaginal thrush, herpes simplex virus, and Kaposi sarcoma (WHO, 2009) which is a reflection of a weakened immune system or loss of the body‟s ability to fight infection.

HIV works by encoding the enzyme reverse transcriptase and thereby making a DNA copy of the viral RNA, which can remain in the nucleus of the infected cell for a long time (Morris and Cilliers 2008; Spencer, 2005). The virus attacks CD4 cells and macrophages and causing it to diminish in number thereby making the patients susceptible to opportunistic infections. As a result CD4 cell count together with viral load is frequently used to assess HIV disease progression (Fenton and Silverman, 2008). The virus is most commonly transmitted via blood or semen during unprotected intercourse with an HIV-infected individual (Fenton and Silverman, 2008). However, sharing contaminated needles and injecting contaminated blood products are also both ways of transmitting HIV (Spencer *et al*., 2007). Other body fluids that contain blood, pre-seminal fluid, vaginal fluid and breast milk are also possible routes for transmission of the virus (Fenton and Silverman, 2008). Mother to child transmission of HIV is a major global concern and can occur before or during birth or through breast-feeding (Semba, 2006).

HIV infected population are prone to liver disease as evidenced from literatures. HIV infection by itself may contribute to hepatic and biliary tract abnormalities including hepatomegaly, liver fibrosis, liver cirrhosis, hepatic/biliary steatosis and elevated liver enzymes. (Ramana *et al*., 2013). Co-infections with other hepatocellular viral infections like Hepatitis B virus and hepatitis C virus may further be responsible to liver related severe morbidity and mortality (Ramana, 2012).

The impact of HIV has been most severe in some of the poorest countries in Africa. At the end of 2009, there were 9 countries in Africa where more than one tenth of the adult population aged 15-49 years was infected with HIV (UNAIDS, 2010). In the same year, an estimated 1.8 million new HIV infections occurred in Africa accounting for 69 percent of new infections worldwide and 370,000 children began their lives with HIV (UNAIDS, 2010). Sub-Saharan Africa is more heavily affected by HIV and AIDS than any other region of the world. In 2008, it was home to two thirds (67%) of all people living with HIV and nearly three quarters (72%) of AIDS-related deaths (UNAIDS and WHO, 2009).

As the HIV/AIDS pandemic continues to expand, the moral imperative to provide safe and efficacious treatment options becomes of paramount interest to the international health-care communities. The use of antiretroviral therapy (ART) has become the cornerstone of the clinical approach available to prevent transmission and slow progression of the infection in people living with HIV/AIDS (PLWHA) worldwide. Efforts have begun for a significant scaling up of the use of antiretroviral drugs (ARVs) in settings such as sub-Saharan Africa where the epidemic has had its most devastating impact.

1.3 Management of HIV and AIDS- Antiretroviral therapy

Antiretroviral therapy (ART) was developed for people living with HIV/AIDS (PLWHAs) to prolong life and reduced mortality and morbidity associated with AIDS. UNAIDS estimated that more than 2.5 million deaths have been averted since the introduction of antiretroviral therapy (UNAIDS, 2010). The main aim of ART is to suppress the plasma viral load and to restore the immune function. (NASCOP, 2002). This concurs with the fact that in the early 1980s when HIV/AIDS epidemic began and the corresponding absence of ART, people with AIDS were not likely to live longer than a few years (Carcelain *et al*., 1999).

There has been increased expansion and access to ART to those eligible for treatment. Antiretroviral therapy coverage rose from 7% in 2003 to 42% in 2008, with especially high

coverage achieved in eastern and southern Africa (48%) (UNAIDS and WHO, 2009). According to UNAIDS and WHO estimates, 47% (6.6 million) of the estimated 14.2 million people eligible for treatment in low and middle-income countries were accessing life-saving antiretroviral therapy in 2010, an increase of 1.35 million since 2009 (UNAIDS and WHO, 2010). In the eastern part of Africa, AIDS-related deaths have fallen by 29% since 2002, a decline which is attributed to the use of antiretroviral drugs (NACC and NASCOP, 2006).

Today, there are more than 31 antiretroviral drugs grouped into five classes and approved by the U.S Food and Drug administration (FDA) to treat HIV infections (WHO, 2009). Each of the five classes attack HIV in a different way as depicted in the mechanism of action in column three of the table 1.

Table 1.1: Classes of ART and their mechanism of action.

ART class	Approval year	Mechanism of Action
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	1987	Inhibit reverse transcription by being incorporated into the newly synthesized viral DNA and preventing its further elongation
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	1997	Inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function.
Protease Inhibitors (PIs)	1995	Target viral assembly by inhibiting protease enzyme used by HIV to cleave nascent proteins for final assembly of new virus.
Fusion or Entry Inhibitors	2003	Prevent HIV from binding to or entering human immune cells.
Integrase Inhibitors	2007	Inhibit integrase enzyme needed by HIV to insert its genetic material into human cells.

Source: WHO (2009).

1.4 Combination antiretroviral therapy (cART)

However, conventional antiretroviral therapies (ARTs) do not completely eradicate HIV from the body and clinical trials are reporting that monotherapies are not completely effective (Saag and Kilby, 1999). Currently, preferred regimens use multi class combination product (involving combination of two or more antiretroviral therapy, an approach called highly active antiretroviral therapy (HAART) approved by the U.S Food and Drug Administration (FDA), which have resulted

in decreased HIV RNA levels and CD4 cell increases in the vast majority of patients (Saag and Kilby, 1999). With the advent of HAART, overall prognosis has improved dramatically with significant impact on the management of HIV infection, suppression of viral replication and reduction of the morbidity and mortality associated with AIDS (Palella *et al*., 1998). The advantage of HAART over monotherapy is a significant reduction in the risk of development of resistant strains commonly seen with monotherapy (Saag and Kilby, 1999). HAART has dramatically decreased the number of hospital admissions and AIDS patients have achieved an impressive improvement in the quality of life (Palella *et al*., 1998).

1.4.1 HAART related liver toxicity

While the use of HAART medications has had a profound impact on the AIDS epidemic in the world, it should be understood that the drugs carry their own side effects. Increasing adverse effects caused by HAART ranging from mild to severe have been well documented in many studies and are a major safety concern (Hawkins, 2010). Of late research has revealed that HIV infected individuals are at greater risks of developing non-infectious complications (Liver disease, cardiovascular disease that may precipitate with the initiation of HAART (Ramana *et al*., 2013).

Hepatotoxicity, liver enzymes elevation and drug interactions are a significant problem in HIV patients on HAART. Hepatotoxicity can influence HIV-1 treatment and can cause an increase in morbidity and mortality (Ramana *et al*., 2013). Protease Inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs) can be hepatotoxic with asymptomatic elevations in liver enzymes, liver dysfunction and liver failure (Martinez, 2004). Hepatotoxicity related to NRTIs was first documented in the early 1990s with zidovudine monotherapy (Gradon *et al*., 1992). Combination therapy was subsequently found to further increase the risk of hepatotoxicity. Asymptomatic enzyme elevations greater than five times normal have been recorded in patients under HAART (Saves *et al*., 1998).

The liver plays a central role in transforming and clearing of chemicals such as drugs and it is susceptible to damage from toxicity of these agents. Due to its unique metabolism and close relationship with the gastrointestinal tract, the liver receives blood coming directly from gastrointestinal organs and then spleen via portal veins which bring drugs and xenobiotics in near-undiluted form (Sulkowski, 2004). Certain medicinal agents, when taken in overdoses and sometimes even when introduced within therapeutic ranges, may injure the liver causing them to be

withdrawn from the market due to hepatotoxicity (Sulkowski, 2004). The National Institutes of Health of USA presented findings on liver toxicity in International AIDS Society (IAS) conference and its retrospective analysis showed that hepatotoxicity is associated with all classes of antiretroviral medications in use (Clifford *et al*., 2003).

Drug induced hepatotoxicity characterized by elevation of AST/ALT levels to at least twice the upper limit of normal (ULN) can occur with drugs from all ARV classes (Sulkowski *et al*., 2000). Several mechanisms are responsible for either inducing hepatic injury or worsening the damage process due to HAART. Many chemicals damage mitochondria causing it to release excessive amount of oxidants which, in turn, injure hepatic cells releasing intracellular enzymes into blood circulation (Martinez, 2004).

Although most liver diseases cause only mild symptoms initially, it is vital that early diagnosis and detection is done by performing full liver function tests (LFTs). Patients with HAART-induced hepatotoxicity may be asymptomatic, with liver injury diagnosed during routine blood testing, while others develop symptoms including nausea, fatigue, itching and jaundice with the latter symptom being significant (O'Brien *et al*., 2003). Liver function tests (LFTs) are carried out to detect the presence of liver disease, distinguish among different types of liver disorders, and gauge the extent of known liver damage and response to treatment (Prognosis) (Abrescia *et al*., 2005).

1.5 Liver function test (LFTs)

LFTs are a group of clinical biochemistry laboratory blood assays designed to give information about the state of a patient's liver (Abrescia *et al*., 2005). Some liver analytes in LFTs are associated with liver functionality e.g. Albumin (ALB) and total proteins (PROT), others are concerned with hepatocellular integrity e.g. aminotransferases (ALT & AST) and some associated with cholestasis - biliary tract blockage e.g. gamma-glutamyl transferase (ɣ-GT) and alkaline phosphatase (ALP) (MoH, 2007). Liver biomarkers are useful in the monitoring, evaluation and management of patients with hepatic dysfunction due to drug toxicity (Abrescia *et al*., 2005). The most important biochemical analytes of the liver significant in diagnosing drug-induced hepatotoxicity are outlined below.

1.5.1 Total protein (PROT)

Plasma proteins are synthesized predominantly in the liver and are the building blocks of all cells and body tissues. In the course of disease PROT concentration and the percentage represented by individual fraction can significantly deviate from normal values (Koller, 1984). Total protein measurements are used in the diagnosis and treatment of a variety of diseases involving the liver (Lindsey, 1986).

1.5.2 Albumin (ALB)

Albumin is a carbohydrate-free protein, representing 55 to 65 percent of the plasma PROT. It maintains the plasma colloidal osmotic pressure, transport and stores a wide variety of ligands and serves as a source of endogenous amino acids. It binds toxic heavy metal ions and many drugs which is why a decrease in albumin in the blood can have important pharmacokinetic consequence (Grant *et al*., 1987). Hyperalbuminemia is of little diagnostic significance except in dehydration but hypoalbuminemia is very common in many diseases. It stems from various factors namely, impaired synthesis as a result of liver disease or due to diminished protein intake and increased catabolism due to tissue damage or inflammation (Grant *et al*., 1987). An albumin measurement also allows for monitoring of the patient‟s response to nutritional support and is useful test of liver functionality (Marshal, 1989). In severe hypoalbuminemia, plasma albumin levels are below 25g/L (Grant *et al*., 1987).

1.5.3 Alanine aminotransferase (ALT)

Alanine aminotransferase (ALT) is an enzyme present in a variety of tissues and its major source is the liver. Measurement of ALT levels is used in diagnosis of hepatic disease where elevated serum ALT is found in hepatitis, cirrhosis, obstructive jaundice, carcinoma of the liver, and chronic alcohol abuse (Sherwin and Sobenes, 1996). Both serum aspartate aminotransferase (AST) and ALT become elevated whenever disease processes affect liver cell integrity. Though ALT is liver specific and its activity persist longer than elevations of AST activity (Sherwin and Sobenes, 1996). Elevated ALT/AST above 40U/L is an indicator for hepatotoxicity which can be categorized as mild/grade 1 (40-84 U/L); moderate/grade 2 (85-174 U/L); severe/grade 3 (175-350U/L) and severe/grade 4 (>350U/L) (MoH, 2007).

1.5.4 Aspartate aminotransferase (AST)

Aspartate aminotransferase is widely distributed in tissue, principally hepatic, cardiac, muscle, and kidney and elevated serum levels are found in diseases affecting these tissues (Nagy, 1984). Hepatobiliary diseases such as cirrhosis, metastatic carcinoma, and viral hepatitis also increase serum AST levels (Nagy, 1984).

1.5.5 Alkaline phosphatase (ALP)

Alkaline phosphatase is a group of phosphatases found in almost every tissue in the body. Normal adult males tend to have ALP higher levels than females, but pregnant females have increased levels due to placental secretion of ALP (Moss *et al*., 1987). Normal ALP levels are elevated during periods of active bone growth, like in young children and adolescents however, abnormal elevation of ALP levels >160 U/L occurs in diseases such as hepatitis, cirrhosis, malignancy, chemical toxicity, and bone diseases such as metastatic carcinoma, rickets, Paget‟s disease, and osteomalacia (Moss *et al*., 1987).

1.5.6 Gamma glutamyl transferase (GGT)

Gamma glutamyl transferase is an enzyme involved in the transfer of γ-glutamyl residue from γ-glutamyl peptides to amino acids, water and other small peptides. GGT activity is found primarily in brain, prostrate, pancreas and liver (Krefetz and McMillin, 2005). Enzymatic activity of γ-GT is often the only parameter with increased values when testing for diseases affecting the mentioned organs and is one of the most sensitive indicators known (Krefetz and McMillin, 2005). γ-GT activities are found in the serum of patients requiring long term medication with Phenobarbital and phenytoin (Krefetz and McMillin, 2005). Clinical applications of assay however are confined mainly to diagnosis and monitoring of hepatobiliary disease (Krefetz and McMillin, 2005).

1.5.7 Total bilirubin (BIL-T)

Bilirubin is formed in the reticulo-endothelial system during the degradation of aged erythrocytes. The heme portion from hemoglobin and from heme-containing proteins is removed, metabolized to bilirubin, conjugated with glucuronic acid for solubilization and subsequent transport through the bile duct and elimination via the digestive tract (Fody, 2005). Elevations of circulating unconjugated bilirubin occur in liver immaturity and several diseases, in which the bilirubin

conjugation is impaired causes. Bile tract obstruction or damage to hepatocellular structure causes increase in levels of both direct and indirect bilirubin in the circulation (Balisteri and Shaw, 1987).

As PLWHA continue to rely on HAART to enhance their quality of life, HAART-related toxicities keeps occurring and are encountered by clinicians (Campbell *et al*., 2009) and there is need to tackle this challenge.

1.6 Interactions of HAART and plant-based extracts

There has been a great deal of interest recently in the role of complementary and alternative medicines for the treatment of various acute and chronic diseases (Aruoma *et al*., 2006). Of the various classes of phytochemicals, interest has focused on the anti-inflammatory and antioxidant properties found in various plants extracts. Recently medicinal plants are being increasingly utilized to treat a wide variety of clinical diseases and numerous cultures still rely on indigenous medicinal plants for their primary health care needs (Gurib-Fakim *et al*., 2010).
South Africa, a country with a strong history of traditional healing, hosts a variety of around 30,000 flowering plant species, accounting for almost 10% of the world‟s higher medicinal plant species (Van Wyk, 2008).

Reynolds *et al*., 2003 observed that access to effective therapies for HIV-positive persons is arguably one of the highest global public health priorities but while providing affordable access to PLWHAs, treatment should be accompanied with alternative therapy to enable patients live longer and healthier. These authors suggested that “the expansion of access to highly active antiretroviral therapy (HAART) should be accompanied by a more evidence-based approach to optimize HIV care guidelines locally” (Reynolds *et al*., 2003).

Consequently, an enhanced appreciation of the potential interactions between HAART and plant-based extracts will be critical for the promulgation of relevant evidence-based practice guidelines. The importance of this will be a distinguishing characteristic and may prove helpful in combating the disease course. Although consideration of the potential for herbal-drug interactions is most often limited to questions of bioavailability, and the metabolic fate of drugs (Reynolds *et al*., 2003).

1.7 *Hypoxis hemerocallidea*

Hypoxis hemerocallidea (*H. hemerocallidea*) belongs to the genus *Hypoxis,* from the large lily family *Hypoxidaceae* and is widely distributed in the southern Africa sub-region (Boukes and van de Venter, 2011). The plant is characterized by strap like leaves, and bright yellow, star- shaped flowers (Musabayane *et al*., 2005). The tuberous rootstock (i.e. the corm) of the plant is commonly referred to as „African potato" and is referred to in various local languages as Afrika patat (Afrikaans), inkomfe (Zulu) and ilabatheka (Xhosa) (Ojewole *et al.,* 2009). There are over 90 *Hypoxis* species spread throughout sub-Saharan Africa, North and Southern America, South East Asia, and Australia. Southern Africa holds a diversity of about 45 of these species (Drewes *et al*., 2008).

Taxonomically, the *H. hemerocallidea* plant falls under the star-lily family or *Hypoxidaceae,* which consists of 8 general and 130 species; with 90 of them belonging to Southern Africa (Drewes *et al*., 2008).

This family usually consists of monocotyledonous plants, which are normally found in the savanna regions of South Africa, Swaziland, Lesotho, Botswana, Mozambique, Zimbabwe and in North-Eastern Africa (Drewes *et al*., 2008; Katerere and Eloff, 2008). In South Africa, *H. hemerocallidea* plant is found growing in the wild areas of the Eastern Cape, KwaZulu-Natal, Gauteng and Limpopo provinces. It can also be found in the mountainous areas of South America, Australia, and in the coasts of Asia (Drewes *et al*., 2008).

The *H. hemerocallidea* plant has been described as a stemless, geophytic, perennial herb with large dark brown to black corms (tubers) and bright yellow flowers; the plant is a herbaceous and a tuberous perennial plant that consists of yellow star-shaped flowers, long strap-like leaves (30 cm long and 3.2 cm wide), brown tuberous rhizomes or corms (up to 10 cm in diameter or length and about a half a kilogram in weight) and lots of adventitious roots that allow them to survive unfavorable conditions as shown in the figures 1.3 and 1.4 (Ndong *et al*., 2006).

Traditionally, after washing with clean water, the plant"s corms are cut into small pieces, boiled for about 20mins, and then the decoction is consumed orally. *H. hemerocallidea* extracts, powders, infusions and decoctions have been used for centuries by southern African traditional healers for the treatment, management and control of an array of human ailments, including cancers, nervous disorders, immune-related illnesses, heart weaknesses and urinary tract infections (Owira and Ojewole, 2009).

Figure 1.3: Photograph showing the *H. hemerocallidea* plant (Drewes *et al.*, 2008).

Figure 1.4: Photograph showing the macroscopic view of the *H. hemerocallidea* corm (Drewes *et al*., 2008).

1.7.1 Chemical constituents of *Hypoxis hemerocallidea*

H. hemerocallidea corm has a catalogue of folkloric and therapeutic uses. It is one of the most popular and ethno-botanically acknowledged medicinal plants in southern Africa (Drewes *et al*., 2008). Attempts have been made in some laboratories to isolate, purify and characterize the chemical constituents of this plant‟s corm that could be responsible for its medicinal properties. One of the most important chemical constituents of the herb which has been confirmed to be abundantly present in extracts of *H. hemerocallidea* is a norlingan diglucoside, hypoxoside, a biologically inactive pro-drug (Nair and Kanfer, 2006), with an uncommon aglycone structure, consisting of diphenyl-1-en-4-yne-pentane skeleton (Nair *et al*., 2007). Hypoxoside is reported to have low toxicity, hence, the traditional consumption of „African potato‟ as a food (Smit *et al*., 1995). In human gut, hypoxoside is converted to rooperol, a biologically active compound, by betaglucosidase enzyme, which is abundantly present in the human gut and rapidly dividing cancer cells (Mills *et al*., 2005).

Both rooperol and its pro-drug, hypoxoside, have been shown to undergo phase 1 hepatic metabolism by cytochrome P_{450} (probably CYP 3A4) enzyme, while their phase II metabolic products, consisting of diglucuronide, disulphate and mixed glucuronide-sulphates, are eliminated by first-order kinetics (Mills *et al*., 2005). Rooperol can be recovered from these metabolites by deconjugation reactions (Albrecht *et al*., 1995). Most of the therapeutic properties of *H. hemerocallidea* extracts observed clinically in man and in laboratory animals to date have been attributed to rooperol (Albrecht *et al*., 1995).

Rooperol has been shown to be antineoplastic, bacteriostatic and bactericidal (Drewes *et al*., 2008). It has been suggested that the antimetastatic activity of rooperol could be mediated through its ability to stimulate the synthesis of collagen type 1 that could impede cell invasions (Dietzsch *et al*., 1999). Recent laboratory investigations have shown that rooperol has a strong antioxidant activity, a strong affinity for phospholipid membranes, and that it inhibits free radical-induced membrane lipo-oxidation (Laporta *et al*., 2007). These findings seem to suggest that rooperol is important in the maintenance of cell membrane stability (Hostetmann *et al*., 2000), a phenomenon which partially explain its activity against neoplastic cells. It is still unclear at the moment, whether the ability of rooperol to inhibit inflammatory processes *in vitro* and *in vivo*, is as a consequence of its direct ability to inhibit the production of pro-inflammatory cytokines, tumour necrosis factor (TNF)-

α, and interleukins; or due to its inhibitory effects on enzymes involved in the synthesis of pro-inflammatory mediators, such as leukotrienes and prostaglandins (Hostetmann *et al*., 2000).

In a study, Van der Merwe *et al*., (1993) showed that rooperol is a potent inhibitor of lipo-oxygenase (an enzyme that catalyses the first-step in the conversion of arachidonic acid to leukotrienes), but not cyclooxygenase (COX), which catalyses the rate-limiting step in the conversion of arachidonic acid to prostaglandins. Another study by Guzek *et al*., (1996), in an investigation which attempted to highlight the potential therapeutic benefit of *H. hemerocallidea* extracts in the treatment of airways inflammatory diseases, showed that rooperol and its derivatives inhibit the production of TNF-α, interleukin1-β and interleukin-6, and also suppress the production of nitric oxide *in vitro*. However, direct inhibitions of COX-1 (constitutive) and COX-2 (inducible) isoforms of the COX enzymes by extracts of *H. hemerocallidea* corm have also recently been reported (Gaidamashivili and Van Staden, 2006).

Despite the prevailing uncertainties about the precise mechanisms by which rooperol exerts its anti-inflammatory effects, it is important to recognize that rooperol shares intimate structural similarity with a well-known, strong antioxidant, nordihydroguairectic acid (Nair *et al*., 2007), and comparably inhibits leukotriene and prostaglandins synthesis in polymorphonuclear leukocyte and platelet microsomes, respectively (Coetzee *et al*., 1996).

On the strength of the available scientific, pharmacological and clinical evidence, several patents have been registered on rooperol, and the extract has also been registered under the trade name „Harzol™" in Germany for the treatment of prostate cancer (Nair *et al*., 2007). At present, there are numerous commercial herbal preparations which contain rooperol or extracts of *H. hemerocallidea* in the market for the treatment, management and control of many modern and 21st century diseases of man (Nair *et al*., 2007).

Among the chemical constituents of *H. hemerocallidea*, phytosterols have been suggested to be partly responsible for some of the observed therapeutic and pharmacological properties of the corm"s extracts. Mohamed and Ojewole (2003) attributed the hypoglycaemic effect of *H. hemerocallidea* extracts observed in streptozotocin induced diabetic rats to phytosterols and sterolins. Phytosterols are known to stabilize plant cell membranes (Nair and Kanfer, 2006), and are also known to have many therapeutic benefits, including enhancement of immune system in immune-compromised individuals (Laporta *et al*., 2007). The antiprostatic adenoma activity attributed to extracts of *H. hemerocallidea*, has also been ascribed to phytosterol glycosides, mainly

β-sitosterol glycosides (Hostetmann *et al*., 2000). These claims, however, remain speculative in view of the fact that daily intake of the same amounts of phytosterols and their glycosides from other plant sources have not produced the same magnitude of therapeutic effects (Hotelman *et al*., 2000).

1.7.2 Pharmacological properties of *Hypoxis hemerocallidea*

In recent years, attempts have been made to investigate the scientific basis of the therapeutic claims attributed to *H. hemerocallidea*. Evidence-based laboratory investigations indicate that aqueous and alcohol extracts of *H. hemerocallidea* possess many interesting pharmacological properties, including anti-nociceptive (in mice), anti-inflammatory and anti-diabetic properties (in rats) *in vivo* (Ojewole, 2006). „Intraperitoneal injections of 50-800 mg/kg body weight of *H. hemerocallidea* extracts produced significant and dose-dependent anti-nociceptive effects against chemically- and thermally-induced nociceptive pain in mice" (Ojewole, 2006). At the same dose level, oral administrations of *H. hemerocallidea* corm extract also „significantly inhibited egg albumin-induced acute inflammation and streptozotocin-induced diabetic rats in a dose-dependent manner" (Ojewole, 2006). These observations, therefore, suggest that extracts of *H. hemerocallidea* could possess anti-inflammatory and anti-diabetic (hypoglycaemic) properties respectively.

As suggested by Ojewole (2006), the extracts of *H. hemerocallidea* could inhibit the synthesis, production and release of inflammatory cytokines and mediators such as prostaglandins. Studies have also indicated that lectin-like proteins purified from aqueous extracts of *H. hemerocallidea* can inhibit cyclooxygenase (COX) enzyme that mediates prostaglandins synthesis *in vitro* (Gaidamashivili and Van Staden, 2006). However, other studies have shown that ethanol extracts of *H. hemerocallidea* have higher inhibitory effects on COX-1 catalysed prostaglandin synthesis than aqueous extracts of the plant"s corms (Steenkamp *et al*., 2006). Aqueous extracts of *H. hemerocallidea* corms have previously been shown to scavenge free radicals (hydroxyl ions) *in vitro* (Mahomed and Ojewole, 2003), and it has been suggested that the ability of the corm"s extracts (both aqueous and ethanol) to suppress inflammation could be mediated via its antioxidant activity which in turn, inhibits COX enzymes (Steenkamp *et al*., 2006).

It has also been reported that lectin-like proteins derived from extracts of *H. hemerocallidea* inhibited the growth of *Staphylococus aureus, in vitro* (Gaidamashivili and Van Staden, 2002). Undoubtedly, agglutinins found in the storage parts (corms) of *H. hemerocallidea*, play a critical role in the plant"s defensive mechanism against pathogenic micro-organisms. This observation

would, therefore, support the age-old usage of *H. hemerocallidea* in the treatment of microbial infective disorders (Hutchings *et al*., 1996; Laporta *et al*., 2007).

Laboratory reports have further shown that both ethanol and aqueous extracts of *H. hemerocallidea* corm inhibit the growth of *Escherichia coli, in vitro* (Steenkamp *et al*., 2006), an observation quite consistent with the previously reported antibacterial activity of *H. hemerocallidea* (Gaidamashivili and Van Staden, 2002). *Escherichia coli* infection is the most common secondary cause of bacterial prostatitis (Steenkamp *et al*., 2006). This observation may therefore, partly also explain the traditional usage of the extracts of this plant‟s corm in the treatment of urinary tract infections (Singh, 1999). Benign prostate hyperplasia (BPH) can cause blockade of the urinary tract in men, and this may lead to chronic bacterial prostatitis. Anecdotal and folkloric reports have documented that *H. hemerocallidea* extracts have been used traditionally in the treatment of prostate hyperplasia (Singh, 1999). The observation that *H. hemerocallidea* extracts can inhibit the growth of *E.coli in vitro*, therefore, seems to support the use of this plant‟s corm in the treatment of prostate hyperplasia.

However, more recent experimental and clinical evidence have suggested that the effects of *H. hemerocallidea* corm extracts on BPH might not only be due to their antibacterial activities, but could also be due to their anti-inflammatory and antioxidant properties (Ojewole, 2002). This body of evidence, therefore, supports the claims that *H. hemerocallidea* extracts may contain chemical compounds that suppress tumour growth, and hence, its use in the treatment of cancers (Nair *et al*., 2007).

It has also been reported that aqueous extracts of *H. hemerocallidea* caused bradycardia and brief hypotension in guinea-pigs and rats *in vitro* and *in vivo*, respectively (Ojewole *et al*., 2006). Although the investigators were unable to establish the precise pharmacological mechanisms underlying their observations, they ruled out involvement of the cholinergic system, since the cardio-depressant effects of the extracts were not modified by atropine pretreatment (Ojewole *et al*., 2006). However in a study involving Chacma baboons, Coetzee *et al*., (1996) established that a purified extract of *H. hemerocallidea* corms (rooperol) increased myocardial contractility *in vivo*. Rooperol caused moderate, transient increased cardiac output, stroke volume and vascular pressures without increased heart rate or filling pressures, suggestive of increased myocardial contractility probably allied to its catechol structure (Coetzee *et al*., 1996).

The cardiovascular observations tend to suggest that extracts of *H. hemerocallidea* corm contain some bioactive chemical compounds with cardiovascular activities. These findings may also lend pharmacological credence to the age-old usage of this plant in the treatment and management of heart ailments and hypertension in some rural communities of southern Africa.

However, what has recently stimulated the greatest interest, not only among traditional healers and their patients, but also in scientific communities, the pharmaceutical industries as well as in government circles, is the claim that *H. hemerocallidea* corm can boost human immune system. Some healthcare providers in South Africa are currently using extracts of *H. hemerocallidea* corms as immune-stimulant preparations for patients living with HIV/AIDS, on the strength of the recommendation of South Africa‟s national Department of Health (Southern Africa Development Community, 2002; Mills *et al*., 2005).

In this regard, the use of this plant‟s corms has been extended to immune-related illnesses, such as common cold, flu and arthritis (Mills *et al*., 2005). Unfortunately, despite the popular belief in the immune-boosting properties of this plant‟s corms, there is absolutely no laboratory or clinical evidence yet to support this immune-stimulant claim, which at present still remains speculative.

1.8 Statement of research problem

The goals of HAART include achieving and maintaining viral suppression, improving immune function and delaying disease progression. This has led to improved wellbeing and longevity of people living with HIV/AIDS (PLWHAs) (Tomkins, 2005). However, adverse effects have been associated with this chemotherapeutic regime for PLWHAs (Charles *et al*., 2014). For instance, hepatotoxicity has been reported in most antiretroviral drugs contributing to more than 50% of acute liver failure cases, a fraction of which require immediate transplantation (d‟Arminio *et al*., 2000).

These adverse effects can lead to non-adherence and interruption or discontinuation of HAART which increases risk for cardiovascular disease, hepatic complications, opportunistic infections and eventually death (Nerad *et al*., 2003, Hammer *et al*., 2008, Stein, 2009). The hepatotoxicity associated with HAART, complicates its management and increases the cost of overall health care making it a double edged sword.

It is anticipated that as the population of PLWHAs remain on HAART for longer period of time, HAART-related metabolic disorders would correspondingly increase. The consequences are quite huge in terms of economic cost and care. Jevtovic (2008) observed that the cumulative long-term toxicities of HAART emerge as a significant complication to the liver.

These adverse effects associated with using HAART for the treatment of HIV infection have further encouraged the utilization of herbal medicines as an alternative medical therapy by PLWHAs (Gurib-Fakim *et al*., 2006). This complementary or alternative therapy is thought to mitigate this toxic effect of HAART regimens (Gurib-Fakim *et al*, 2006).

1.9 Research questions

i. Does *H. hemerocallidea* mitigate liver injuries following HAART?
ii. Would vitamins C and E serve as a better antioxidant supplements in HAART-induced liver injuries?
iii. Does liver function tests (LFTs) reveal information on the liver following HAART and *H. hemerocallidea*?

1.10 Objectives of study

The objectives of this study are presented in line with three major focus areas:

a. To determine the histological effects of highly active antiretroviral therapy on the liver of experimental animals.
b. To determine the effect of co-administration of crude aqueous extract of Hypoxis hemerocallidea and highly active antiretroviral therapy on the liver of experimental animals.
c. To assess the changes in liver enzymes in HAART treatment alone and co-administration of Hypoxis hemerocallidea in experimental animals.

CHAPTER TWO

MANUSCRIPT FROM RESEARCH

Hepatic histomorphological and biochemical changes following highly active antiretroviral therapy in an experimental animal model: does *hypoxis hemerocallidea* exacerbate hepatic injury?

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Abstract

As the roll-out of antiretroviral therapy continues to drive downwards morbidity and mortality in PLWHAs, organ toxicities (especially the liver) are frequently becoming a major concern for researchers, scientists and healthcare planners.

This study was conducted to investigate the possible protective effect of *Hypoxis hemerocallidea* (*H. hemerocallidea*) against HAART-induced hepatotoxicity. A total of 63 pathogen-free adult male Sprague-Dawley rats were divided into 9 groups and treated according to protocols.

While no mortality was reported, animals treated with adjuvant HAART and *H. hemerocallidea* recorded least %body weight gain. Significant derangements in serum lipid profiles were exacerbated by treatment of with *H. hemerocallidea* as LDL (increased $p<0.03$), TG (increased $p<0.03$) with no change in total cholesterol levels. Adjuvant *H. hemerocallidea* with HAART recorded reduced LDL ($p<0.05$ and 0.03), increased HDL ($p<0.05$) and TG ($p<0.05$ and 0.001). Markers of liver injury assayed showed significant increase ($p<0.003$, 0.001) in AST in *H. hemerocallidea* alone as well as HAART+ vitamins C and E groups respectively. Adjuvant HAART and *H. hemerocallidea* and vitamins C and E also caused significant declines in ALT and ALP levels. Serum GGT were not markedly altered. Histopathological derangements ranged from severe hepatocellular distortions, necrosis and massive fibrosis following co-treatment of HAART with vitamins C and E as well as HAART alone. These results warrant caution on the adjuvant use of *H. hemerocallidea* with HAART by PLWHAs as implications for hepatocellular injuries are suspect with untoward cardiometabolic changes.

Introduction

The acquired immune-deficiency syndrome (AIDS) is a significant threat to the health of mankind and the search for effective therapies to treat AIDS is of paramount importance. The development and evolution of anti-human immune deficiency virus (HIV), which causes AIDS has tremendously improved over the last 2 decades with resultant significant increase in life expectancy among HIV-infected patients (Azu, 2012; Charles *et al*., 2014). Highly active antiretroviral therapy (HAART), a combination of this chemotherapeutic regime for people living with HIV/AIDS (PLWHAs), suppresses viral replication but its major drawback is adverse effects of toxicities to organs (Charles *et al*., 2014).

Hepatotoxicity has been associated with most antiretroviral drugs like zidovudine, stavudine or didanosine (Abrescia *et al*., 2005). The liver functions in the formation of excretory products, the degradation of complex cellular materials, and the synthesis of plasma proteins. Because of this functional complexity, any change in liver function and histoarchitecture, becomes an important consideration for study of abnormality (Soriano *et al*, 2008).

Besides the high cost of HAART regimens, the adverse effects associated with using chemotherapy for the treatment of HIV infection have further encouraged the utilization of herbal medicines as an alternative medical therapy by PLWHAs (Ji *et al*., 2001). This complementary or alternative therapy is thought to mitigate this toxic effect of HAART regimens (Gurib-Fakim *et al*, 2010).

Hypoxis hemerocallidea (*H. hemerocallidea*) commonly called African potato has a long history of traditional use for a diversity of ailments (Drewes *et al,* 1984) and more recently has been the subject of several scientific studies. In many parts of Africa the corms of this attractive yellow flowered herb have been used in the treatment of urinary diseases, prostate hypertrophy, and internal cancer (Nair *et al*, 2007) and more recently as immune boosters for HIV-AIDS. Its traditional usage dates back many generations (Awad and Fink, 2000) and anecdotal evidence indictaes that the plant can be poisonous (Arnold *et al*, 2000). *H. hemerocallidea* is noted for the occurrence of a hypoxoside which is a secondary metabolite of the plant (Nair *et al*, 2007) that is hydrolyzed into rooperol-the active and powerful antioxidant component of the corm (Laporta *et al*, 2007) in the large intestine.

Pharmacokinetic studies have indicated that rooperol can be found in feces, and metabolites are found in the serum and urine as its glycosides, sulfates, mixed glucuronides, and sulfuronides (Kruger *et al,* 1994). These metabolites, when conjugated back to rooperol, were found to be

cytotoxic to cancerous cells (Nair *et al*, 2007). The glycoside has low toxicity and the corm containing it is also used as food (Drewes *et al,* 1984) and has been well used for traditional and pharmaceutical purposes (Awad and Fink, 2000).

With increasing interest in the use of phytosterols (one of phytochemical components of *H. hemerocallidea*) for the reduction of serum cholesterol and for immune boosting, there has been a resultant increase in scientific investigations (Moreau *et al*, 2002) surrounding these benefits. Interestingly, there has been a surge in commercially available herbal medicines containing sterols with *H. hemerocallidea* extract enrichments claimed to be efficacious against a variety of diseases. However, the scientific validation of these claims remains to be verified despite its anti-inflammatory, antimicrobial, antidiabetic, anticonvulsant and anticancer properties reported by various authors (Ncube *et al*, 2011; Ojewole *et al*, 2006; Steenkamp *et al*, 2006) but none on any antiretroviral based therapy. As a result, there is paucity of literature explaining its attenuating influence on the liver associated with HAART.

Therefore, this work is aimed at investigating the role of crude aqueous extracts of *H. hemerocallidea* in the histoarchitecture of the liver, glycogen distribution, degree of fibrosis and hepatocellular functional indices of animal experimental protocol following highly active antiretroviral therapy.

Materials and methods

Chemicals and drugs

Lamivudine (3TC), Stavudine and Nevirapine (Aspen) and vitamin C (L-ascorbic acid) were procured from Pharmacare Ltd, Port Elizabeth, South Africa and are of analytical grade. Vitamin E solution was obtained from Kyron Prescription CC, Benrose in Johannesburg.

Plant

Fresh corms of *H. hemerocallidea* were purchased from a local „Muthi" shop in Umbilo Road, Durban, KwaZulu-Natal, between June and July, 2014. The corms were authenticated at the Department of Life Science, Westville Campus, University of KwaZulu-Natal, South Africa.

Preparation of corm aqueous extract

H. hemerocallidea fresh corms were extracted according to the procedure of Ojewole *et al*, 2009. They were washed with water, cut into smaller pieces, air dried at room temperature (25-28^{0}C) and ground into powdered form in a commercial blender. The milled corm was soaked in hot distilled water and extracted twice, on each occasion with 2.5 liters of hot distilled water (at 90–100^{0}C) for 12 hours. The combined extract soluble were concentrated to dryness under reduced pressure in a rotary evaporator at 70 ± 1^{0}C. The resulting crude aqueous extract was freeze dried, finally giving of a dark brown, and powdery aqueous extract residue. Without any further purification, aliquot portions of the aqueous extract residue were weighed and dissolved in distilled water (at room temperature) for use on each day of our experiments.

Ethical approval

The study protocol was approved by the University of KwaZulu Natal Animal Ethics Committee (Ethical clearance number: 100/14/Animals). The animals received humane care in accordance with the Principle of Laboratory Animal Care of the National Medical Research Council and the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institute of Health Guide, 1985).

Animal treatment and experimental design

Sixty three adult male Sprague-Dawley rats aged 9-10 weeks old and weighing between 256-312g were used for this study. The animals were bred and maintained at the Animal House of the Biomedical Resources Unit, University of KwaZulu-Natal. All the rats were housed in well ventilated plastic cages (7 animals per cage) having dimensions of (52cm long × 36cm wide and 24cm high). They were maintained under standardized animal house conditions (temperature: 21–23°C; light: approximately 12 h light per day) and were fed with standard rat pellets (from Meadow feeds a Division of Astral Operations Limited, Durban, South Africa) and given tap water *ad libitum*. The animals were randomly distributed to nine treatment groups: A, B, C, D, E, F, G, H and I with seven animals per group and treated as follows:

Group A: HAART (a cocktail of Lamivudine, Stavudine and Nevirapine) using recommended human therapeutic doses and accordingly adjusted to the equivalent animal dose was administered as a daily dose.

Group B: received HAART and *H. hemerocallidea* (100 mg/kg body weight)

Group C received HAART and *H. hemerocallidea* (200 mg/kg body weight)

Group D received HAART and Vitamin C (250 mg/kg body weight)

Group E received HAART and Vitamin E 40 mg/kg body weight (Bansal *et al*., 2005)

Group F: Combination of HAART, Vitamins C and E.

Groups G and H received *H. hemerocallidea* extract alone at doses of 100 and 200 mg/kg respectively.

Groups I served as the control administered 0.9% normal saline.

All administration was done daily by oro-gastric gavage except for vitamin E which was administered subcutaneously. At the end of the treatment period (56 days), the animals were killed 24 hours after the last treatment under excess Halothane ® anesthesia.

Body and Liver weight

Body weights of animals were recorded on the first day before treatment (initial), thereafter weekly and on the day of sacrifice (final). Liver weight (LW) was measured by an electronic balance (Mettler Toledo; Microsep (Pty) Ltd, Greifensee, Switzerland).

Assessment of Liver function and lipid profile

Blood samples were collected through cardiac puncture and allowed to cloth for 30 minutes and centrifuged for 15 minutes at 3000 revolutions per minute. The serum was decanted into Eppendorf tubes and prepared for biochemical analyses.

Biochemical analyses of the serum enzymes for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) were spectrophotometrically determined by the method of Reitman and Frankel (1957).

Determination of Serum Lipids

Low density lipoprotein (LDL), high density lipoprotein (HDL), Triglycerides (TG) and total cholesterol (CHOL) were determined by enzymatic methods according to Diniz *et al*., (2006) using commercial diagnostic kits (Randox, UK).

Histopathological examination of liver tissues

Twenty four hour after the last treatment the animals were given humane sacrifice under excess Halothane® anesthesia and the liver were removed and weighed. They were examined for gross

pathology and immediately fixed in 10% neutral buffered formalin. After proper fixation, the tissues were dehydrated in graded series of alcohol, cleared in Xylene and embedded in paraffin wax using a cassette.

For routine histological study, the liver tissues were sectioned at 5µm thickness using Leica RM 2255 microtome and stained with hematoxylin and eosin (H&E) for general assessment of liver structure. For histochemical studies, the tissues were stained with Periodic acid Schiff (PAS) for glycogen, neutral polysaccharides and basement membrane, and Masson trichrome (MT) for the assessment of possible fibrosis in the liver (John and Marilyn, 2007). The stained slides were then cover slipped using DPX mounting glue directly over the tissue section ensuring no air bubbles were trapped. Thereafter, the slides were left overnight to dry for examination under light microscope.

The sections were examined using a binocular microscope, Nikon Eclipse 80i, Tokyo, Japan (used to acquire the images). An independent histopathologist blinded to the treatment groups reported on the qualitative assessments of the slides.

Statistical analysis

Continuous variables (Liver and body weights, Liver function test and Lipid profile level), were analyzed by one–way analysis of variance (ANOVA) followed by Dunnett‟s multiple comparison post-test using Graph pad prism ® statistical software 6.02. The results are expressed as mean ± SD (standard deviation). Values were considered significant at $p < 0.05$.

Results

Mortality:

No animal died during the experimental period.

Body weight and organ (Liver) weight changes

While the final body weights of rats were in all groups higher than their corresponding initial body weight, the percentage weight gain was maximal in group F (HAART with vitamins C and E), then groups A and I and B all recording 48.66 %, 46.38 % and 44.08 %, 42.48% respectively. Least weight gain was observed in group D animals treated with HAART and vitamin C (29.89%) (Table 3.1). Similarly, liver weight changes were not statistically significant except for group B that recorded a slight increase ($p<0.05$) and group F that recorded lowest weight when compared with

the control. Organ-body weight ratios were significantly decreased in groups D, E, F, G and H ($p<0.05$) (Table 1).

Table 1: Body and liver weight changes in experimental and control groups of animals

GROUP	Initial (g)	Final (g)	BW diff (g)	% BW diff	LW (g)	LBWR
A	260.00±6.06	381.00±11.21	121·00	46·38	12.83±1.31	0.035
B	285.86±3.80	407.29±13.69	121·43	42·48	13.78±1.06	0.036
C	311.43±3.56	427.00±09.94	115·57	37·11	14.18±3.41	0.035
D	279.57± .16	363.14±11.50**	83·57	29·89	12.05±1.49	0.028*
E	281.57±2.88	382.71±05.51	101·14	35·92	10.05±0.98*	0.028*
F	261.00±7.49	388.00±08.28	127·00	48·66	10.08±0.76*	0.026**
G	271.57±7.14	359.71±12.31**	88·14	32·46	10.66±0.72*	0.027*
H	273.00±6.29	371.86±18.43*	98·86	36·21	09.41±1.27**	0.026**
I	256.71±8.65	369.86±12.66*	113·15	44·08	13.16±2.38	0.035

*$p<0.05$, **$p<0.03$, BW= body weight of rats, LW= liver weight of rats, LBWR= liver- body weight ratio.

Serum AST, ALT, ALP and GGT in experimental groups

There were changes in the functional hepatotoxicity indices recorded via AST, ALT ALP and GGT. Though AST levels were higher in all groups except A and E, it was significantly elevated in groups F and H (HAART with vitamin C&E and *H. hemerocallidea* 200mg/kg) respectively ($p<0.001$, 0.03) compared with the control. There was statistically significant decrease in ALT levels in groups A, B, C, D and E ($p<0.03$) whereas the levels in groups F, G and H were lower than that of control ($p>0.05$). Similarly, ALP levels of animals treated with HAART, HAART + *H. hemerocallidea* (both doses), and HAART + vitamin E ($p<0.001$), HAART + vitamin C, *H. hemerocallidea* (both doses) ($p<0.03$) were significantly lower compared with the control. The results of the GGT levels were not statistically significant in all groups when compared with the control group ($p>0.05$) (Table 2).

Table 2: Effect on Serum AST, ALT ALP and GGT in the experimental and control group of animals

GROUP	AST (IU/L)	ALT (IU/L)	AST/ALT Ratio	ALP (IU/L)	GGT (IU/L)
A	92.00±3.23	48.67±11.91***	1.89	116.67±41.55***	2.00±0.89
B	106.33±7.45	52.00±2.37***	2.04	95.00 ± 5.59***	3.00±0.00
C	108.00±9.30	51.33±8.96***	2.10	88.00 ± 4.98***	3.00±0.89
D	104.67±4.03	55.33±7.61***	1.89	122.67 ±16.79**	3.33±0.52
E	94.33±13.55	44.00±3.10***	2.14	99.67 ± 4.03***	3.00±0.89
F	128.33±6.83***	75.67±17.6	1.70	167.00 ± 5.87	2.67±0.52
G	105.33±11.67	75.00±8.53	1.40	160.67 ± 36.88*	3.00±0.89
H	116.00±13.00**	75.67±8.31	1.53	145.33 ± 23.62**	2.67±1.37
I	96.33±9.48	79.67±9.97	1.21	218.00 ± 71.52	2.33±0.52

*p<0.05, **p<0.03, ***p<0.001, AST=alanine amino aspartate, ALT= alanine aminotransferase, ALP=alkaline phosphatase, GGT=gamma-glutamyl transferase.

Effect on lipid profile

Although there was marginal upward difference in total cholesterol levels in all groups, these were not significantly different (p>0.05). There were statistically significant increase in the serum levels of LDL in groups F and H (p<0.03), whereas groups treated with HAART, HAART + vitamin C (p<0.001), HAART + *H. hemerocallidea* 100mg/kg, HAART + vitamin E (p<0.05) as well as HAART + *H. hemerocallidea* 200mg/kg (p<0.03) all recorded significant decline in the parameter compared with the controls. HDL levels in groups A, C, E (p<0.05) as well as in groups D, F and H (p<0.03) were all significantly lower than the control group. While triglyceride (TG) levels in groups A, C, D, E (p<0.001), B (p<0.05) statistically declined, levels were however increased in the groups administered HAART + vitamins C and E (p<0.001) as well as *H. hemerocallidea* 200mg/kg (p<0.03) (Table 3.3).

Table 3: Lipid profile of the experimental and control groups of animals

GROUP	LDL (mmol/L)	HDL (mmol/L)	CHOL (mmol/L)	TG (mmol/L)
A	-0.10 ± 0.02***	1.00 ± 0.05*	1.10 ± 0.09	0.45 ± 0.09***
B	-0.23 ± 0.13*	0.94 ± 0.06	1.07 ± 0.14	0.68 ± 0.16*
C	-0.12 ± 0.09**	0.98 ± 0.03*	1.10 ± 0.09	0.52 ± 0.13***
D	-0.09 ± 0.06***	1.04 ± 0.11**	1.17 ± 0.14	0.45 ± 0.14***
E	-0.17 ± 0.03*	0.97 ± 0.05*	1.03 ± 0.05	0.51 ± 0.04***
F	-0.62 ± 0.01**	1.06 ± 0.05**	1.13 ± 0.05	1.50 ± 0.04***
G	-0.27 ± 0.03	0.92 ± 0.05	1.07 ± 0.05	0.90 ± 0.01
H	-0.63 ± 0.13**	1.06 ± 0.06**	1.07 ± 0.05	1.39 ± 0.30**
I	-0.38 ± 0.17	0.67 ± 0.47	1.03 ± 0.14	1.03 ± 0.23

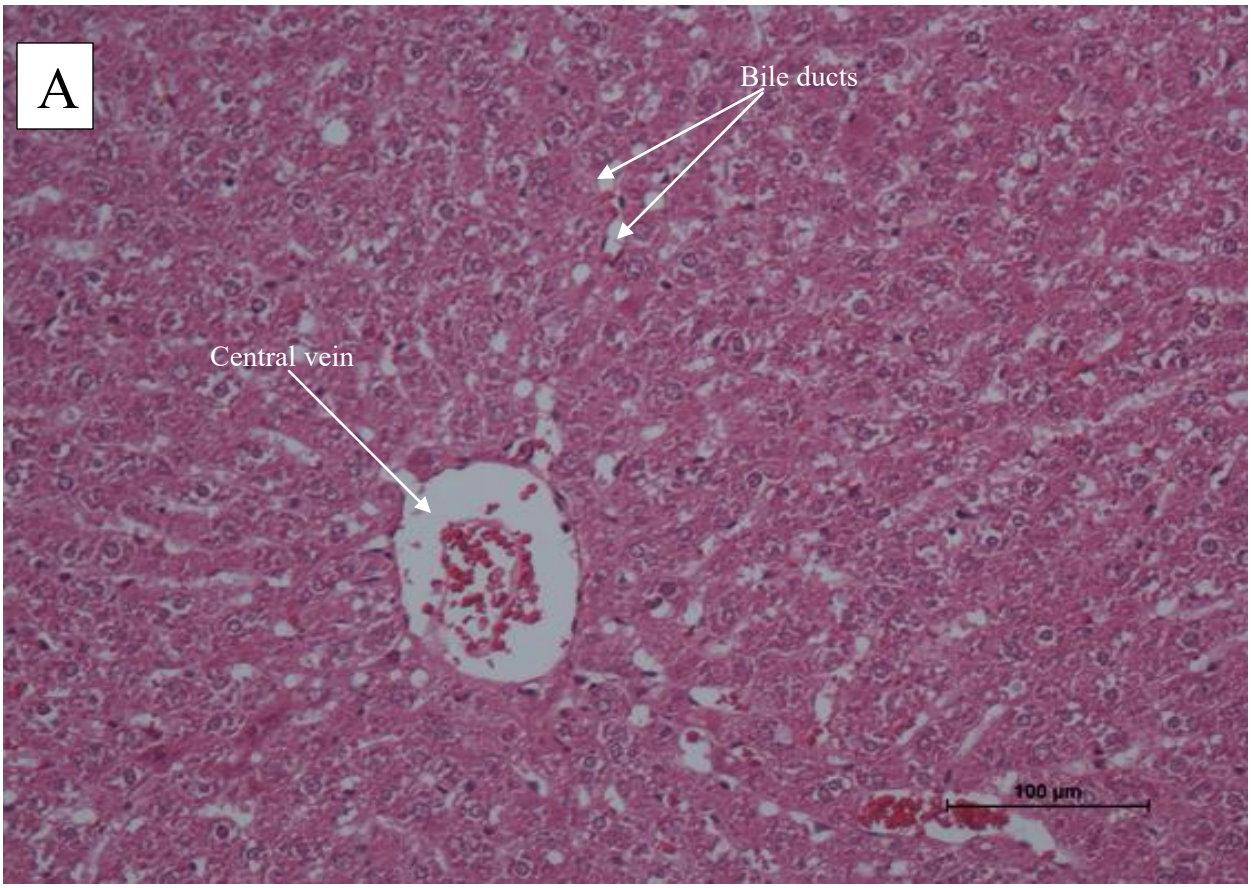
*p<0.05, **p<0.03, ***p<0.001. LDL=low density lipoprotein, HDL=high density lipoprotein, CHOL=total cholesterol, TG=triglycerides.

Histopathology of the liver

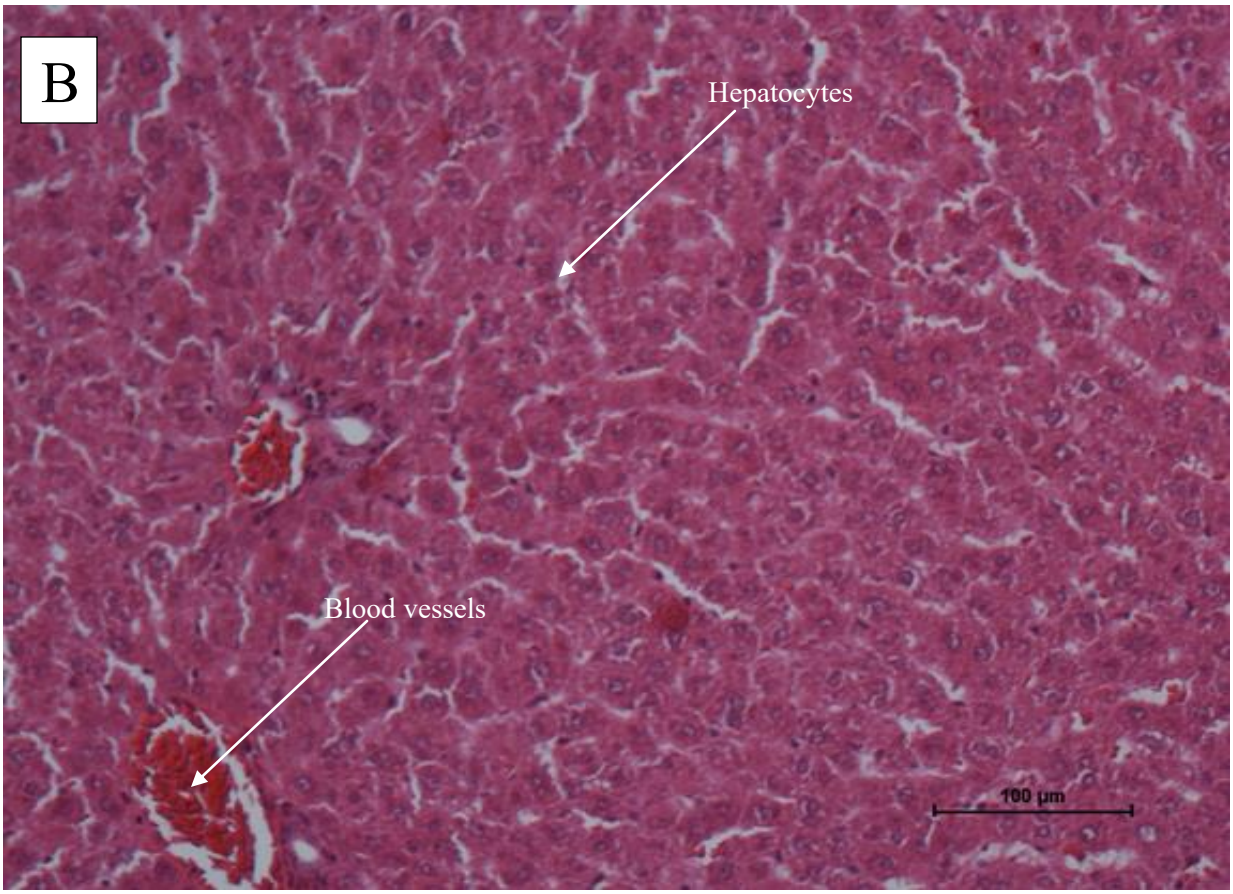
Whilst various stains were deployed to investigate qualitative changes in the liver, our report will be described according to the staining technique. In H&E stained slides, control sections of liver showed typical hepatocellular architecture with central vein (containing blood) and cords of hepatocytes arranged peripherally in a radiating fashion. The outlines of hepatocytes and sinusoidal spaces are clearly seen with no obvious pathologies. This trend was observed in liver sections from groups E (HAART + vitamin E). Histopathological assessment of other groups revealed various degrees of distortions. Liver tissues of group B animals showed mild distortion in the radial arrangements of hepatocytes and sinusoids with some form of ballooning of hepatocytes. Whereas in group C rats, some of the central veins appeared eroded with gradual loss of polyhedral arrangement of cords of cells. There was extensive necrosis of hepatocytes and sinusoidal spaces with marked loss of architecture in group D sections of liver. Some of the hepatocyte nuclei appeared enlarged. In sections of liver of group F animals, there was complete loss of architecture with hepatocytes appearing as isolated cell with (or without) prominent nucleoli. Groups treated with *H. hemerocallidea* alone (both doses) showed liver sections that generally appeared enlarged in sinusoidal appearance with necrotic distortions (Plates 1).

Photomicrograph of the liver sections stained with PAS reveal normal liver architecture, containing a large amount of glycogen with bright pink hepatocytes, neutral polysaccharides and basement membranes in the sections of animals treated with HAART + *H. hemerocallidea* (both doses), with vitamin C and E (Plates 2 A, D, E, F, G, H & I). The PAS stain demonstrates glycogen. However, there was distortion of hepatocellular cord in the animals administered with HAART and concomitantly with *H. hemerocallidea* (both doses) (Plates 2 B &C).

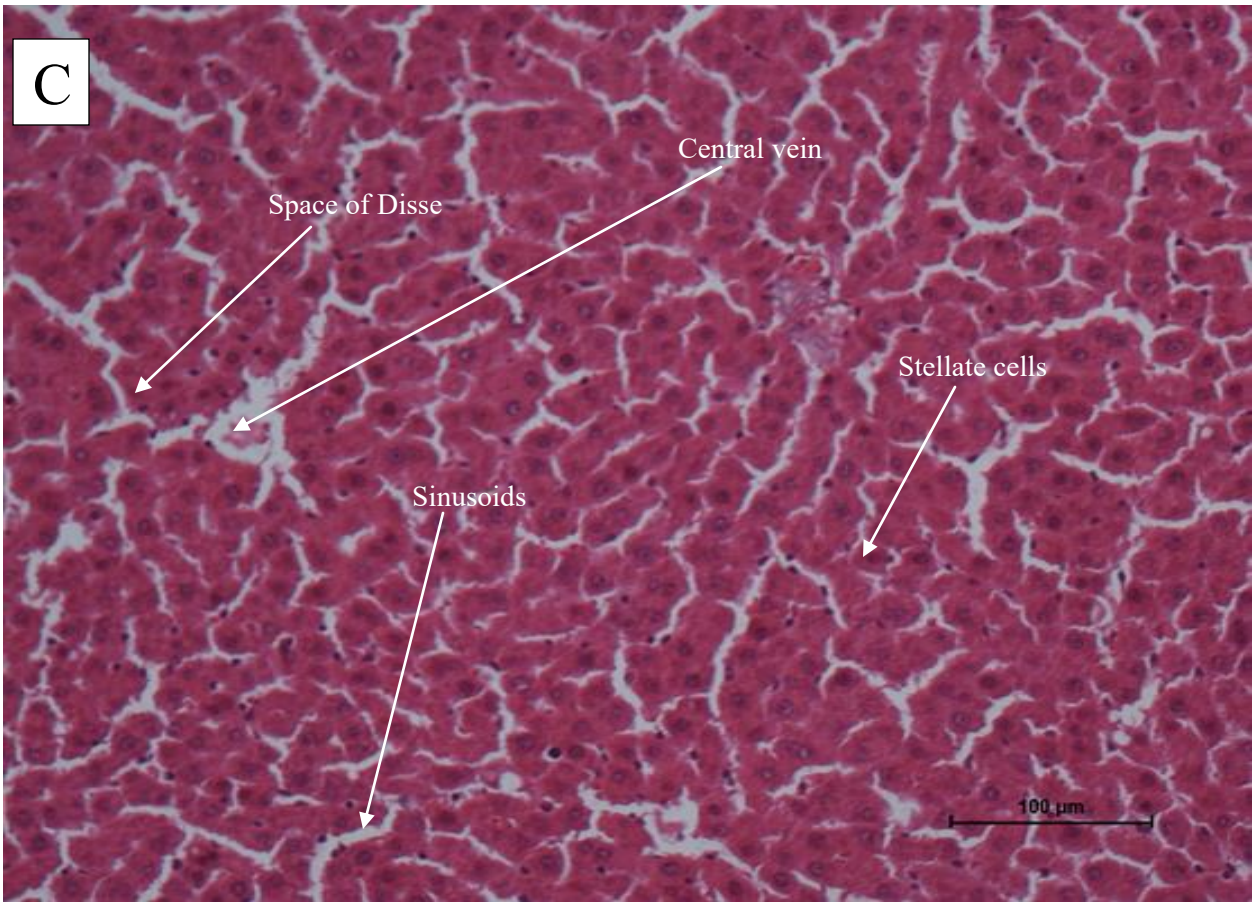
Photomicrograph of the liver sections stained with Masson Trichrome (MT), show normal architecture of the liver stained blue while the cytoplasm of hepatocytes are stained red, nuclei can be seen as dark red to black structures within cells (Plates 3 A, E, F, G, H & I). Also, the liver sections showing fibrous tissue, as it is evident, the normal architecture of the liver is destroyed in this animals administered with HAART concomitantly with *H. hemerocallidea* (both doses), nodules surrounded by fibrous bands (Plates 3 B & C).



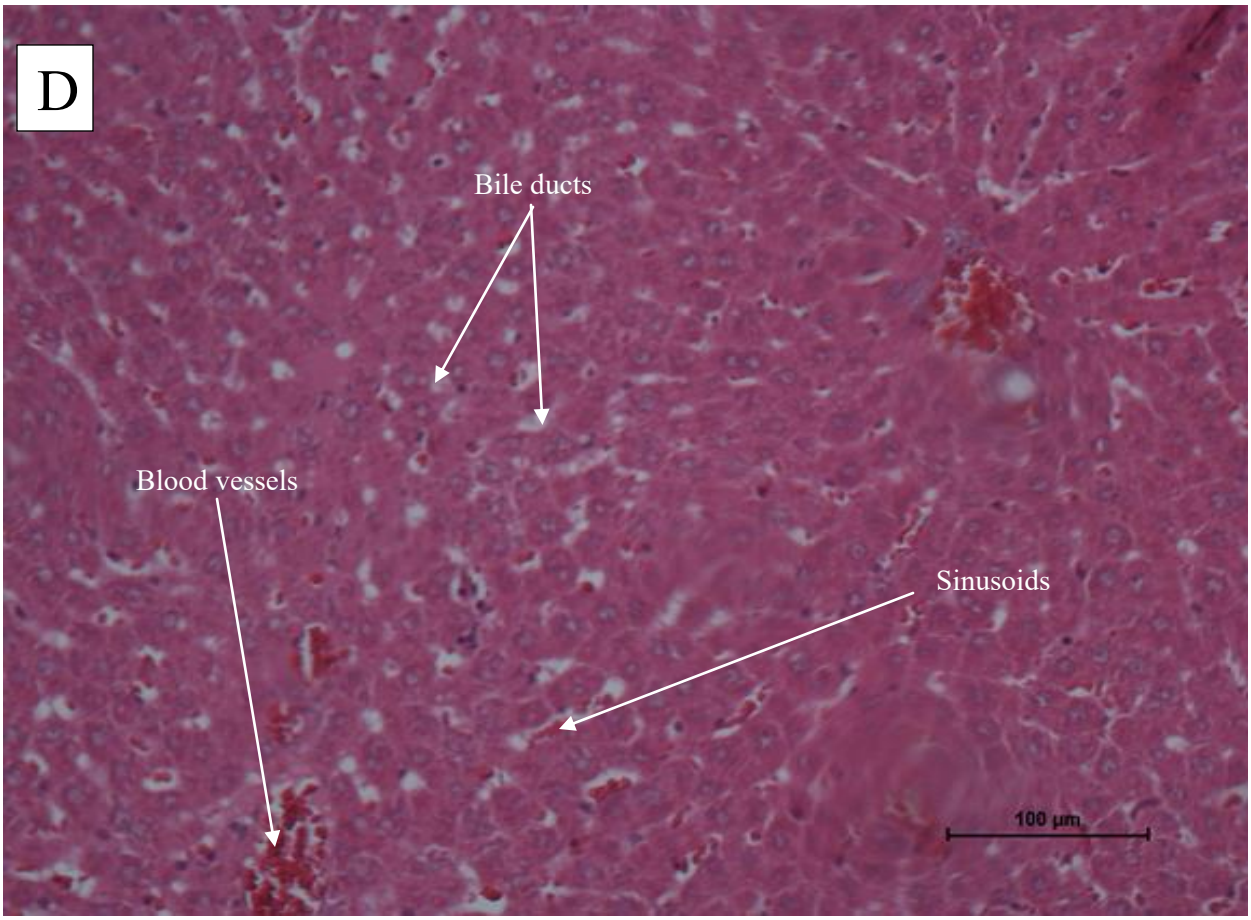
Plates 1 (A): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART cocktail. H & E stains. Note the swollen of the central vein and congested bile ducts.



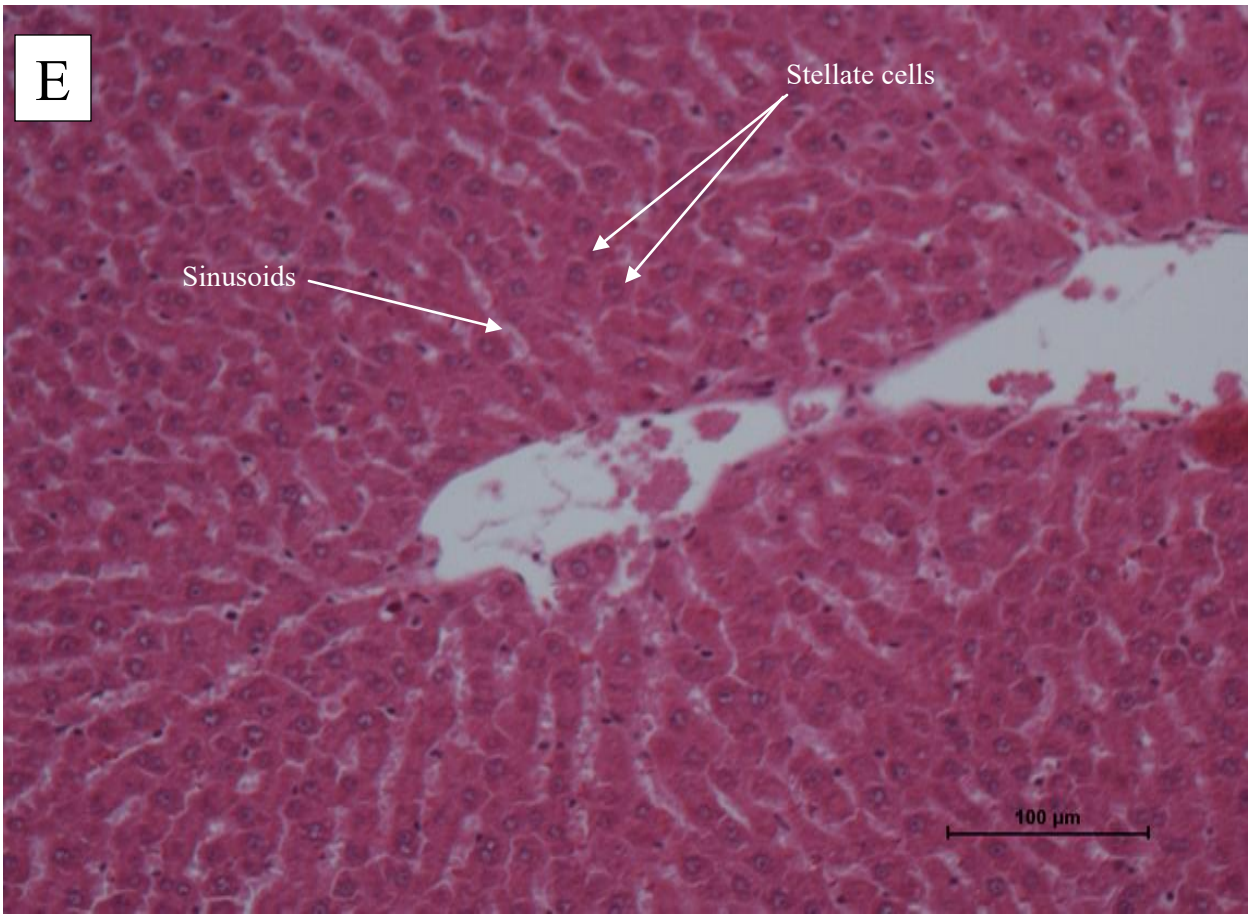
Plates 1 (B): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and low dose of *H. hemerocallidea* extract. H & E stains. Note the cellular distortion of the hepatocytes of and the occlusion of the blood vessels.



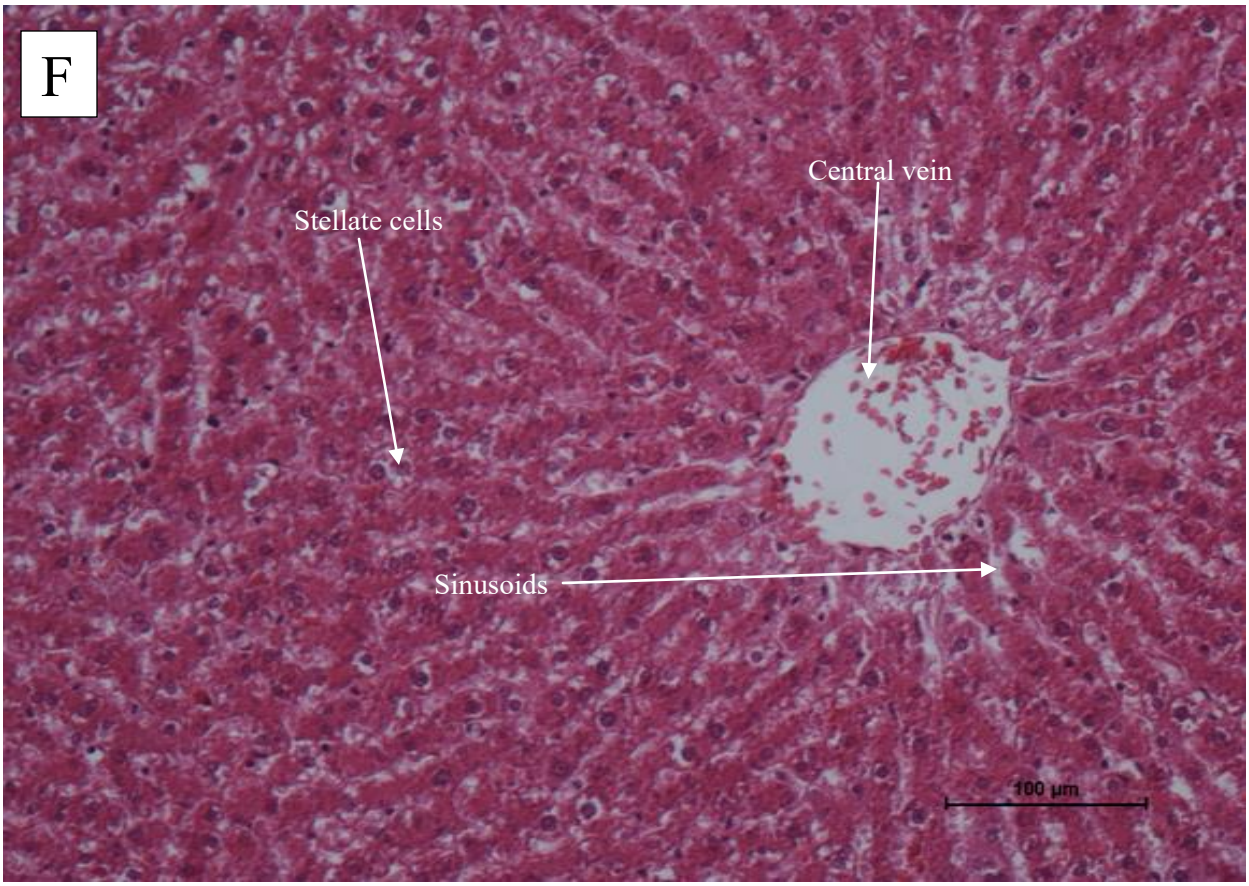
Plates 1 (C): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and high dose of *H. hemerocallidea* extract. H & E stains. Note the disorganisation of the stellate cells and sinusoids and generalized hyperplasia.



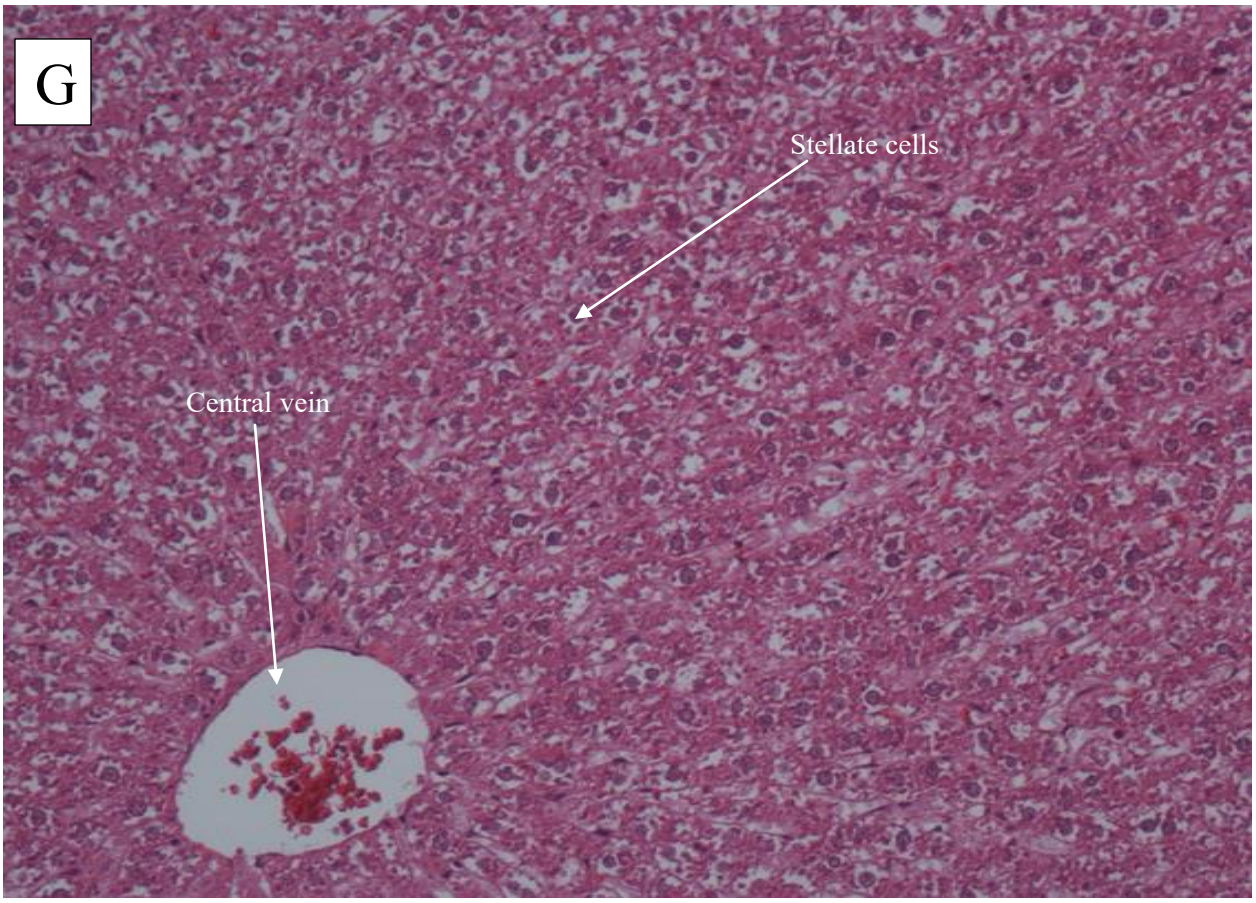
Plates 1 (D): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and vitamin C. H & E stains. Note the displacement of the sinusoids and generalized hypercellularity.



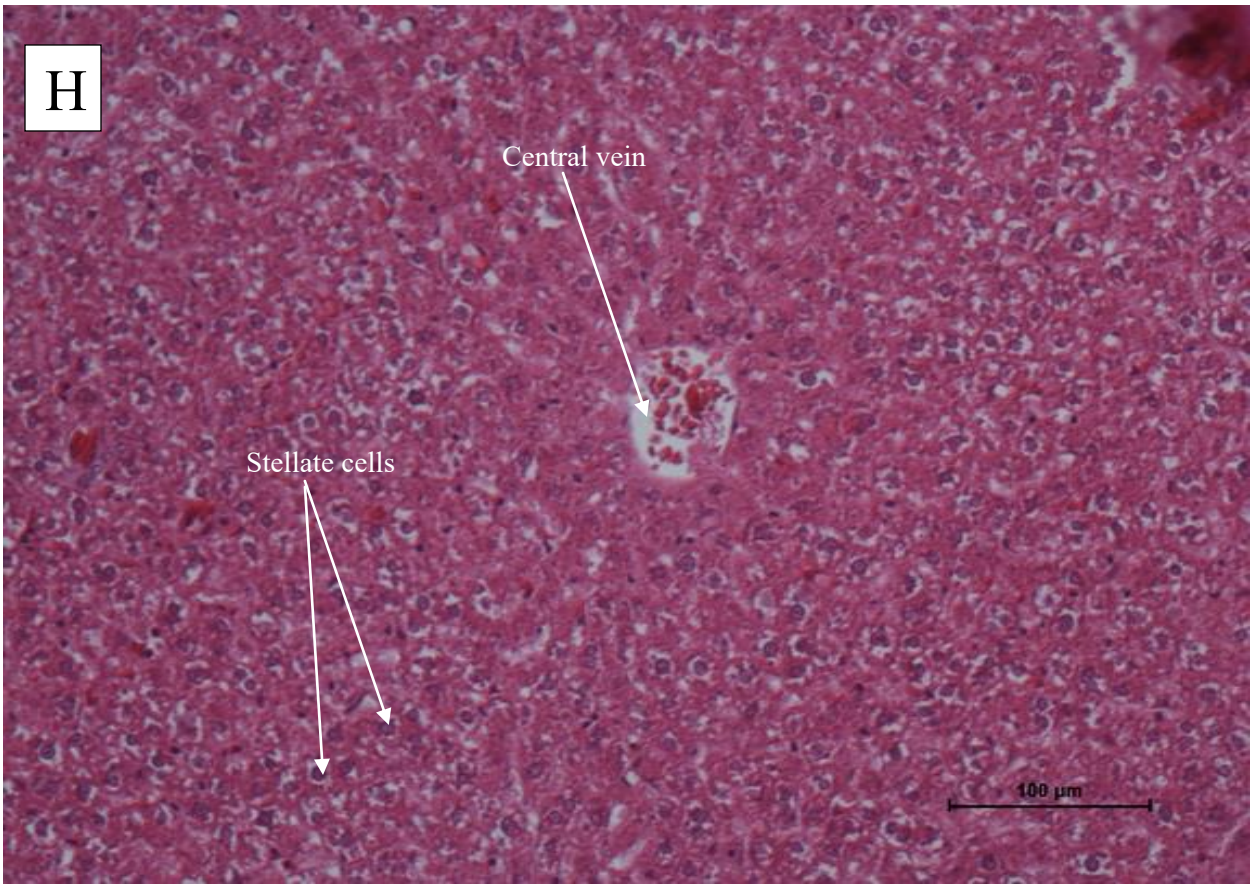
Plates 1 (E): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and vitamin E. H & E stains. Note the disorganisations of the sinusoids and stellate cells as well as atrophy of the central vein.



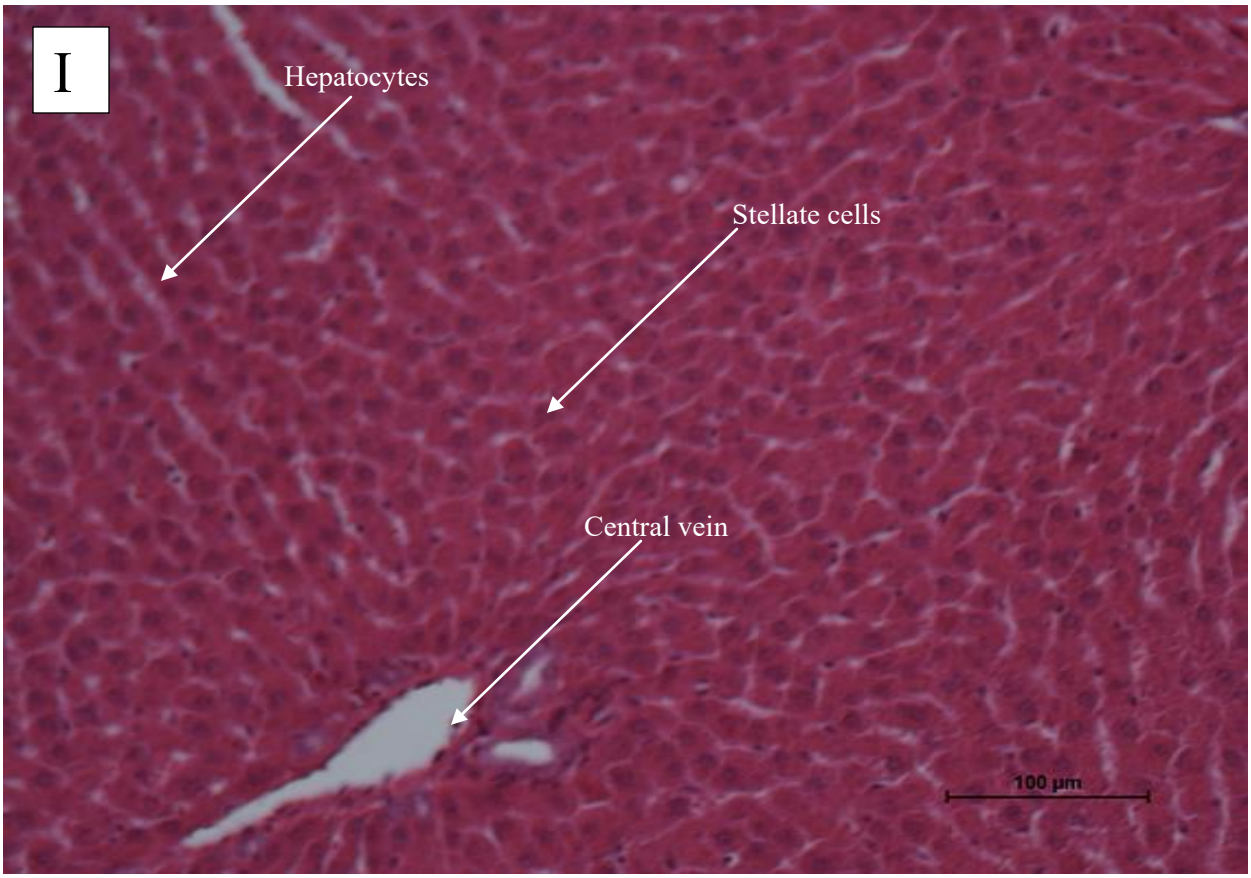
Plates 1 (F): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and vitamin (C and E). H & E stains. Note the cellular distortions of the sinusoidial cells and stellate cells and hyperplasia of the central vein.



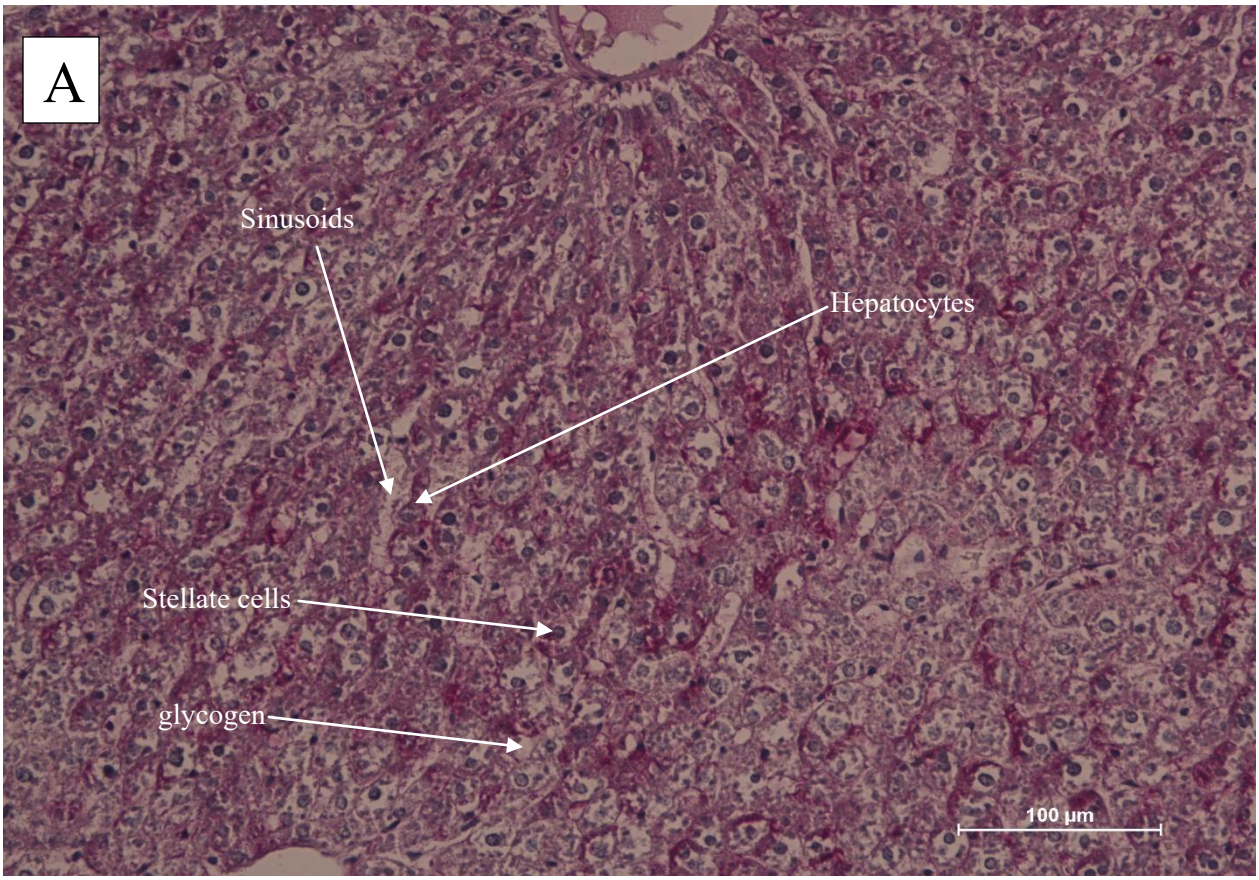
Plates 1 (G): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with low dose of *H. hemerocallidea* extract alone. H & E stains. Note: Histoarchitecture is essential normal.



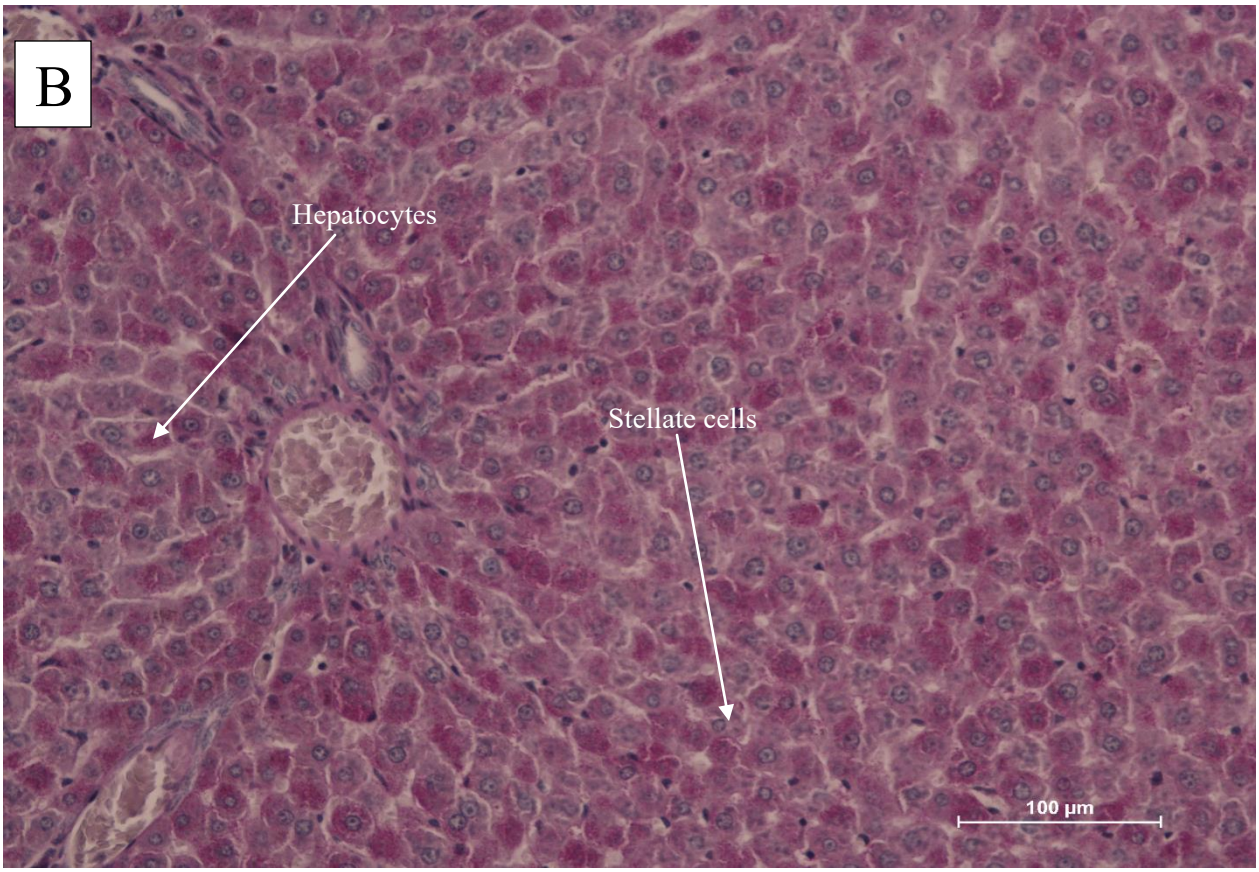
Plates 1 (H): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with high dose of *H. hemerocallidea* extract alone. H & E stains. Note the derangement of the stellate cells and occluded central vein.



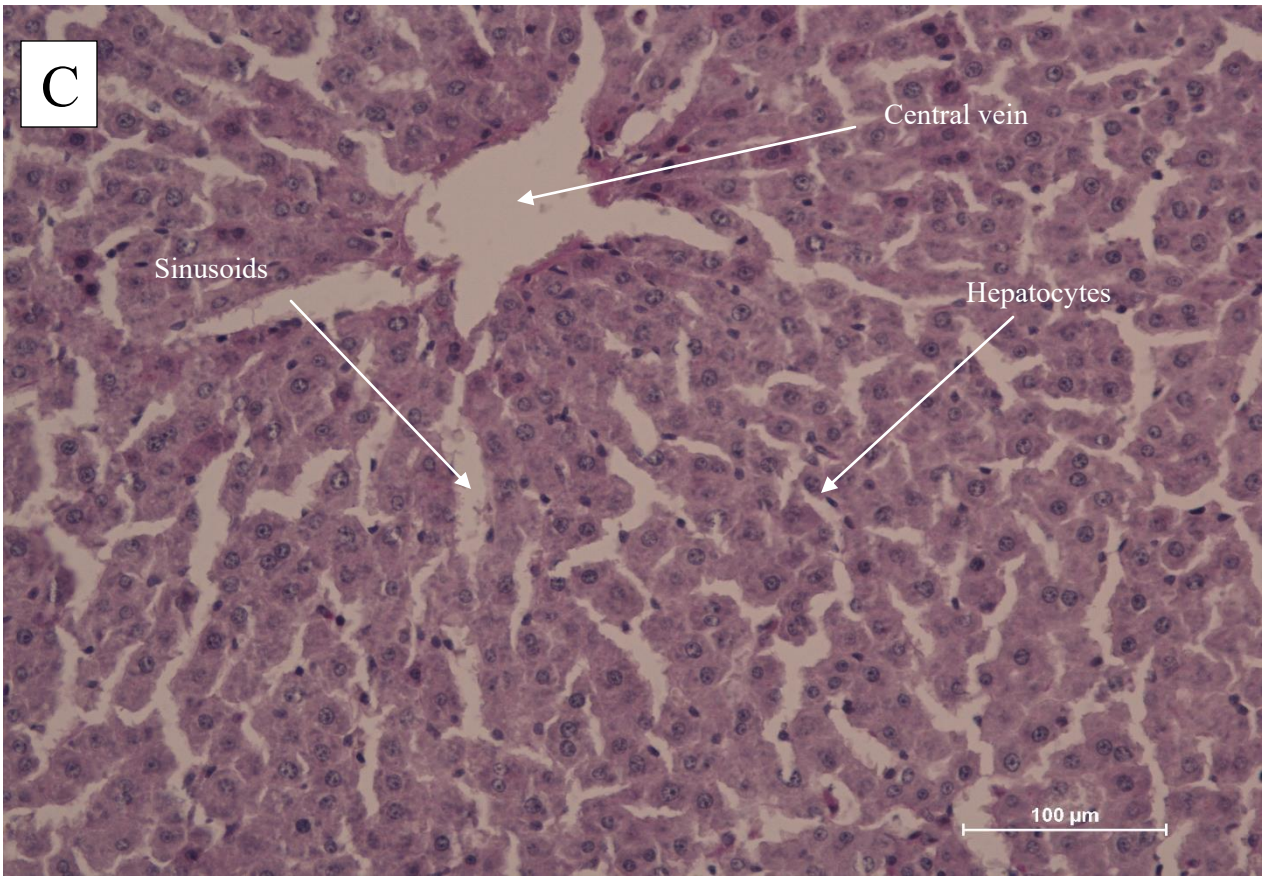
Plates 1 (I): Photomicrograph of cross section of liver of Sprague-Dawley rats in control. H & E stains. Note: Typical hepatocellular architecture with central vein containing blood.



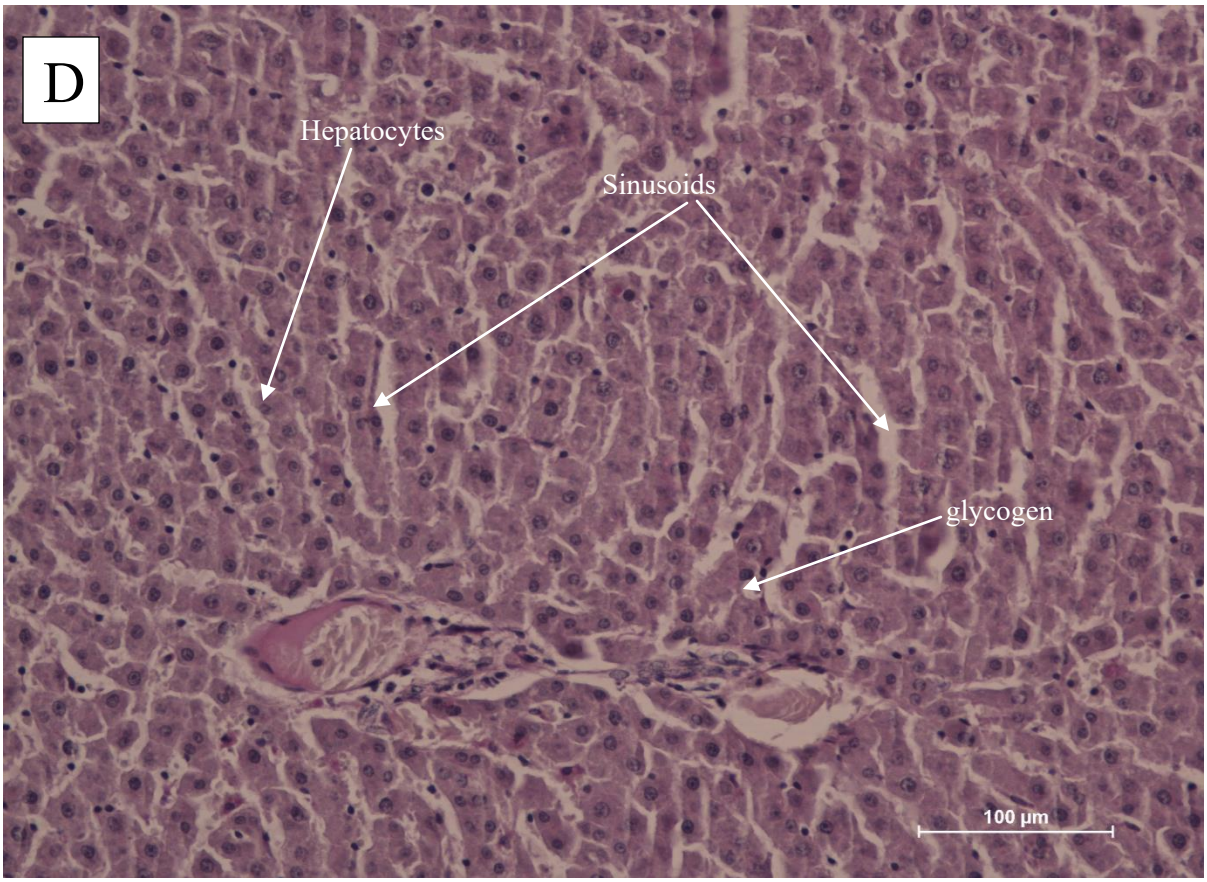
Plates 2 (A): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART cocktail. PAS stains. Note: Liver architecture showing glycogen and bright pink hepatocytes



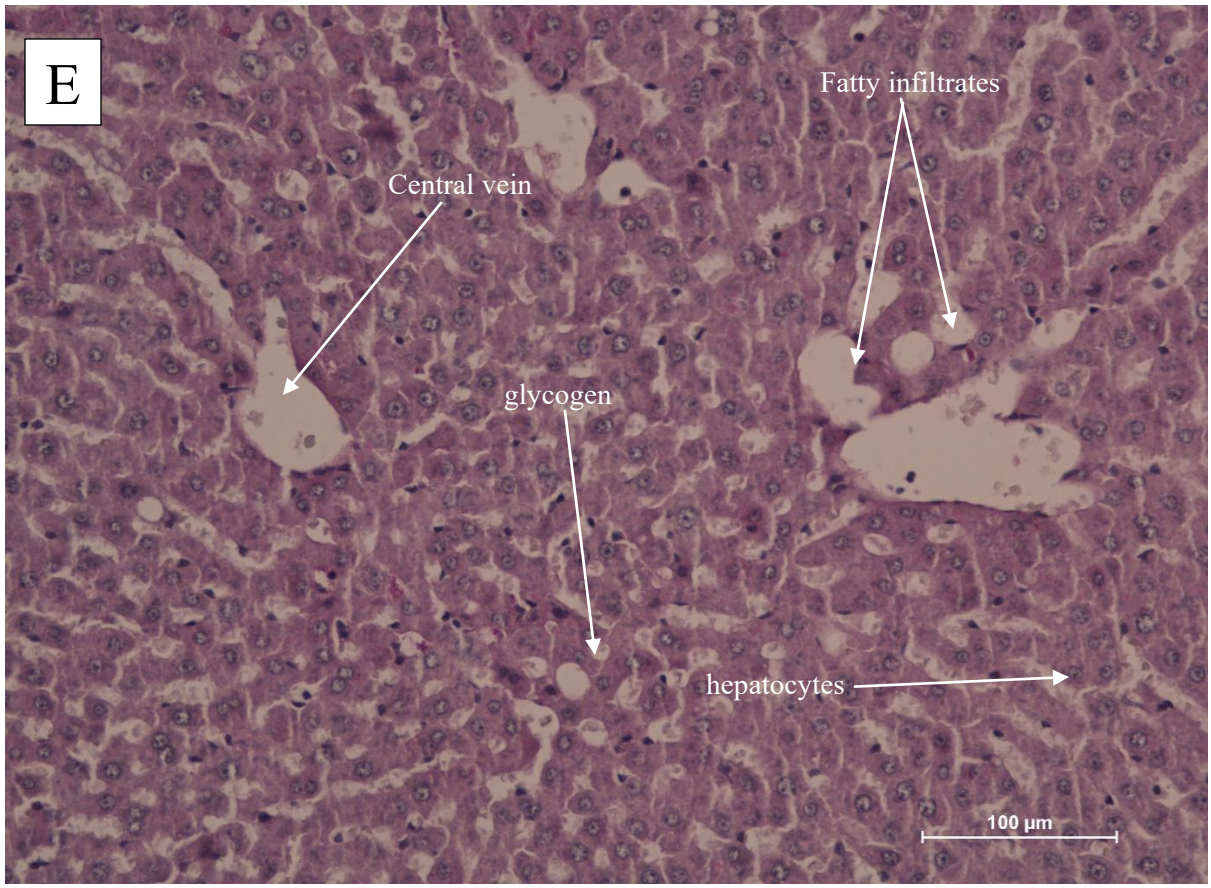
Plates 2 (B): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and low dose of *H. hemerocallidea*. PAS stains. Note: Distorted hepatocytes in the cords.



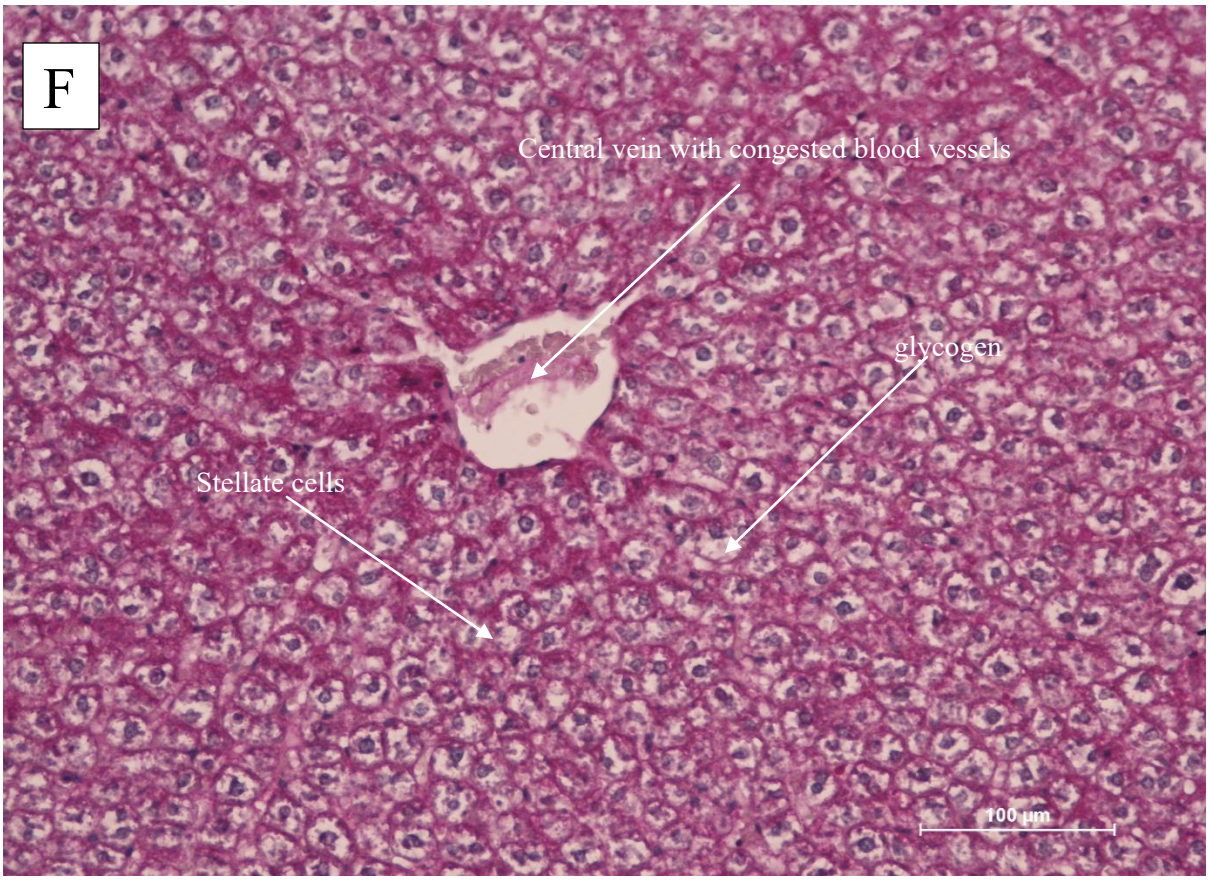
Plates 2 (C): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and high dose of *H. hemerocallidea*. PAS stains. Note: Distorted hepatocytes in the cords.



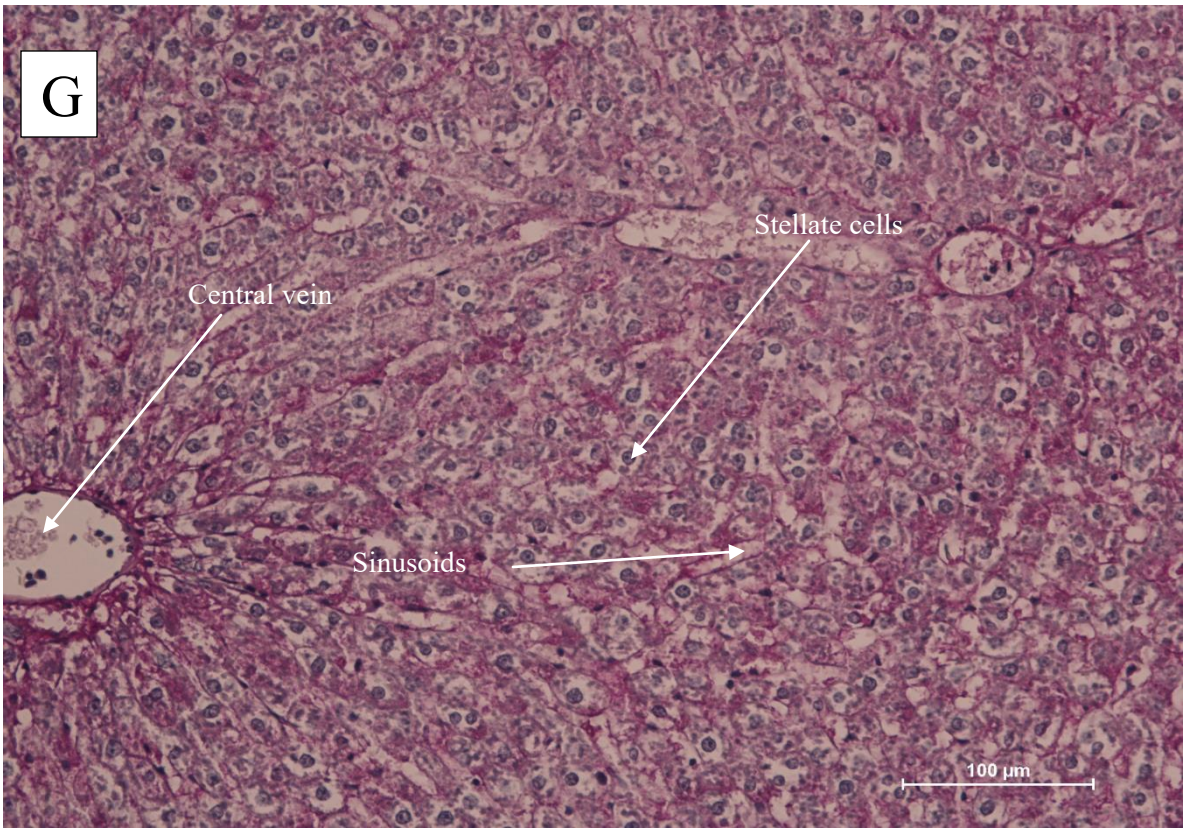
Plates 2 (D): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and vitamin C. PAS stains. Note: Liver architecture with glycogen and bright pink hepatocytes.



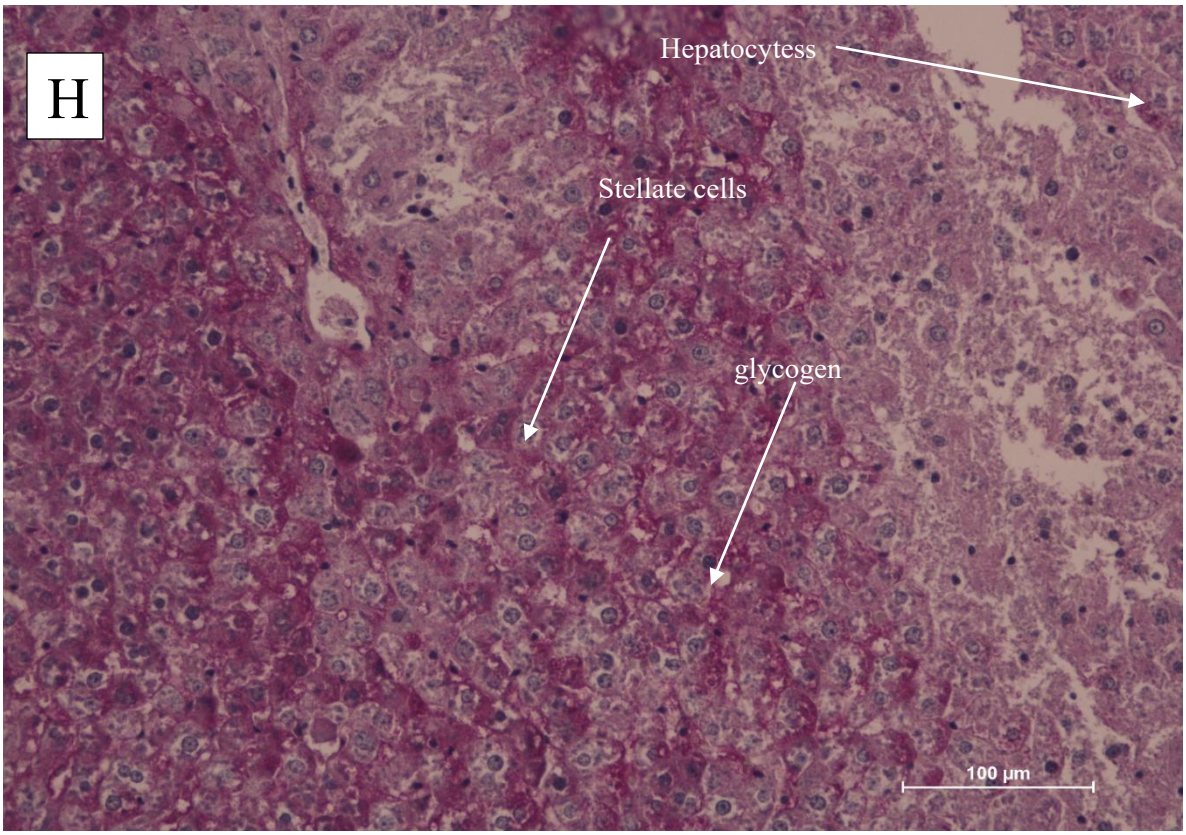
Plates 2 (E): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and vitamin E. PAS stains. Note: Liver architecture with glycogen and bright pink hepatocytes.



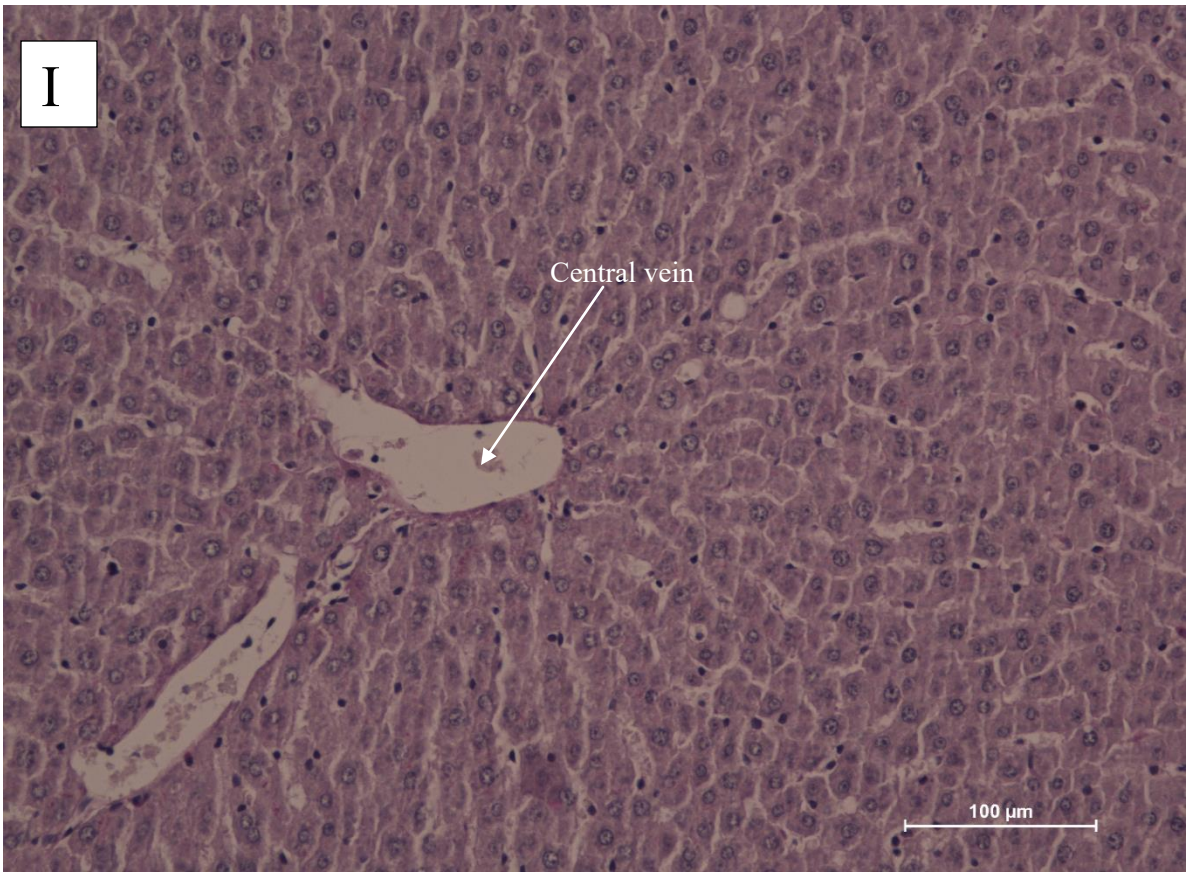
Plates 2 (F): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and vitamin (C and E). PAS stains. Note: Liver architecture with glycogen, stellate cells and swollen central vein with congested blood vessels.



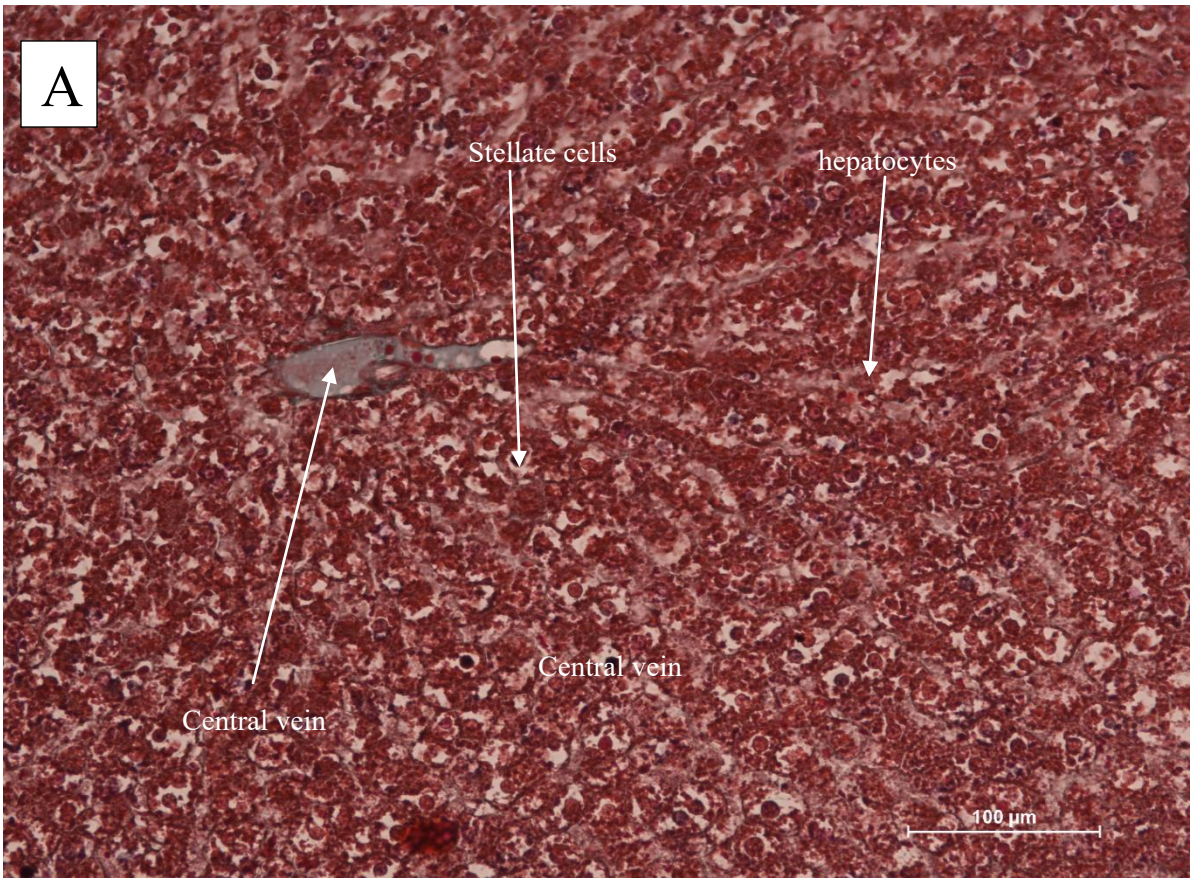
Plates 2 (G): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and low dose of *H. hemerocallidea* extract alone. PAS stains. Note: Liver showing normal histoarchitecture.



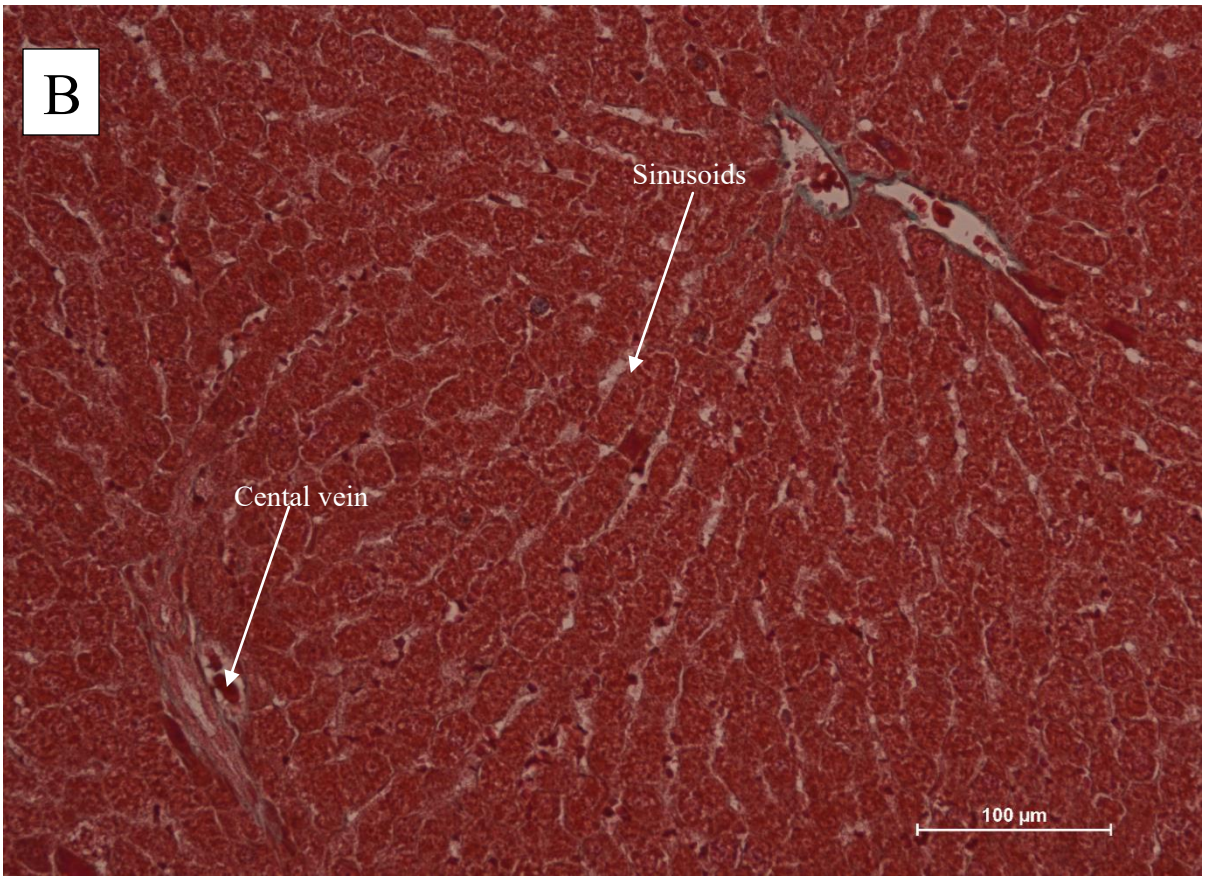
Plates 2 (H): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and high dose of *H. hemerocallidea* extract alone. PAS stains. Note:Liver architecture with glycogen and bright pink hepatocytes.



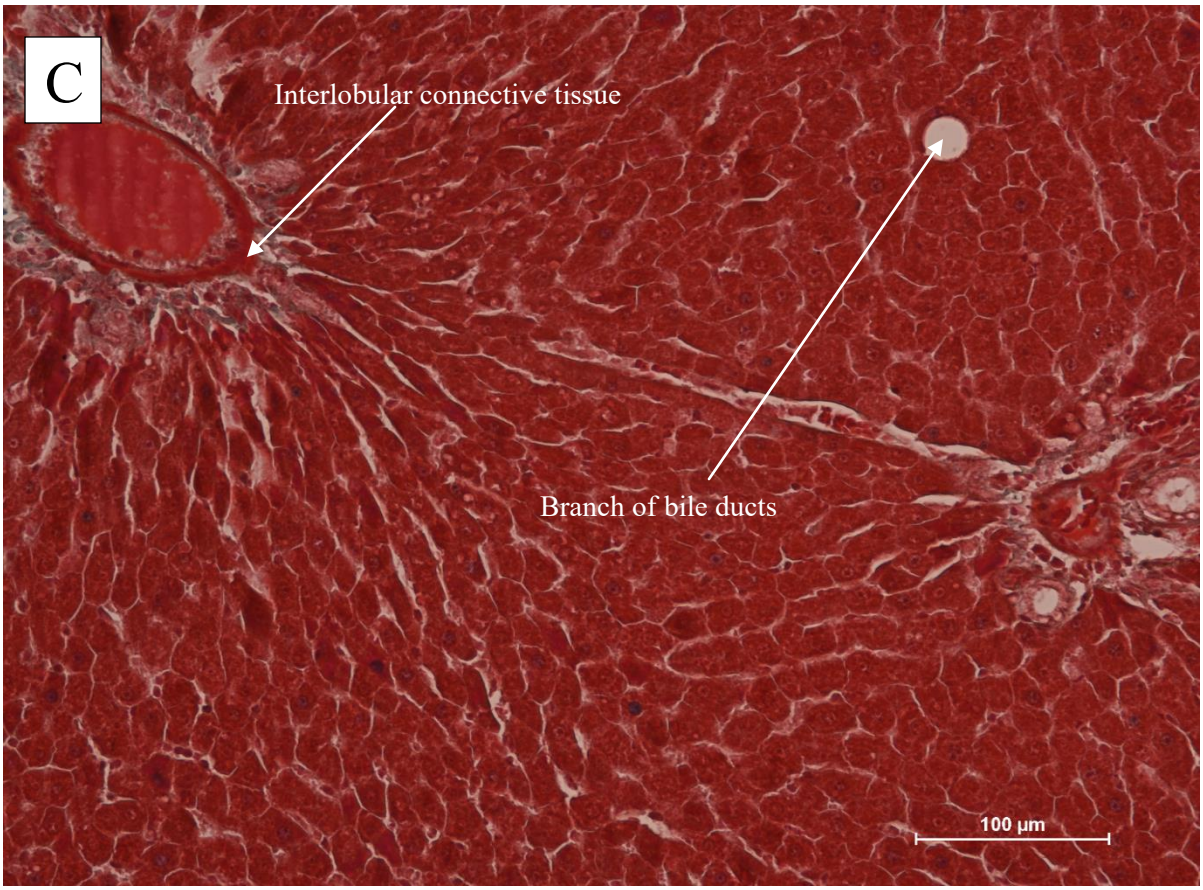
Plates 2 (I): Photomicrograph of cross section of liver of Sprague-Dawley rats. Control group. PAS stains. Note: Normal liver histoarchitecture.



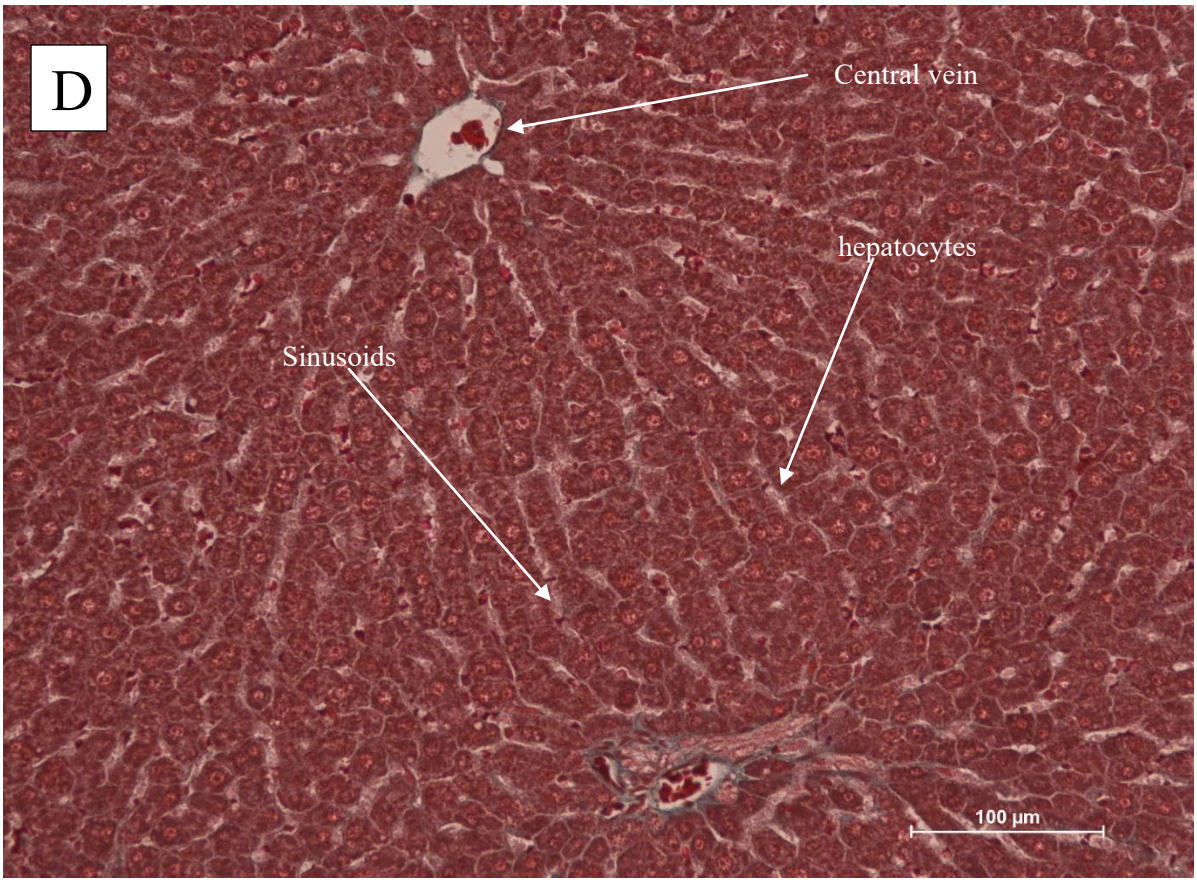
Plates 3 (A): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART cocktail. Massons Trichrome. Note: liver architecture with hepatocytes stained red with dark red nuclei.



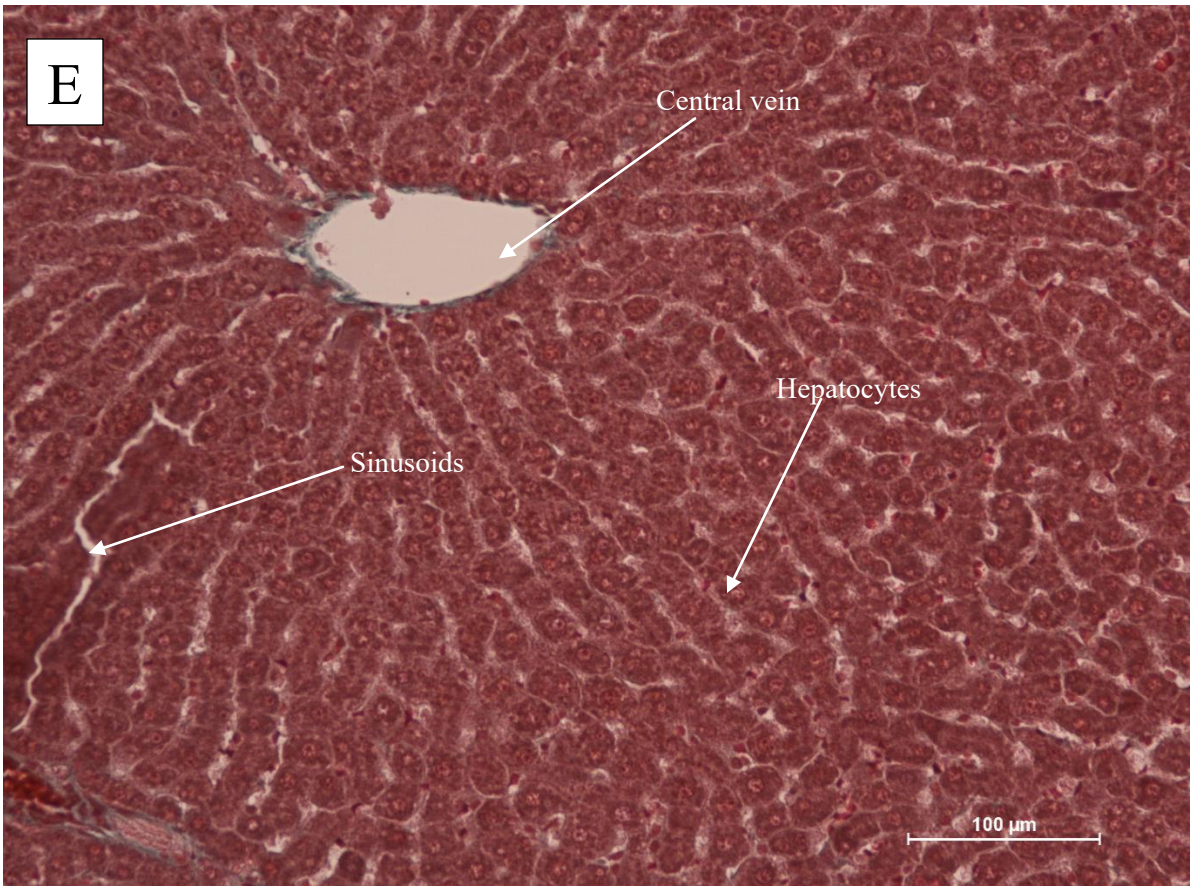
Plates 3 (B): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and low dose of *H. hemerocalliea.* Massons Trichrome stains. Note: Abnormal and distorted liver architetcture is seen.



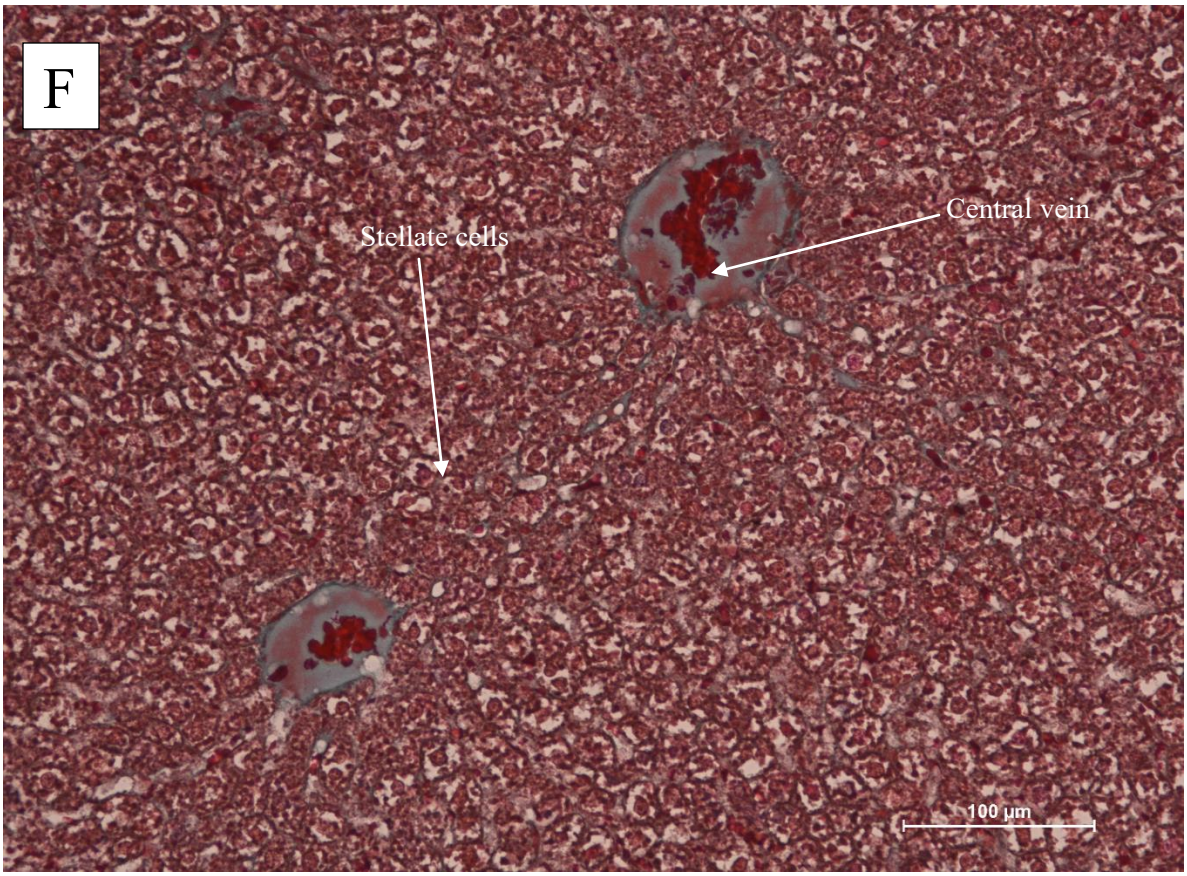
Plates 3 (C): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and high dose of *H. hemerocalliea.* Massons Trichrome stains. Note: Abnormal and distorted liver architetcture is seen.



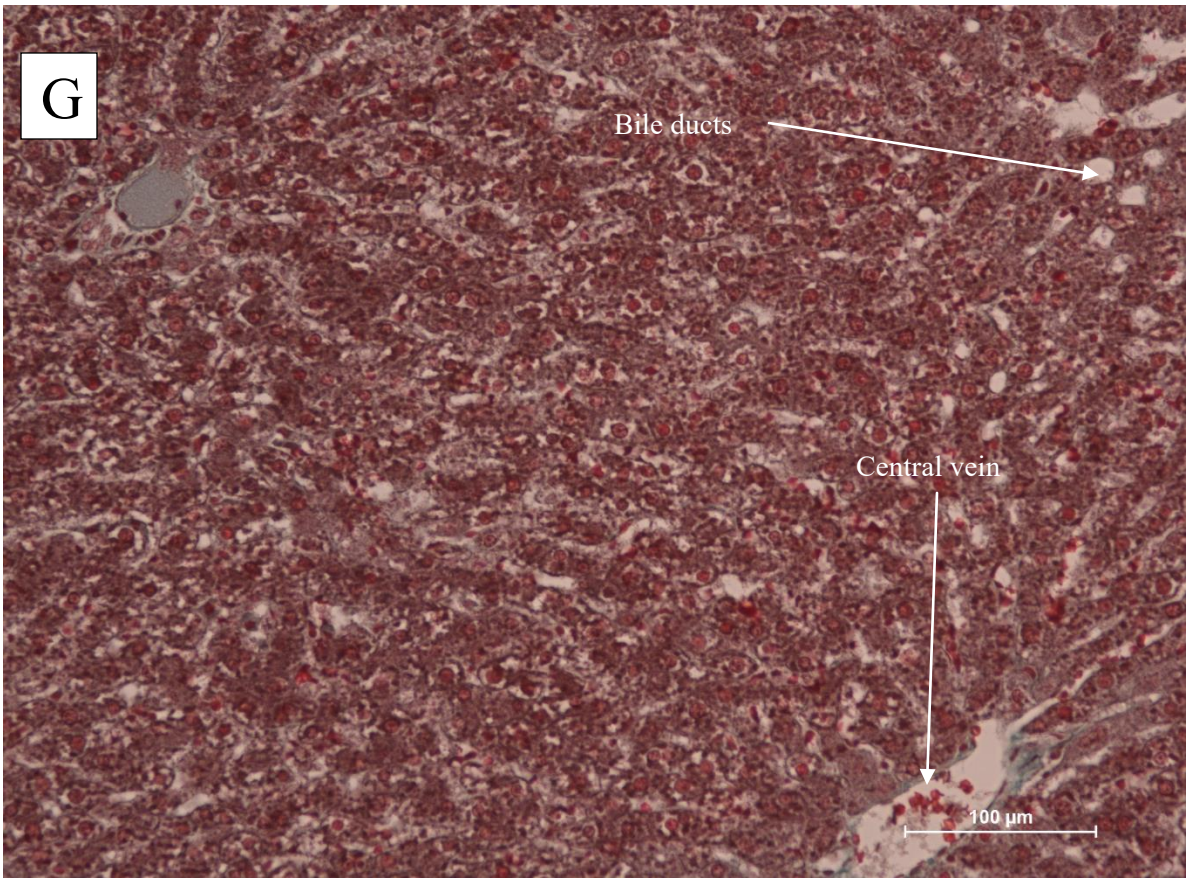
Plates 3 (D): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and vitamin C. Massons Trichrome stains. Note: liver architecture with radially arranged hepatocytes stained red with dark red nuclei.



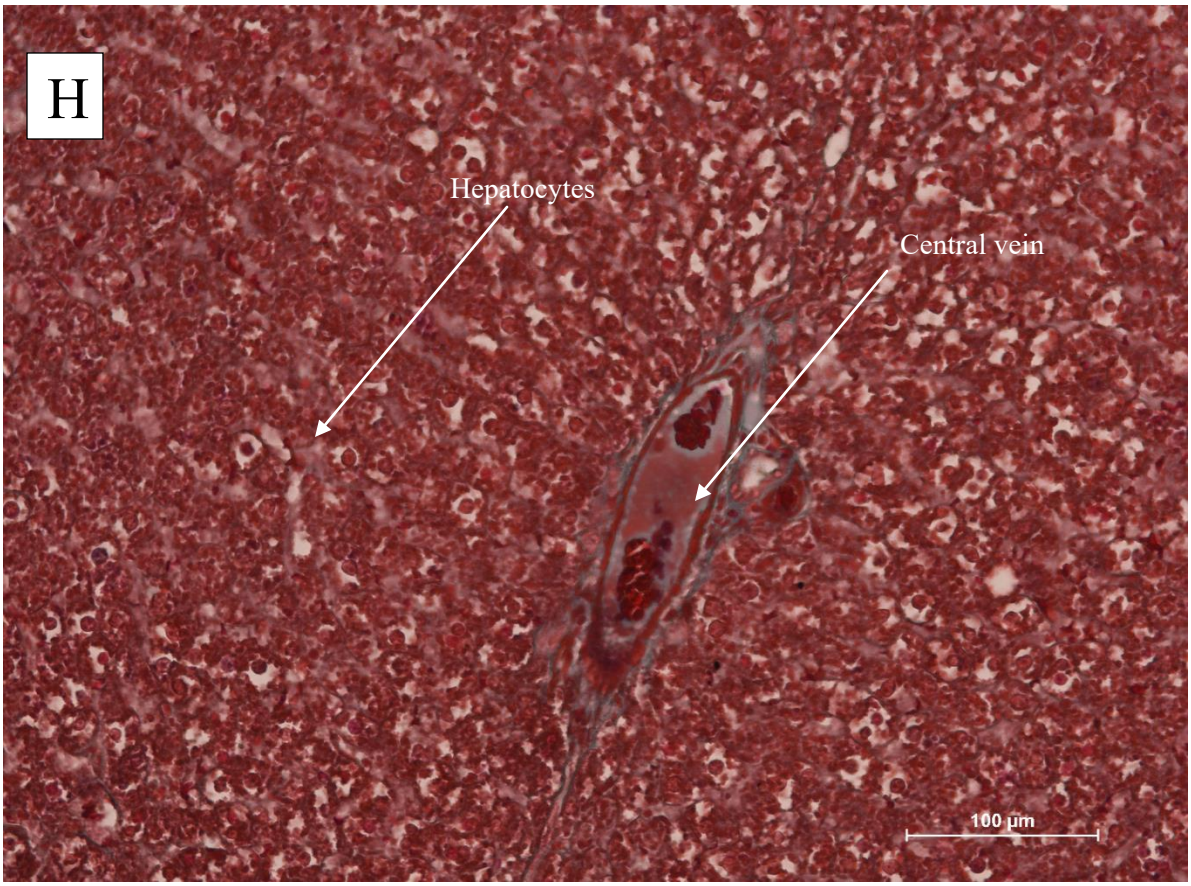
Plates 3 (E): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and vitamin E. Massons Trichrome stains. Note: liver architecture with radially arranged hepatocytes stained red with dark red nuclei.



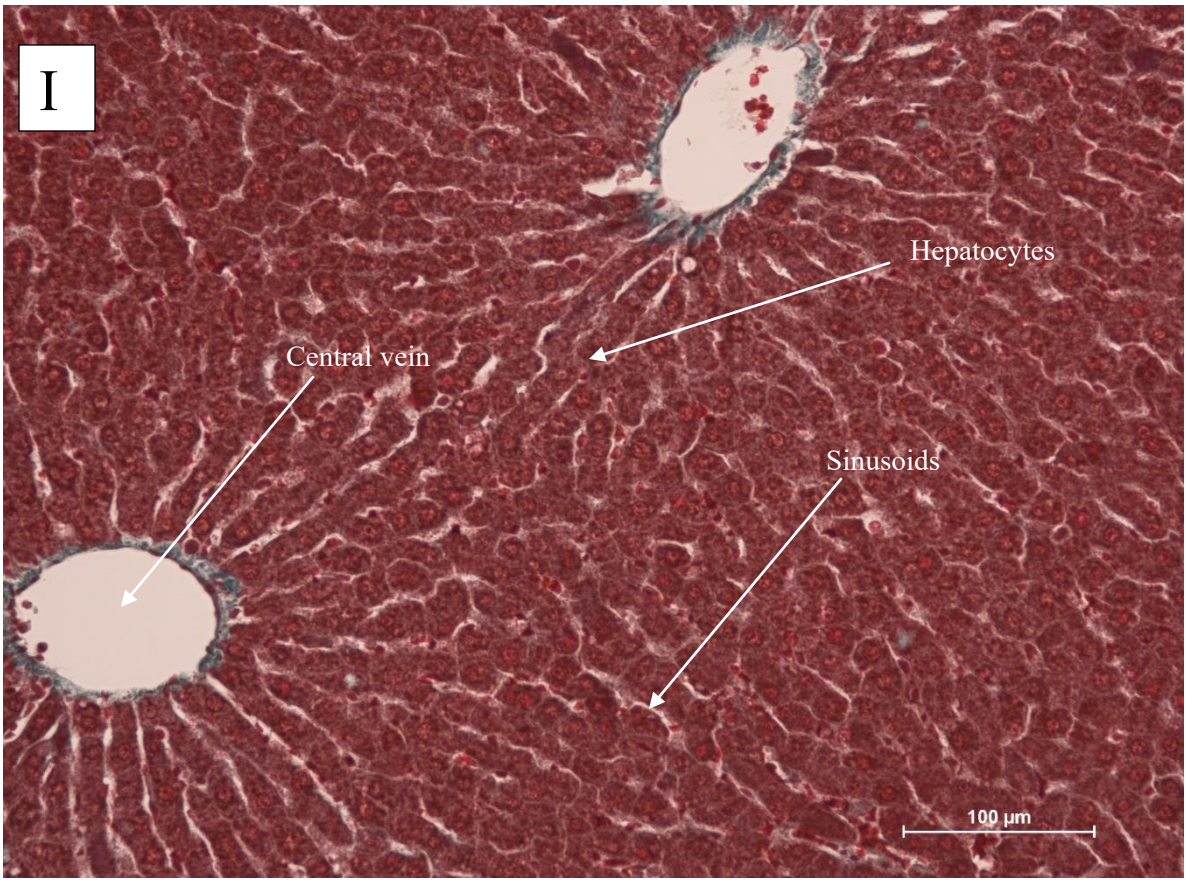
Plates 3 (F): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with HAART and vitamin (C and E). PAS stains. Massons Trichrome stains. Note: liver architecture with distorted central vein and congested stellate cells.



Plates 3 (G): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with low dose of *H. hemerocallidea* extract alone. Massons Trichrome stains. Note: Liver histoarchitecture showing central vein and bile ducts.



Plates 3 (H): Photomicrograph of cross section of liver of Sprague-Dawley rats treated with high dose of *H. hemerocallidea*. Massons Trichrome stains. Note: Liver histoarchitecture showing swollen central vein and distorted hepatocytes.



Plates 3 (I): Photomicrograph of cross section of liver of Sprague-Dawley rats. Control group. Massons Trichrome stains. Note: Normal Liver histoarchitecture and radially arranged hepatocytes.

Discussion

The liver performs numerous vital metabolic functions, including biochemical, synthetic and excretory functions. This central role played by liver in the clearance and transformation of chemicals exposes it to toxic injury (Naruse *et al,* 2007; Saukkonen *et al,* 2006). Our current study revealed that the administration of HAART and *H. hemerocallidea* concomitantly lead to slight changes in the biochemical markers, reduction in qualitative evaluated glycogen and tissue fibrosis in this protocol. This in addition to its inability to mitigate hepatocellular morphological changes further supports its herbal-drug interaction (HDI) effects. It is possible that unfavorable HDI between *H. hemerocallidea* and HAART could affect efficacy or safety of the later.

As people living with HIV/AIDs continue to benefit decreased morbidity and mortality due to HAART, organ toxicities (especially the liver) are frequently becoming a major concern in view of associated consequences that may predispose to metabolic complications. The result of this study showed that animals on HAART as well as adjuvant treatment with *H. hemerocallidea* recorded the least body weight gain indicating that *H. hemerocallidea* exacerbated rather than mitigate the probable cause. This is in tandem with the marker of toxicity, the liver-body weight ratio (Table 1) which is a sensitive and effective indicator of toxicity and thus important in the identification of target organ by toxicants (Asagba *et al*., 2007).

The aminotransferases are the most frequently utilized and specific indicators of hepatocellular necrosis. These enzymes- aspartate aminotransferase (AST) and alanine amino transferase (ALT) catalyze the transfer of the amino acids of aspartate and alanine respectively to the keto group of ketoglutaric acid. ALT is primarily localized to the liver but the AST is present in a wide variety of tissues like the heart, skeletal muscle, kidney, brain and liver (Friedman *et al,* 2003; Rosen and Keefe, 2000; Thapa and Anuj, 2007).

Our study revealed an observed increase in the AST levels in the treated groups except in group F (HAART with vitamin C and E) animals which was statistically elevated compared with the control, this difference may result from herbal-drug interaction (HDI). Whereas the ALT levels of animals treated with HAART, HAART with *H. hemerocallidea* (both doses), HAART with vitamin C and E concomitantly statistically decreased as compared with the control. AST is present in both the mitochondria and cytosol of hepatocytes, ALT is localized to the cytosol (Rosen and Keefe, 2000; Thapa and Anuj, 2007).

The activity of AST and ALT in the serum at any moment reflects the relative rate at which they enter and leave circulation. Mild elevations are usually seen in drug toxicity, extrahepatic biliary atresia (EHBA), fatty liver, cirrhosis, non-alcoholic steato hepatitis (NASH), myositis, duchenne muscular dystrophy and even after vigorous exercise (Thapa and Anuj, 2007). Hepatic causes of increased serum ALT activity, with or without increased AST activity, include hepatocellular necrosis, injury, or regenerative/reparative activity (Boone *et al,* 2005; Hall, 2001; Meyer and Harvey, 2004; Zimmerman, 1999). Decreases in ALT activity have been observed with concurrent hepatic microsomal enzyme induction in the rat (Boone *et al,* 1998) and the decreased ALT levels in animals treated with HAART, HAART with *H. hemerocallidea* (both doses),

HAART with vitamin C and vitamin E concomitantly were not considered indicative of hepatic injury because no substantive concurrent changes in liver histology or liver weight were observed except for the animals that were concomitantly administered HAART and high dose *H. hemerocallidea* that shows severe distortions and decreased sinusoidal spaces. The ratio of AST to ALT is of use in drug-induced toxicity and alcoholic liver disease. The lack of ALT rise is probably due to pyridoxine deficiency. In viral hepatitis the ratio is usually less than one. The ratio invariably rises to more than one as cirrhosis develops possibly because of reduced plasma clearance of AST secondary to impaired function of sinusoidal cells.

ALP is an indicator for hepatobiliary injury most found in circulation originates from liver or bone (Hagerstrand, 1975; Boone *et al,* 2005). Current study revealed decreased *H. hemerocallidea* activity in the serum of all animals administered with HAART alone and/or with *H. hemerocallidea* (both doses) and vitamin C and vitamin E. Increased ALP activity from the liver in the absence of cholestasis has been reported in dogs with increased endogenous or administered glucocorticoids (Meyer and Harvey, 2004; Wiedmeyer *et al,* 2002) and in rats and dogs with concurrent microsomal enzyme induction (Amacher *et al,* 2001). In rats, intestinal ALP is the major circulating isoenzyme, so a transient increase in serum ALP may occur postprandially, whereas fasting and hypothyroidism, pernicious anemia, zinc deficiency and congenital hypophosphatasia can result in a decrease in serum ALP (Boone *et al,* 2005).

Gamma-glutamyl transferease (GGT) is supplemental indicator of hepatobiliary injury, especially when evaluated with changes in ALP activity. Its activity in the current study shows no significant changes. GGT is a canalicular enzyme whose serum activity is increased in cholestatic liver disease. Increases in circulating GGT activity can arise from impaired bile flow and biliary epithelial

necrosis (Hall, 2001; Leonard, 1984; Boone *et al,* 2005). In liver disease GGT activity correlates well with alkaline phosphatase levels but rarely the GGT levels may be normal in intra hepatic cholestasis like in some familial intrahepatic cholestasis (Jansen and Muller, 2000). Most enzyme elevations associated with HAART are moderate, not accompanied by jaundice, and usually reversible (Wit *et al,* 2002).

As earlier reported by Carr *et al,* (1998) that shortly after the introduction of combination antiretroviral therapy, a syndrome of subcutaneous lipoatrophy, central adiposity, dyslipidemia, and insulin resistance, termed HIV-associated lipodystrophy (HIVLD) was noted. This was initially associated with PI exposure, but subsequently exposure to NRTIs (Feeney and Mallon, 2011), particularly thymidine analogue NRTIs (tNRTIs) such as Stavudine (Mallal *et al*., 2000) and Zidovudine (Bogner *et al*., 2001) were also recognized as being central to the development of this syndrome.

In this current study not all lipid parameters were significantly altered as there was no significance difference in the total cholesterol levels ($p<0.05$) while there were significant increase ($p<0.05$) in the serum level of LDL, HDL and except in group G (*H. hemerocallidea* low dose) as compare with the control animals, however the increase was not in folds. More so, the level of triglycerides (TG) statistically declined in groups administered with HAART, HAART with *H. hemerocallidea* and HAART with vitamins C and E, whereas its level increases in the group administered with 200 mg *H. hemerocallidea* alone and HAART concomitantly with vitamin C and E as shown in table 1, these effect are suspected to be as result of herbal-drug interaction and drug-drug interactions. In addition to HIVLD, in the general population there is concern about the increasing prevalence of the metabolic syndrome among HIV infected individuals. The pattern of abdominal obesity, low HDL-C, high triglycerides, and insulin resistance, all seen in HIVLD, are also components of the metabolic syndrome (Grundy *et al*., 2004). An interesting finding which points towards a hepatic cause of dyslipidemia is the fact that hepatitis C virus (HCV) co-infection appears to protect against the development of HAART-associated dyslipidemia (Di Giambenedetto *et al*., 2004; Cooper *et al*., 2007; Feeney and Mallon, 2011). This may be due to alterations in hepatocyte lipid secretion – mono-infection with HCV genotype is associated with lower serum cholesterol but a marked increase in hepatic steatosis. The ultimate result of abnormally functioning subcutaneous adipose tissue is reduced storage capacity for circulating lipids resulting in increased circulating free fatty acids, reduced adiponectin secretion, and lipid accumulation in non-adipose tissues such as liver

(hepatic steatosis) (Feeney and Mallon, 2011) and hepatic triglyceride accumulation which was even lower as compared to the control animals in this current study.

Morphological studies have identified the hepatic tissue as site of attack by HAART, resulting in the derangement on the hepatocellular cords and sinusoidal spaces. More so, hepatocellular injury has been identified as a potential flaw with most antiretroviral regimen (especially with Nevirapine and Stavudine) (Wyatt *et al*., 2009). Therefore, the deviations in the hepatocellular-architecture in HAART-treated group as well as the inability of adjuvant *H. hemerocallidea* to mitigate the derangements rather resulting into hepatocellular distortions suggests that the later having herbal-drug interaction (HDI). Our result on co-administration of vitamins C and E with HAART does not support the concurrent use of these vitamins as supplements by PLWHAs. While there are merits in its use as discussed extensively in Azu (2012), accumulating evidence from our laboratory (Azu *et al*., 2014) on the exacerbation of organ injuries has been made previously. It is believed that concurrent administration vitamins C and E alter bioavailability, metabolism and pharmacokinetics of certain HIV medications raising concerns about the possibility of HAART and vitamin supplementation leading to increased toxicity.

More so, the administration of HAART and *H. hemerocallidea* concomitantly leads to reduction in qualitative evaluation of glycogen and tissue fibrosis in this protocol. This in addition to its inability to mitigate hepatocellular morphological changes further supports its HDI effects. It is possible that unfavorable drug-drug interactions between *H. hemerocallidea* and HAART could affect efficacy or safety of the later are possible. Mills *et al*., 2005 reported that interactions between extracts of *H. hemerocallidea* and antiretroviral drugs inhibit CYP3A4, an isoform of cytochrome P450 and drug transporter protein (P-glycoprotein). Many antiretroviral drugs are substrate of CYP3A4 and some herbal preparations are known to alter blood levels of these drugs through their effects on CYP3A4 and p-glycoprotein (Mills *et al*., 2005). It is therefore reasonable to argue that *H. hemerocallidea* could potentially interact with HIV drug metabolizing enzymes thus patients taking *H. hemerocallidea* extracts concurrently with antiretroviral drugs, may be at risk of developing adverse effects which may lead to treatment failure and hepatocellular toxicity.

Conclusion

While HAART continues to be fundamental in the management of HIV/AIDS in sub-Saharan Africa, the complex issues of toxicities, resistance as well as herbal-drug interactions would continue to be hindrance to achieving the desirable goals. It has emerged from this study that

hepatotoxicity result based on the histopathological and with little changes in the biochemical assessments following HAART with concomitant *H. hemerocallidea* adjuvants. It is therefore advisable to take caution in Hypoxis-HAART combination use as this may impede the ultimate goal of HAART. It will be important, therefore, to conduct more in vivo study in healthy volunteers to ascertain the true inhibitory effects of these herbal medicines and products in humans.

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CHAPTER THREE

SYNTHESIS, CONCLUSION AND RECOMMENDATION

3.1 Synthesis

A number of metabolic consequences have been described in individuals receiving HAART. These consequences include derangements in lipodystrophies, insulin resistance, glucose intolerance, lactic acidaemia, hepatotoxicity and renal toxicity (Sattler, 2003; Carr, 2003).

These metabolic complications have been associated with each class of ARV drug (nucleoside reverse transcriptase inhibitors [NRTIs], non-nucleoside reverse transcriptase inhibitors [NNRTIs] and PIs) (Sattler, 2003; Carr, 2003). Many putative mechanisms have been investigated in these classes.

For instance, hepatotoxicity related to NRTIs has been documented with zidovudine monotherapy (Rodriguez *et al*., 1998). Combination therapy was subsequently found to further increase the risk of hepatotoxicity where asymptomatic enzyme elevations greater than five times normal were recorded in 30% of patients undergoing antiretroviral treatment (Spengler, 2002). All the nucleoside analogues have been implicated but hepatotoxicity were more prevalent with stavudine (d4T), didanosine (ddl) and zalcitabine (ddC) (Spengler, 2002). These drugs have a higher affinity for mitochondrial polymerase (Moyle, 2000).

Human mitochondrial DNA polymerase, an enzyme that is required for normal mitochondrial replication, is inhibited by nucleoside analogues. Depletion of mitochondrial DNA impairs the cellular respiratory chain and inhibits pyruvate and fatty acid oxidation pathways (Lewis *et al*., 1995). Mitochondria appear enlarged and swollen with matriceal densities and occasional vacuoles on electron microscopy (Lewis *et al*., 1995). With more severe NRTIs mitochrondrial toxicity, microvesicular hepatic steatosis, giant mitochondria and intrahepatic cholestasis are characteristic on light microscopy (Burkus, 2002). Clinically, severe NRTI mitochondrial toxicity is manifested by the development of hepatomegaly and steatosis and occasionally may be associated with lactic acidosis and liver failure (Burkus, 2002). Non-nucleoside analogue reverse transcriptase inhibitors (NNRTIs) have also be reported to cause hepatotoxicity with a significant number of patients developing abnormal liver function test such as asymptomatic aminotransferase elevations and hypersensitivity reaction (Spengler, 2002). The hypersensivity reaction can develop in the first few weeks of therapy and may cause symptoms of rash, fever, hypotension along with hepatic dysfunction (Wassef and Keizer, 1992). The histological features observed in patients with HAART

hepatotoxicity include bile duct injury and proliferation, hepatocellular necrosis and Mallory bodies, ballooning of hepatocytes, kupffer cell activation and peri-cellular fibrosis (Kemmer *et al*., 2000). The use of *H. hemerocallidea* in treatment for PLWHAs is increasing at an apparently much greater rate (Nair *et al*, 2007). However, reports have been documented of the potential drug-drug interactions between highly active antiretroviral therapy and *H. hemerocallidea* (Mills *et al*., 2005). The interactions occur secondary to the effect of HAART on the cytochrome P450- enzyme system (Mills *et al*., 2005). Many antiretroviral drugs are substrates of CYP450 enzymes and some herbal extracts are known to be inhibitors of CYP3A4 which is an isoform of CYP450 and drug transporter protein (Mills *et al*., 2005). Inhibition of the CYP3A4 will result in inhibition of antiretroviral drug metabolism which potentially lowers the efficacy of the drug resulting in toxicity (Mills *et al*., 2005). Thus, knowledge of these drug-drug interactions between *H. hemerocallidea* and antiretroviral regimens are critical in determining whether they can be administered concomitantly in PLWHAs and cautionary caveats should be taken in evaluating this in terms of safety and efficacy.

3.2 Conclusion

While studies to elucidate histomorphological changes in the liver following highly active antiretroviral therapy is limited, this research have highlighted that hepatotoxicity is a serious complication in patients taking HAART. These perturbations were not attenuated by adjuvant treatment with *H. hemerocallidea* whereupon the histological structures of the liver where disrupt leading to a more compromised integrity.

3.3 Recommendations

In view of the findings of this research on hepatotoxicity of HAART regimen, we believe that although the occurrence of hepatic derangements are prevalent, HIV patients who are clinically and virologically stable should continue with HAART unless severe or complex complications emerge. If they occur, adequate treatment options for the adverse events are prescribed like regimen change, temporarily withdrawal or complete stoppage after a thorough clinical evaluation. Secondly, in view of the possible drug-drug interactions likely between *H. hemerocallidea* and HAART regimen, it is recommended that further studies be carried out on the specific active component of *H. hemerocallidea* on this protocol and detailed pharmacokinetic properties thereof. This in addition to other investigational parameters would shed light on the possible mechanisms of action of *H. hemerocallidea* in this model.

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