EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF AQUEOUS SEED EXTRACT OF *NIGELLA SATIVA* IN HIGHLY ACTIVE ANTIRETROVIRAL THERAPY ADMINISTERED RATS

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF MASTER OF SCIENCE IN MEDICAL BIOCHEMISTRY

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A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial fulfillment of the Requirements of the Degree of Master of Science in Medical Biochemistry

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This is to certify that the thesis prepared by **Kissi Mudie**, entitled: "***Evaluation of Hepatoprotective Activity of Aqueous Extract of Nigella sativa in Highly Active Antiretroviral Therapy Administered Rats***": and submitted in partial fulfillment of the requirements for the Degree of Master of Science (Medical Biochemistry) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Background: Liver is a metabolically active organ responsible for many vital life functions. It performs many activities that are critical for survival. Due to its important activities, the liver is exposed to a number of insults and is one of the body's organs most subject to injury. Although antiretroviral drugs have significantly improved morbidity and mortality in HIV-infected patients, these benefits are compromised by numerous side effects, adverse clinical events and toxicities. The most common and troublesome toxicity of antiretroviral drugs is hepatotoxicity. In spite of tremendous advances in modern medicine, there are hardly any reliable drugs that protect the liver from damage and/or help in regeneration of hepatic cell. It is, therefore, necessary to search for effective and safe herbal drugs for the treatment of liver disease to replace currently used drugs of doubtful efficacy and safety.

Aim of the study: to investigate the hepatoprotective activity of aqueous extract of *Nigella sativa* seed in highly active antiretroviral therapy (Lamivudine, Zidovudine and Efavirenz) administered rats.

Materials and Methods: thirty six rats weighed between 150-200g were randomly divided into six groups and each group comprised of six rats. Rats in group I were administered with distilled water. Rats in group II were administered with highly active antiretroviral therapy only. Rats in groups III - VI were administered 100, 200, 400 and 800 mg/kg *N. sativa* plus highly active antiretroviral therapy respectively. The treatments were given orally for 28 consecutive days. On the 29th day, all rats were sacrificed under light diethyl ether anaesthesia; blood samples were collected for the assessment of biochemical parameters, while liver tissue was used for histopathological assessment.

Results: Serum levels of liver enzymes ALT, AST, ALP, and GGT were significantly ($p<0.05$) increased and albumin concentration was significantly decreased in animals treated with highly active antiretroviral therapy as compared to the normal control. Histopathological observations also revealed severe damage in the structure of liver tissue in animals administered with highly active antiretroviral therapy. Treatment of highly active antiretroviral therapy exposed animals with *N. sativa* showed marked improvement in both biochemical and histopathological findings. Rise in liver enzymes was almost restored to normal in animals treated with *N. sativa.*

Conclusion: *N. sativa* through its antioxidant activity effectively protects highly active antiretroviral therapy induced liver toxicity.

Key Words: HAART, Nigella sativa, Liver enzymes, hepatoprotective

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LIST OF ABBREVIATIONS

ALP:	Alkaline phosphatase
ALT:	Alanine aminotransferase
ANOVA:	analysis of variance
ART:	antiretroviral therapy
AST:	Aspartate aminotransferase
CAM:	Complementary and alternative medicine
DNA:	Deoxyribonucleic acid
dsDNA:	Double strand DNA
DPX:	Dibutylphthalate in xylene
EPHI:	Ethiopian Public Health Institute
fL:	Femtolitre
GGT:	Gamma glutamyl transferase
gp:	glycoprotein
HAART:	highly-active antiretroviral therapy
LDH:	Lactate dehydrogenase
MDH:	Malate dehydrogenase
NADH:	Nicotinamide adenine dinucleotide
NRTI:	nucleoside reverse transcriptase inhibitor
NNRTI:	nucleoside reverse transcriptase inhibitor
PI:	Protease inhibitor
RT:	Reverse Transcriptase
SEM:	Standard error of mean
TMMRD:	Traditional and Modern Medicine Directorate

1. INTRODUCTION

1.1. Liver

Liver is the largest organ in human body. It is located below the diaphragm in the right upper quadrant of the abdominal cavity. An adult's liver weighs approximately 3 pounds and extends approximately from the right 5th rib to the lower border of the rib cage. The internal structure of the liver is made of around 100,000 small hexagonal functional units known as lobules. Each lobule consists of a central vein surrounded by 6 hepatic portal veins and 6 hepatic arteries. These blood vessels are connected by many capillary-like tubes called sinusoids, which extend from the portal veins and arteries to meet the central vein like spokes on a wheel. Each sinusoid passes through liver tissue containing 2 main cell types: kupffer cells and hepatocytes. Kupffer cells are a type of macrophage that capture and break down old, worn out red blood cells passing through the sinusoids whereas; hepatocytes are the working cells of the liver that have a unique capacity to reproduce in response to liver injury. Hepatocytes make up 70 – 80% of the cytoplasmic mass of the liver. The hepatocytes of the liver are tasked with many of the important metabolic jobs that support the cells of the body (Ramadori *et al.,* 2008).

1.2. Functions of Liver

The liver is a metabolically active organ responsible for many vital life functions. Liver plays a great role in carbohydrate, protein and fat metabolism, synthesis of bile components, detoxification of blood and storage of vitamins and minerals. It also performs many activities that are critical for survival such as synthesis of blood clotting factors, creation of proteins necessary for growth and metabolic processing of most drugs and toxins. It also has a surprising role in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction (Ahsan *et al.,* 2009).

Our digestive system breaks down carbohydrates into the monosaccharide glucose, which cells use as a primary energy source. Blood entering the liver through the hepatic portal vein is extremely rich in glucose from digested food. Hepatocytes absorb much of this glucose and store it as the macromolecule glycogen, a branched polysaccharide that allows the hepatocytes to pack away large amounts of glucose and quickly release glucose between meals. The absorption and release of glucose by the hepatocytes helps to maintain homeostasis and protects the rest of the body from dangerous spikes and drops in the blood glucose level (Highleyman and Franciscus, 2012).

Amino acids entering the liver require metabolic processing before they can be used as an energy source. Hepatocytes first remove the amine groups of the amino acids and convert them into ammonia and eventually urea. Urea is less toxic than ammonia and can be excreted in urine as a waste product of digestion. The remaining parts of the amino acids can be broken down into ATP or converted into new glucose molecules through the process of gluconeogenesis (Neff *et al.*, 2005).

Fatty acids in the blood passing through the liver are absorbed by hepatocytes and metabolized to produce energy in the form of ATP. Glycerol, another lipid component, is converted into glucose by hepatocytes through the process of gluconeogenesis. Hepatocytes can also produce lipids like cholesterol, phospholipids, and lipoproteins that are used by other cells throughout the body. The liver also controls the production, metabolism, and excretion of cholesterol, which is an important component of cell membranes and certain hormones (Ahsan *et al.,* 2009).

The liver produces and excretes bile (an alkaline compound) required for emulsifying fats and help the absorption of vitamin K from the diet. The emulsification of fats by bile turns the large clumps of fat into smaller pieces that have more surface area and are therefore easier for the body to digest (Anthea *et al*., 1993).

Liver also stores vitamins and minerals - such as vitamins A, D, E, K, and B12, and the minerals iron and copper - in order to provide a constant supply of these essential substances to the tissues of the body (Highleyman and Franciscus, 2012).

As blood from the digestive organs passes through the hepatic portal circulation, the hepatocytes of the liver monitor the contents of the blood and remove many potentially toxic substances before they can reach the rest of the body (Anthea *et al*., 1993).

The liver functions as an organ of the immune system through the function of the Kupffer cells that line the sinusoids. Kupffer cells are a type of fixed macrophage that form part of the mononuclear phagocyte system along with macrophages in the spleen and lymphnodes. Kupffer cells play an important role by capturing and digesting bacteria, fungi, parasites, worn-out blood cells, and cellular debris (Ramadori *et al.,* 2008).

The liver is responsible for the production of several vital protein components of blood plasma: prothrombin, fibrinogen, and albumins. Prothrombin and fibrinogen proteins are coagulation factors involved in the formation of blood clots. Albumins are proteins that maintain the isotonic environment of the blood so that cells of the body do not gain or lose water in the presence of body fluids (Highleyman and Franciscus, 2012).

The liver also produces insulin-like growth factor 1 (IGF-1), a polypeptide protein hormone that plays an important role in childhood growth and continues to have anabolic effects in adults (Ramadori *et al.,* 2008). Due to these important activities, the liver is exposed to a number of insults and is one of the body's organs most subject to injury.

1.3. Antiretroviral Drugs

Antiretroviral drugs are medication for treatment of infection by retroviruses, primarily HIV. Several classes of antiretroviral drugs have been developed to treat HIV infection: nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry/fusion inhibitors, and integrase inhibitors. These drugs block various steps of the HIV replication cycle (**Figure 1.1**).

1.3.1. Nucleoside reverse transcriptase inhibitors

Nucleoside reverse transcriptase inhibitors (NRTIs inhibit further action of the reverse transcriptase enzyme when they incorporate themselves into the viral DNA being generated. They mediate reverse transcriptase inhibition through incorporation into the

nascent DNA strand during reverse transcription. This incorporation causes the termination of transcription, thereby blocking viral replication. This class of drugs includes zidovudine (ZDV or azidothymidine (AZT), lamivudine (deoxythiacytidine or 3TC), didanosine (dideoxyinosine or ddI), abacavir (ABC), zalcitabine (ddC), stavudine (d4T), emtricitabine (Emtriva) and tenofovir (TDF) (Chang and Schiano, 2007; Michaud *et al.*, 2012).

1.3.2. Nonnucleoside reverse transcriptase inhibitors

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) stop reverse transcriptase enzyme from transcribing RNA into DNA by binding directly to the enzyme and rendering it inactive. NNRTIs inhibit reverse transcription by a different mechanism (i.e., through binding to noncatalytic enzyme sites). NNRTI inhibition is usually mediated through steric hindrance that impedes structural changes in HIV reverse transcriptase. This class of antiretroviral agents includes nevirapine, efavirenz and delavirdine, and etravirine (Goldsby *et al*., 2004; Aranzabal *et al*., 2005).

1.3.3. Protease inhibitors

Protease inhibitors (PIs) prevent the cleavage of precursor proteins necessary for the assembly and release of HIV virions during the last stage of the viral reproductive cycle. PIs act on the viral protease, inhibiting the maturation of new viral particles, therefore attacking already formed HIV before initiation of the next cycle of infection (Michaud *et al.*, 2012). Currently approved PIs include indinavir, nelfinavir, amprenavir, ritonavir, saquinavir, lopinavir /ritonavir and fosamprenavir. Newer PIs include atazanavir, tipranavir and darunavir (Stevens, 2010; Chang and Schiano, 2007).

1.3.4. Entry/Fusion inhibitors

Fusion inhibitors such as enfuvirtide (T20) block entry of HIV into host cells by preventing fusion of the HIV membrane with the target cell membrane. Maraviroc, an HIV entry inhibitor, prevents the usage of the coreceptor, CCR5 and entry of the viral particle to the target cell. It blocks the binding of HIV to the chemokine coreceptor necessary for penetration of the host cell (Michaud *et al.*, 2012; Stevens, 2010).

1.3.5. Integrase inhibitors

Integration is a unique and essential step in viral replication. The high efficacy of raltegravir has been related to its favorable physical-chemical characteristics and to the inhibition of the integrase stage in viral replication. Raltegravir inhibit integration of HIV's DNA into the host genome (Goldsby *et al*., 2004; Stevens, 2010).

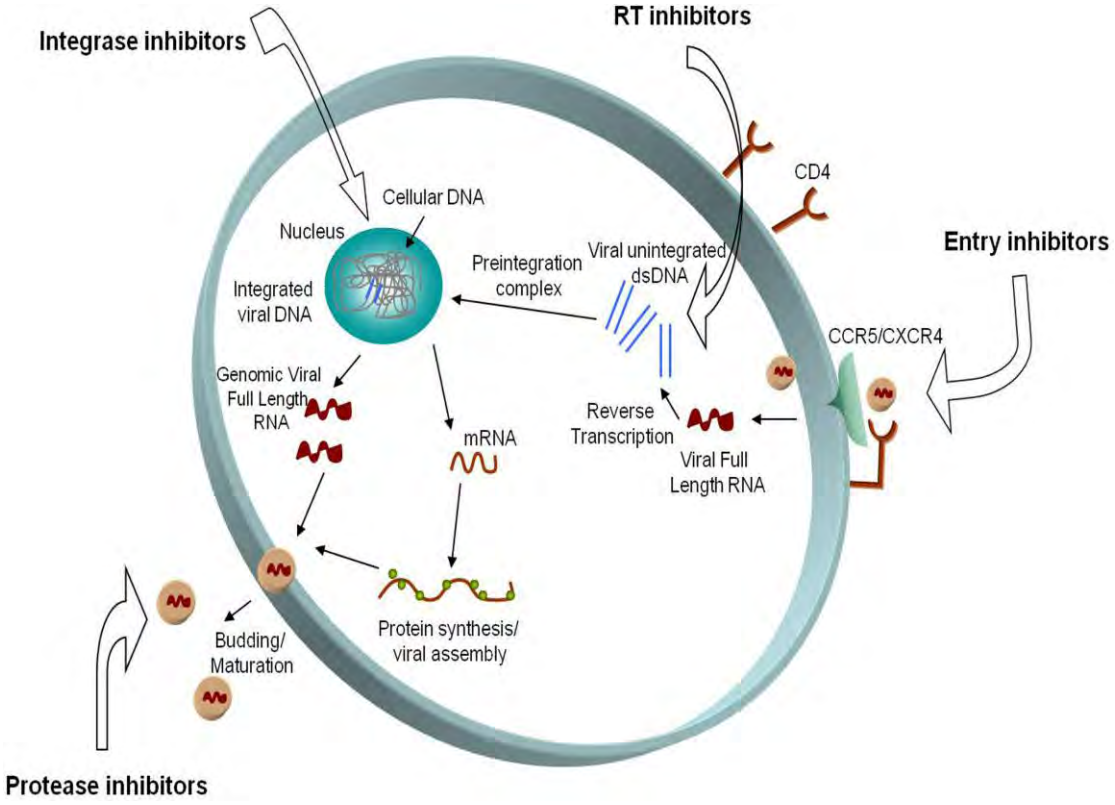

Figure 1.1. *Depiction of the HIV life cycle and antiretroviral drug targets (*Michaud *et al.*, 2012). (dsDNA=double strand DNA; mRNA=messenger RNA; CCR5/CXCR4= chemokine receptors; RT= reverse transcriptase)

Studies have shown that treatment with multiple drugs is more effective in killing the virus and avoiding viral resistance than treatment with a single drug. Potent regimens involving a combination of drugs from at least two of the drug classes mentioned earlier are the standard of treatment and are referred to as highly active antiretroviral therapy (HAART). Currently preferred treatment protocols use combinations of two NRTIs and either a NNRTI or a protease inhibitor (Kayode *et al.,* 2011; Nubila *et al*., 2012; Apostolova *et al*., 2010).

In the years before HAART was available, death from opportunistic infections caused by AIDS was the leading concern for HIV-infected patients (Neff *et al.*, 2005). Availability of HAART has significantly improved the outcome of HIV/AIDS, in terms of prevention of opportunistic infections. HAART has allowed patients infected with HIV to restore and retain immune function, increased CD4+ cell counts, and delayed in progression to AIDS (Awodele *et al*., 2011; Kalyesubula *et al*., 2011). HAART have also had a significant impact in reducing perinatal transmission of HIV (Neff *et al.*, 2005).

Due to the special characteristics of the HIV/AIDS, the development of antiretroviral drugs was particularly rapid and focused essentially on clinical efficacy, that is, reduction in mortality (Apostolova *et al*., 2010).

Although antiretroviral drugs have significantly improved morbidity and mortality in HIV-infected patients, these benefits are compromised by numerous side effects, adverse clinical events and toxicities. Viral resistance to the drugs may also develop, and some patients may be unable to take the drugs because they cannot tolerate the side effects (Kayode *et al*., 2011; Barrose, 2011). Some of the clinical events include AIDS-related insulin resistance, lipodystrophy syndrome, gastrointestinal symptoms and hyperglycaemia. Acute and chronic toxicities associated with these drugs include hypersensitivity reactions, neurotoxicity, nephropathy, liver damage, the appearance of body fat redistribution syndrome and the different metabolic alterations that accompany it. There is also a problem with cross-resistance among antiretroviral drugs of the same class (Liu *et al.,* 2009).

1.4. Liver Toxicity Associated with Antiretroviral Drugs

To maintain a healthy liver is a crucial factor for overall health and well-being, however; it is continuously and variedly exposed to environmental toxins and abused by poor drug habits and alcohol which can eventually lead to various liver ailments like hepatitis, cirrhosis and alcoholic liver disease (Panda *et al*., 2009).

The manifestations of drug-induced hepatotoxicity are highly variable, ranging from asymptomatic elevation of liver enzymes to fulminant hepatic failure. The occurrence of

drug-associated hepatotoxicity is a major problem in all phases of clinical drug development and the most frequent cause of post-marketing warnings and withdrawals (Nichols *et al.*, 2008).

Since the introduction of HAART, HIV infection has become a chronic disease. This has resulted in an increased prevalence and incidence of comorbidities among HIV-infected persons, requiring the use of more medications for longer periods. Therefore, it is not surprising that HAART-associated toxicity, especially liver toxicity, has perhaps become one of the main limitations to treatment (Aranzabal *et al*., 2005; Setzer *et al*., 2008).

Clinical manifestations of hepatotoxicity that have been previously reported include fatal portal hypertension, dress syndrome (drug rash, eosinophilia and systemic symptoms), and lipodystrophy syndrome consisting of central obesity, buffalo hump, wasting of extremities, hyperlipidemia, and insulin resistance (Ugiagbe and Ugiagbe, 2012).

Almost all antiretroviral drugs belonging to all available classes are responsible for an intrinsic liver toxicity, which is increased by the combined use of at least three different antivirals, in the so-called HAART (Labarga *et al*., 2007; Manfredi *et al*., 2005), despite the probability and extent of injury varies substantially with the individual agents (Ocama *et al.*, 2008).

All three classes of antiretroviral drugs: PIs, NRTIs and NNRTIs have been associated with hepatotoxicity. All Protease inhibitors (PIs) are metabolized by the cytochrome P450 3A4 system and have been associated with hepatotoxicity. Among the PIs, high-dose ritonavir is associated with the highest incidence of hepatotoxicity, with most studies demonstrating a 3–9% incidence of severe hepatotoxicity. Tipranavir, a newer PI, has been associated with reports of severe hepatotoxicity (Chang and Schiano, 2007).

As a class, NRTIs have been associated with hepatic steatosis and lactic acidosis. The spectrum of hyperlactataemia associated with NRTIs ranges from asymptomatic mild lactate elevation to a rare but potentially fatal lactic acidosis syndrome (LAS). LAS is characterized by lactate levels >5 mM, metabolic acidosis and liver dysfunction which can lead to death or the need for liver transplantation. Though the incidence of LAS is

rare (1.3–3.9 cases per 1000 patient-years), mortality is high and approaches 100% in some series. Once LAS is identified, prompt discontinuation of NRTI is warranted. Current recommendations advise against co-administration of didanosine and stavudine due to an increased risk of lactic acidosis (Chang and Schiano, 2007).

Liver toxicity has been addressed in the context of every antiretroviral regimen, but the risk of hepatotoxic events seems to be higher with NNRTIs (Aranzabal *et al*., 2005). During the first few weeks of therapy including NNRTIs, liver injury may appear in the setting of a hypersensitivity reaction to these drugs, along with rash and fever (Pineda and Macias, 2005).

Of NNRTIs, nevirapine warrants particular attention with regard to hepatotoxicity. Nevirapine toxicity may manifest as a rash-associated hypersensitivity reaction within the first few weeks of starting therapy in 2.3% of patients. A second, late onset toxicity related to cumulative dose over time is more commonly observed than a hypersensitivity reaction (Chang and Schiano, 2007). Cases of fulminant liver failure have also been reported in HIV-infected patients on nevirapine therapy, including pregnant women receiving multiple doses for the prevention of mother-to-child HIV transmission. Likewise, healthcare workers taking nevirapine for post-exposure prophylaxis after occupational HIV exposures have developed life-threatening hepatotoxicity (Pineda and Macias, 2005).

Efavirenz (EFV) is the most widely used NNRTI and although considered a safe drug, there is growing concern that EFV-containing therapies are associated with lipid and metabolic disorders, psychiatric symptoms and hepatotoxicity. Up to 10% of HIV patients treated with EFV exhibit increases in liver enzymes that may require the treatment to be discontinued. The study also showed that EFV reduces the proliferation and viability of hepatic cells *in vitro* through an acute mitotoxic effect, which may be relevant to the understanding of the hepatotoxicity associated with this drug. Moreover, since EFV is usually administered in conjunction with two NRTI, which are well known to have deleterious effects on mitochondria, it is tempting to speculate about potential

drug combinations in which these and other serious side effects may be enhanced (Apostolova *et al.*, 2010).

Liver toxicity is also frequently and significantly occurred in the setting of HIV infection that is treated with antiretroviral therapy due to its broad spectrum of supporting factors including concurrent chronic hepatitis, underlying diseases, opportunism and their pharmacologic therapies, alcohol and substance abuse (which is particularly frequent among HIV-infected people) (Manfredi *et al*., 2005).

Over 94% of patients with hepatotoxicity were asymptomatic; where the majority of HIV patients on HAART had asymptomatic enzyme elevations. Hence, there is a need for regular monitoring of liver function tests, at short intervals, in HIV patients starting HAART because of the risk of early hepatotoxicity and asymptomatic presentations (Ugiagbe and Ugiagbe, 2007).

At present, there is no agreement on how to manage patients suffering from ART-associated liver toxicity. An algorithm has been proposed, which indicates different schedules of behavior depending on the presence of clinical symptoms. According to this algorithm, it is recommended that ART be suspended in the case of grade 3 or 4 toxic hepatitis; regardless of the symptoms the patient presents (Awodele *et al*., 2011).

1.4.1. Mechanisms of HAART hepatotoxicity

The mechanisms involved in HAART derived liver toxicity are poorly understood. Elevations in serum liver enzyme levels have been described in relation to all the major classes of antiretroviral drugs. The underlying mechanisms proposed have included mitochondrial toxicity relating to several NRTIs and hypersensitivity reactions relating to NNRTIs (Gil *et al*., 2007).

The mitochondrion is a major target of drug-induced cytotoxicity, which occurs through a wide variety of mechanisms such as inhibition or uncoupling of oxidative phosphorylation, oxidative stress and/or opening of the mitochondrial permeability transition pore (Apostolova *et al*., 2010).

Many important adverse effects associated with HAART are known to be the consequence of mitochondrial toxicity, but they have been mainly attributed to the inhibition by NRTI of mitochondrial DNA polymerase γ, the enzyme responsible for mitochondrial DNA (mtDNA) replication. Decreased mtDNA copy numbers result in an impaired synthesis of mtDNA-encoded respiratory chain subunits and a secondary defect of oxidative phosphorylation (Setzer *et al.*, 2008).

The mitochondrial toxicity of NRTIs in the liver has been associated with steatosis, hepatic steatosis, acute organ failure, hyperlactatemia and lactic acidosis (Antoniades *et al.*, 2004).

Nucleoside reverse transcriptase inhibitors, such as zidovudine, didanosine or stavudine, may cause mitochondrial dysfunction, leading to lactic acidosis and steatohepatitis, which may result in liver failure. Subsequent work in HIV negative rat models treated with AZT confirmed the presence of the same type of toxicities in the rat. In all cases, the toxicity of AZT was correlated with abnormal mitochondria and mitochondrial DNA depletion, when examined (Lynx *et al*., 2006).

The pathogenic mechanism for liver toxicity associated with NNRTIs is not known. The effects on mitochondria of NNRTIs, which do not inhibit polymerase γ, are less well documented, though some elements of the toxicity attributed to these drugs resemble disorders induced by mitochondrial dysfunction (Pineda and Macias, 2005).

The cellular and molecular mechanisms underlying the detrimental effects of EFV remain largely unknown, but clinically relevant concentrations of EFV induce a rapid mitotoxic effect in human hepatic cells by a mechanism independent of mitochondrial DNA replication. EFV reduced cellular proliferation and viability in a concentration-dependent manner, triggered apoptosis via the intrinsic pathway and modified several parameters of mitochondrial function, including the induction of oxidative stress and a significant increase in mitochondrial mass. The fact that some of these harmful effects were partially reversed by an antioxidant treatment suggests that ROS generation is implicated in their manifestation (Apostolova *et al*., 2010).

1.4.2. *Cytochromes P450*

Enzymes belonging to the large family of P450s protect the organism by transforming liposoluble molecules into more hydrosoluble ones. CYP1, CYP2, and CYP3 are the main families involved in the majority of biotransformation reactions of clinically used drugs, including many antiretroviral agents. In fact, CP450s are the major enzyme system involved in the metabolism of NNRTIs, protease inhibitors, the CCR5 coreceptor antagonist maraviroc, and the integrase inhibitor elvitegravir. Variable expression and activity of P450s contribute to inter- and intraindividual variations in drug clearance, efficacy, and toxicity. P450 isoforms differ among other ways in their degree of tissue expression, their tissue selectivity, selectivity toward their substrates, and the reactions they catalyze. Each isoform has an affinity for certain substrates; activity can be altered by the co-administration of other substrates and by selective inhibitors or inducers. In addition, polymorphisms in some genes that code for P450 enzymes significantly contribute to interindividual variability in drug response (Michaud *et al*., 2012).

Some drugs associated with liver injury have been shown to be metabolized by CYPs to reactive metabolites that react with cellular macromolecules and subsequently disrupt hepatocellular homeostasis. Flavonoids seem to exhibit antioxidant and hepatptrotective properties possibly due to inhibition of cytochromes P450 (CYP) 1A1, CYP1A2 and CYP1B1 (Quintieri *et al*., 2011).

In some cases drug toxicity was reduced rather than increased when CYPs were induced, indicating that their metabolism is a detoxification step rather than an initiating event leading to toxicity. Inhibition of hepatic CYPs led to significantly enhanced rather than decreased cytotoxicity and CYP induction caused a decrease in cytotoxicity, indicating that the toxicity was mainly caused by the parent forms of both compound and not the downstream metabolites (Shi *et al*., 2011).

1.5. TRADITIONAL MEDICINE

Traditional medicine is defined as the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses (WHO, 2000).

Traditional medicine also refers to health practices, approaches, knowledge, and beliefs incorporating spiritual therapies, plant, animal and mineral-based medicines, manual techniques and exercises. These are applied singularly or in combination to treat, diagnose, and prevent illnesses or maintain health (Pekala, 2007).

Traditional medicine that has been adopted by other populations (outside its indigenous culture) is termed alternative or complementary medicine (WHO, 2000). These therapeutic approaches treat diseases or illnesses that fall outside of the realms of conventional medicine (Liu *et al*., 2009).

Traditional, complementary and alternative medicine (TCAM) broadly comprises herbal remedies, spiritual practices and prayer, traditional Chinese medicines, acupuncture, acupressure, chiropractic care, massage therapy, meditation, visualization, therapeutic touch and micronutrients (vitamins, minerals, and multivitamins) (Peltzer *et al.*, 2010).

Despite Western medicine becoming more widespread in Ethiopia, Ethiopians tend to rely more on traditional medicine. Conventional medical services remain concentrated in urban areas and have failed to keep pace with the growing population, keeping health care access out of reach for most people living in Ethiopia (Kassaye *et al.,* 2006). Because traditional medicine is culturally entrenched, accessible, and affordable, most of the Ethiopian population relies on traditional remedies as a primary source of health care (WHO, 2008).

1.5.1. Herbal Medicine

Herbal medicine was defined by the World Health Organization (WHO) as herbs, herbal materials, herbal preparations and finished herbal products that contain as active ingredients parts of plants, or plant materials, or combinations thereof (WHO, 2000).

The World Health Organization (WHO) estimates that 4 billion people, which are 80% of the world's population, presently use herbal medicines for some aspect of primary health care in conjunction with conventional medicines (WHO, 2008).

As conventional medical care (CMC) co-exists with traditional medicine systems in many regions of Africa and elsewhere, people may use medicine from one system exclusively or they may acquire medicine from each health system and use it simultaneously or sequentially (Langlois-Klassen *et al.*, 2007).

The introduction of HAART has led to reduction in AIDS-related morbidity and mortality. However; the management of HAART side effects has remained a challenge in many resource limited settings hence caused the increased use of complementary and alternative medicine (Namuddu *et al.,* 2011).

In Africa, the majority of HIV patients rely on traditional herbal medicine (THM) for management of side effects and other primary health care needs (Namuddu *et al.,* 2011; Mills *et al.*, 2005). This is because African traditional healers are not only more available and accessible than health care professionals (HCPs), but the majority of the local population also strongly believes in the usefulness and power of traditional medicine (Homsy *et al*., 2004). Many patients take a broad range of natural health products (NHPs) in addition to their conventional therapeutic products (Onifade *et al*., 2011).

Studies in South Africa have shown that herbal remedies are good supplements to antiretroviral therapy because of their immune boosting properties. A study in western Uganda found that 38% of HIV positive patients used traditional medicines and antiretroviral drugs at the same time for the management of HIV infection (Bepe *et al*., 2011).

Some HIV-infected people use herbs for potential cure or symptom treatment. Some clinical studies have shown that herbal medicines might have the potential to alleviate symptoms, reduce viral load, and increase CD4+ cells for HIV-infected individuals and AIDS patients (Liu *et al.,* 2009).

Some herbal remedies have been documented to be beneficial when used with conventional medicines. Coumarin derived herbal remedies decreased drug resistance resulting from HIV mutation associated with non-nucleoside analogue-nevirapine. Some herbal remedies have also shown to decrease toxicity associated with HAART (Onifade *et al*., 2012).

1.5.2. Hepatoprotective activity of medicinal plants

In spite of tremendous advances in modern medicine, there are hardly any reliable drugs that protect the liver from damage and/or help in regeneration of hepatic cells. Conventional drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects. It is, therefore, necessary to search for effective and safe drugs for the treatment of liver disease to replace currently used drugs of doubtful efficacy and safety (Panda *et al.,* 2009).

In the absence of reliable liver-protective drugs in modern medicine, a large number of medicinal preparations are recommended for the treatment of liver disorders and quite often claimed to offer significant relief. Attempts are being made globally to get scientific evidences for these traditionally reported herbal drugs (Jain *et al*., 2009).

Many folk remedies from plant origin were tested for their potential antioxidant and hepatoprotective liver damages (Ahsan *et al*., 2009). Many active plant extracts are frequently utilized to treat a wide variety of clinical diseases including liver disease. *Aloe vera*, *Eclipta alba*, *Phyllanthus niruri*, S*olanum indicum* have been shown to possess anti-hepatotoxic properties. *Phyllanthus niruri* and *Maytenus emerginata* exhibit the excellent hepatoprotective properties as indicated by maximum prevention of increased serum biochemical parameters on paracetamol induced toxicity (Parmar *et al.,* 2010).

Phytochemical screening of Bauhinia Purpurea demonstrated the presence of flavonoids, saponins, condensed tannins, steroids, and phenolic compound. Furthermore, Bauhinia Purpurea successfully reversed Paracetamol induced hepatotoxic effect, which is supported by the extract ability to bring down the elevated levels of ALT, AST and ALP, suggesting that these biochemical restorations could be due to the extract's inhibitory effects on cytochrome P450 or/and promotion of the paracetamol glucuronidation (Yahya *et al.,* 2013).

Accumulating evidence has demonstrated that oxidative stress plays a critical role in the initiation and progression of a variety of liver disorders, and many natural antioxidants have been tried to prevent oxidative stress mediated liver injury. The main advantage of natural compounds or extracts is their mild action in comparison to chemically synthesized drugs (Xie *et al*., 2012).

1.5.3. *Nigella sativa*

Nigella sativa (N. sativa) is a dicotyledon belonging to the botanical family of Ranunculaceae of herbaceous plants and known as black cumin seed (Tikur Azmud in Amharic; Gura (Oromiffaa) and Awoseta (Tigrigna). *N. sativa* is cultivated in many provinces of Ethiopia. It grows to a maximum height of 60cm, has blue flowers and finely divided foliage. Small caraway-type seeds are produced within the flowers (Gall and Shenkute, 2009; Michel *et al*., 2010; Roshan *et al*., 2010). The seeds of *N. sativa* are the source of the active ingredients of the plant (Salem, 2005).

Figure 1.2*: Photo of N. sativa plant showing the leaf, stem, flower and Seed*

1.5.3.1. Ethnomedical uses of *Nigella sativa*

Nigella sativa has been used for centuries as a spice, food preservative, preparation of candy and curative or medicinal remedy for various ailments, including infectious diseases (Tonkal, 2009; Raval *et al.,* 2010; Ali and Blunden, 2003). In Ethiopia the seed of *N. sativa* is used in flavoring bread and local beverages. Black seed powder is added to berbere sauce ("wot") to mask the pungency of pepper (Natural Standard, 2013).

Seed of the *N. sativa* has been used for medicinal purposes for centuries in Asia, Middle East, and Africa. It has been traditionally used as a natural remedy for a number of ailments that include headache, stomachache, asthma, chest congestion, hypertension, diabetes, inflammation, cough, bronchitis, fever, dizziness, and influenza and for general well-being (Michel *et al.,* 2010)

Nigella sativa L. seeds were a good source of polyunsaturated fatty acids (PUFAs), phytosterols (PSs) and phospholipids (PhLs) for the human diet. These seeds could be used by the food industry for formulating functional foods enriched with PUFAs and PSs. In pharmaceutical applications, *N. sativa* conjugated sterols could be used as precursors for the hemisynthesis of many hydrosoluble steroids. Since, *N. sativa* L. seeds are a good source of PhLs and aroma compounds, and therefore, they could be utilized in biscuit manufacturing and in food flavoring (Paarakh, 2010).

1.5.3.2. Chemical compostion, Phytochemistry and Pharmacology of *Nigella sativa*

Seeds of the *N. sativa* contain 37% oil and 4.1 % ash (calcium salts), protein (16-19.9%), carbohydrates (33-34%), fibre (4.5-6.5%), saponins (0.013%), moisture (5-7%) (El Tahir and Bakeet, 2006; Raval *et al.,* 2010).

Phytochemical screening of the seeds of *N. sativa* have led to the discovery of many active principles of the *N. sativa* like: Nigellicine, nigellidine, nigellimine-N-oxide, thymoquinone, dithymoquinone, thymohydroquinone, nigellone, thymol, arvacrol, oxy-coumarin, 6-methoxycoumarin, 7-hydroxy-coumarin, alpha-hedrin, steryl-glucoside as well as rich amounts of flavinoids, tannins, essential fatty acids, essential amino acids, ascorbic acid, iron and calcium (Paarakh, 2010).

Several studies have demonstrated that components of *N. sativa* seeds display a remarkable array of biochemical, immunological and pharmacological actions (**Table 1.1**).

Table 1.1: Biochemical, Immunological and Pharmacological actions of *N. sativa* plant

No.	Uses of the plant	References
1	Analgesic	Ali and Blunden, 2003; Al-Ghamdi, 2001
2	anti-allergic	Kalus *et al.*, 2003
3	anti-asthmatic	Boskabady *et al*., 2007
4	anti-bacterial	Singh *et al.,* 2005
5	anti-cancer	Raval *et al.,* 2010; Ali and Blunden, 2003
6	anti-diabetic	Akash *et al.,* 2011; Meddah *et al.*, 2009; Kanter *et al*., 2004
7	anti-fungal	Aljabre *et al.*, 2005; Singh *et al.*, 2005; Khan *et al.,* 2003
8	antihelminthic	Tonkal, 2009; Paarakh, 2010, Ali and Blunden, 2003
9	antihistaminic	Al-Ali *et al.*, 2008
10	anti-hypertensive	Dehkordi and Kamkhah, 2008
11	anti-inflammatory	Burits and Bucar, 2000; Al-Ghamdi, 2001
12	anti-oxidant	Meziti *et al.*, 2012; Abdel-Wahhab and Aly, 2005; Singh *et al*., 2005,
13	anti-oxidative stress	Roshan *et al.*, 2010; Abdelmeguid *et al.,* 2010
14	anti-tumor	Majdalawieh *et al*., 2010
15	anti-ulcer	El-Dakhakhny *et al*., 2000; Ali and Blunden, 2003
16	cardioprotective	Paarakh, 2010; Ebru *et al.*, 2008
17	dyslipidemia and hyperglycaemia	Rchid *et al.,* 2004; Zaoui *et al.*, 2002
18	**hepatoprotective**	Meral *et al.*, 2001; Mahmoud *et al.,* 2002; Coban *et al.*, 2010; Turkdogan *et al.*, 2003
19	Immunomodulator	Majdalawieh *et al.*, 2010; Salem, 2005
20	nephroprotective	Paarakh, 2010; Ali and Blunden, 2003

1.5.3.3. Hepatoprotective activity of *Nigella Sativa*

Nigella sativa has been extensively studied pharmacologically to justify its broad traditional therapeutic value, from which, it was found to have hepatoprotective and immunopotentiating properties. Study by Michel *et al.*, (2010) investigated that aqueous extract have protected against CCl_4-induced acute hepatotoxicity through restoration of the anti-oxidative defense system and down-regulation of the pro-inflammatory pathway. Other study revealed that the seeds, and the major active constituent thymoquinone, exhibited hepatoprotective effect against liver damage induced by carbon tetrachloride and tetra-butyl hydrogen peroxide (Mahmoud *et al.*, 2002).

Most of the hepatoprotective drugs belong to the group of free radical scavengers, and their mechanism of action involves membrane stabilization, neutralization of free radicals and immuno-modulation (Mahmoud *et al.*, 2002). Findings prove the hepatoprotective properties of *N. sativa* seed possibly through immune-modulator and antioxidant activities (Turkdogan *et al.*, 2003).

Nigella sativa exerts a protective and therapeutic effect on cholestatic liver injury in bile duct ligated rats possibly through attenuation of enhanced neutrophil infiltration (Cemek *et al*., 2006) and oxidative stress in the liver tissue (Coban *et al*., 2010).

Treatment of rats with a suspension of the seeds of *N. sativa* orally for 4 weeks protected against CCl_4-induced hepatotoxicity as reflected by the significant decreases in the plasma levels of ALT and AST enzymes together with significant decreases in the plasma level of lipid peroxides and significant increases in the erythrocytes' content of glutathione peroxidase and superoxide dismutase (El Tahir and Bakeet, 2006)

1.6. Significance of the Study

Hepatotoxicity is the most common and troublesome toxicity induced by antiretroviral drugs (Sule *et al.*, 2012). The research findings suggested the need of hepatoprotective agents in the treatment regimen when administering antiretroviral drugs, to minimize hepatic damage (Kayode *et al*., 2011).

On the other hand; in spite of tremendous advances in modern medicine, there are hardly any reliable drugs that protect the liver from damage and/or help in regeneration of hepatic cell (Parmar *et al.,* 2010). Conventional drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects. It is, therefore, necessary to search for effective and safe herbal drugs for the treatment of liver disease to replace currently used drugs of doubtful efficacy and safety (Panda *et al.,* 2009).

Hence, this study is aimed to investigate the hepatoprotective activity of aqueous extract of *N. sativa* seed in HAART administered rats.

2. OBJECTIVES OF THE STUDY

2.1. General objective

The general objective of this study is to investigate the hepatoprotective activity of aqueous extract of *N. sativa* seed in highly active antiretroviral therapy administered rats.

2.2. Specific objectives

- To assess the effect of *N. sativa* aqueous seed extract on the biochemical parameters
- To examine the effect of aqueous seed extract of *N. sativa* on the histopathology of the liver
- To identify secondary metabolites (phytochemicals) of *N. sativa*

3. MATERIALS AND METHODS

3.1. Study Design

The study was carried out by randomized experimental animals.

3.2. Study Area

The study was conducted at Ethiopian Public Health Institute (EPHI), Traditional and Modern Medicine Research Directorate (TMMRD), Addis Ababa, Ethiopia.

3.3. Plant Collection and Preparation of the Extracts

Seed of *N. sativa* was purchased from Goro district, Bale zone 530 kms southeast of Addis Ababa in September 2013. The taxonomic identity of the plant was verified at the Department of Biology, Addis Ababa University. Voucher specimen of the plant (k-001/2013) was kept at the national herbarium, Science faculty, Addis Ababa University.

The plant material was brought to the Directorate of Traditional and Modern Medicine Research, where the study was conducted. The plant material was then carefully washed with distilled water to remove any extraneous materials, dried under shade at room temperature, grounded to a coarse powder using electronic grinder and the aqueous extract of the seeds of the plant was prepared by decoction as follows:

200g of the powdered seed of *N. sativa* was weighed by analytical balance. 1500mL of distilled water was added and boiled for 15 minutes with continuous stirring. After cooling, the solution was decanted and the supernatant solution was filtered with $0.1mm^2$ mesh gauze. The filtrate was transferred into a petridish and was frozen in a deep freezer overnight. On the next day the freezed extract was allowed to dry in a freeze dryer (lyophilizer) under vacuum pressure at lower temperature (-40^0C) and lower pressure ($133x10^{-3}$mbar) for a week to obtain a freeze dried product. After the extract was dried, it was collected in air tight plastic containers, weighed, labeled and put in a desiccator for subsequent experiment (Michel *et al*., 2010; Dehkordi and Kamkhah, 2008).

The weight of the dry extract was expressed as percentage of the total mass of dry plant powder to determine the percentage yield.

3.4. Phytochemical Screening Test

A preliminary qualitative phytochemical screening of the plant material was carried out employing the standard procedures (Debella, 2002; Khan *et al*., 2011; Tiwari *et al*., 2011; Savithramma *et al*., 2011; Edeoga *et al*., 2005) to reveal the presence of saponins, flavonoids, alkaloids, tannins, phenols, and glycosides.

3.4.1. Detection of alkaloids: Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a white coloured precipitate indicates the presence of alkaloids.

Dragendroff's Test: Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of yellow orange precipitate indicates the presence of alkaloids.

3.4.2. Detection of tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

3.4.3. Detection of saponins

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. If foam produced persists for ten minutes it indicates the presence of saponins.

3.4.4. Detection of phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

3.4.5. Detection of flavonoids

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

3.4.6. Test for cardiac glycosides (Keller-Killani test): Five ml of each extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

3.5. Preparation of Highly Active Anti-retroviral Therapy

The three antiretroviral drugs used for the study (Lamivudine, Zidovudine and Efavirenz) were obtained from department of pharmacy, Black Lion Hospital, Addis Ababa.

The drugs were combined at the doses of 26.46 mg/kg Lamivudine (3TC), 52.91 mg/kg Zidovudine (ZDV) and 52.91 mg/kg Efavirenz (EFV). The doses of these drugs were calculated by converting human dose into animal dose by the formula:-

Human Equivalent Dose (HED in mg/kg) = Animal Dose (mg/kg) x Animal Km ÷ Human Km, where Km is a correction factor reflecting the relationship between body weight and body surface area. Km of rat=6 and Km of human = 37 (National Health Research Institute, 2008).The drugs were prepared by grinding the tablets into fine powder and dissolved in distilled water.

3.6. Extract preparation for the experiment

The graded concentrations of 100, 200, 400 and 800 mg/kg were prepared from *N. sativa* aqueous extract. HAART and *N. sativa* aqueous extract were mixed together before administration. Only fresh drugs (prepared daily) were used.

3.7. Experimental Animals preparation

The experimental animals used in this study were 36 Albino Wistar rats of both sexes, each weighing 150–200g and aged three months. The rats were obtained from TMMRD, EPHI. All rats were maintained under the controlled conditions of temperature (25 ± 2◦C), humidity, and light (12 hours of light and dark) in the Animal House of TMMRD, EPHI.

The animals had free access to food and clean tap water. The animals were housed in standard environmental conditions in stainless steel cages. The rats were acclimatised for 7 days before the start of the experiment. During the acclimatization the animals fed with Standard pelleted rat chow and water *ad libitum*.

Ethical approval for this study was obtained from the Research Ethics Committee of the Department of Biochemistry, Addis Ababa University.

A pilot experiment was conducted to determine the ranges of the median lethal doses of aqueous seed extract of *N. sativa*. Intragastric administration of extracts at doses of 100, 200, 400, 800 and 1200 mg/kg were used for the experiment.

3.8. Animal grouping and Drug dose

A modified method described by Peters and Robinson, 1992 was used for this test. In this study, thirty six Albino rats were randomly allotted into one of the six experimental groups, and each group consisted of six rats:

Group I received only distilled water and served as a normal control (2mL).

Group II received only HAART and served as a positive control (2mL.)

Group III received combination of HAART and (100 mg/kg) *N. sativa* seed extract.

Group IV received combination of HAART and (200 mg/kg) *N. sativa* seed extract.

Group V received combination of HAART and (400 mg/kg) *N. sativa* seed extract.

Group VI received combination of HAART and (800 mg/kg) *N. sativa* seed extract.

Animals were deprived of food before drug administration after which they were allowed access to food.

A volume of 2mL of each treatment was administered for each rat by oral intubation (blunt intragastric catheter or gavage) once a day in the morning at 9.00 a.m. for 28 consecutive days. The blunt intragastric catheter was cleaned, placed in an oven and sterilized after each administration to avoid any contamination. Toxicity signs and mortality were monitored daily.

3.9. Blood Sample Collection

At the end of the experiment, animals were fasted overnight and anesthetized with diethyl ether. Immediately each animal was placed in supine position on operating board. The extremities of the animals were stretched and fixed on a dissecting board. The abdominal cavity was opened and blood sample was withdrawn by cardiac puncture using sterile needle of 5ml syringe. The blood samples for biochemical assay were placed into test tubes without anticoagulant (Gabriel *et al*., 2008).

3.9.1. Biochemical Assay

Principles

3.9.1.1. Alkaline phosphatase

Alkaline phosphatase (ALP) measurements are most useful in diagnosing or monitoring hepatobiliary diseases, particularly extrahepatic obstruction due to stones or pancreatic cancer causing cholestasis, and bone diseases associated with increased osteoclastic activity. ALP is measured by the reagent rate analysis of p-nitrophenylphosphate, with cofactors zinc and magnesium, to form p-nitrophenol (**Figure 3.1**). The rate of increase of p-nitrophenol formation, measured at 405 nm is proportional to the activity of alkaline phosphatase in the sample (Tietz, 2001; Sule *et al*., 2012).

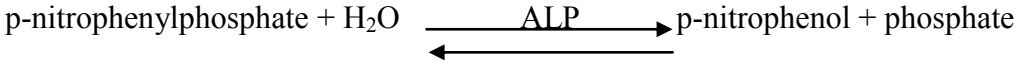

Figure 3.1: Scheme of reaction catalyzed by ALP

3.9.1.2. Alanine aminotransferase

Alanine aminotransferase (ALT) is an enzyme involved in the metabolism of the amino acid alanine. ALT works in a number of tissues, but its highest concentrations are in the liver. Injury to the liver results in release of the enzyme into the blood (Sule *et al*., 2012).

ALT present in the sample catalyzes the transfer of the amino group from L-alanine to α-ketoglutarate forming pyruvate and L-glutamate. Pyruvate in the presence of NADH and lactate dehydrogenase (LDH) is reduced to L-lactate. In addition, NADH is oxidized to NAD+ (**Figure 3.2**). The rate of decrease in absorbance of the reaction mixture at 340 nm, due to the oxidation of NADH is directly proportional to the ALT activity.

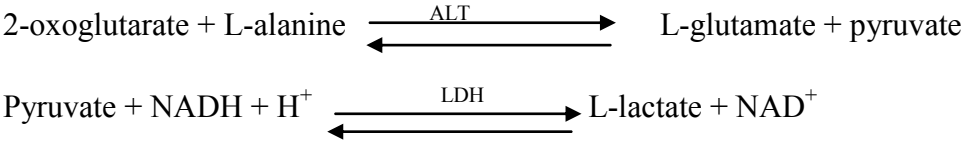

Figure 3.2*: Scheme of reaction catalyzed by ALT*

3.9.1.3. Aspartate aminotransferase

Aspartate aminotransferase (AST) catalyzes the transfer of the amino group from L-aspartate to α-ketoglutarate to yield oxaloacetate and L-glutamate. Malate dehydrogenase (MDH) catalyzes the reduction of oxaloacetate with simultaneous oxidation of NADH to NAD+ (**Figure 3.3**).

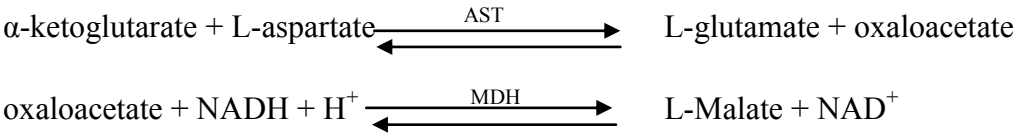

Figure 3.3: *Scheme of reaction catalyzed by AST*

AST is measured by the reagent rate analysis the coupled reaction with malate dehydrogenase (MDH) to reduce NADH (measured at a wavelength of 340nm) to NAD^+. The rate of decrease in absorbance at 340 nm due to NADH depletion is proportional to the AST activity in the sample (Tietz, 2001).

3.9.1.4. Gamma glutamyltransferase

Gamma glutamyltransferase (GGT) is involved in amino acid transport across the membranes (**Figure 3.4**). It is found mainly in biliary ducts of the liver, kidney and pancreas. Enzyme activity is induced by a number of drugs and in particular alcohol. GGT increased in liver diseases especially in obstructive jaundice. GGT levels are used as a marker of alcohol induced liver disease and in liver cirrhosis.

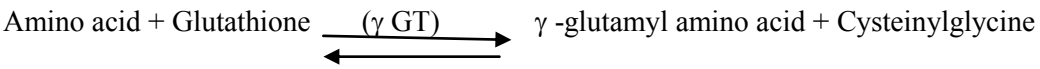

Figure 3.4: *Scheme of reaction catalyzed by GGT*

3.9.1.5. Total Bilirubin

Bilirubin is the degradation product from the heme portion of hemoglobin. Bilirubin is an endogenous anion derived from hemoglobin degradation from the RBC. Hemoglobin is degraded primarily in the spleen to biliverdin. The resulting molecule when biliverdin is reduced is bilirubin, a yellow-pigmented molecule. Bilirubin becomes bound to albumin and transported to the liver where hepatocytes conjugate the bilirubin with glucuronic acid. Conjugated bilirubin passes into the intestine through the bile duct where it is reduced to urobilinogen. Some urobilinogen is reabsorbed and excreted in the urine (Thapa and Walia, 2007).

Total bilirubin is measured as an end point chemical reaction (modified Jendrassik-Grof assay) using diazotization to produce azobilirubin. Increases in absorbance generated by blue colored azobilirubin and measured at 546 nm is proportional to the concentration of total bilirubin in the sample (Sule *et al*., 20120).

3.9.1.6. Albumin

The largest group of solutes in plasma contains three important proteins: albumins, globulins, and clotting proteins (fibrinogen). Albumins are the most common group of proteins in plasma and consist of nearly two-thirds of them (60-80%). They are produced in the liver. The main function of albumins is to maintain the osmotic balance between the blood and tissue fluids and is called *colloid osmotic pressure*. In addition, albumins

assist in transport of different materials, such as vitamins and certain molecules and drugs (Tietz, 2001).

Albumin is measured as an end point chemical reaction with albumin binding to bromocresol green (BCG), which is an anionic dye, in an acidic environment. The increase in absorbance at 620 nm of the green colored product is proportional to the albumin concentration in the sample (Sule *et al*., 2012; Parmar *et al*., 2010).

Procedure

The biochemical assay was conducted according to the standard procedures (National HIV/AIDS Laboratory, 2007) as follows:

Initially the blood samples were allowed to clot. Then the clotted blood was centrifuged (using Humax 4k bench top centrifuge) at 5000 revolution per minute (rpm) for 10 minutes to obtain the serum. Then 40μL of serum sample was pipetted with a working reagent containing TRIS buffer and Substrate (2-oxoglutarate, NADH) in to the measuring cuvette. The mixture was incubated for 1min. The absorbance of the reaction mixture in the measuring cuvette was measured in every 2second interval for 1min. Results were determined via a calibration curve which was generated by the instrument by calibration of a master curve generated from calibrator. Finally Automated Clinical Chemistry Analyzer (Cobas Integra 400 plus, supplied by Roche company, Germany) automatically calculated all the biochemical parameters (ALP, ALT, AST, GGT, total Bilirubin and albumin concentration) and displayed the results on computer screen.

3.10. Histopathology of the Liver

3.10.1. Animal Dissection, Tissue Sampling and Fixation

Animals of each group were sacrificed while under diethyl ether ansthesia. Animals lay up on a clean paper towel and had all four extremites pinned to thin corkboard. A vertical midline incision with scissors cut from the neck to pubis and opens the peritoneum. Then 3-4mm wide strips of tissue samples were randomly taken from right lobe of liver were cut lengthwise. These tissue samples were transferred by a blunt forceps to a test tube

containing 10% buffered formalin that completely immerses the tissues for the purpose of fixation (Yahya *et al*., 2013).

3.10.2. Dehydration and Infiltration

Following washing, tissues were dehydrated in a series of an increase graded ethanol (70%, 80%, 95% and 100%). Xylene was used to remove ethanol from the tissue and replace it with fluid that is miscible with paraffin (Yahya *et al*., 2013; Adjene and Igbigbi, 2012).

3.10.3. Embedding

The tissues were embedded in paraffin wax with the help of electro-thermal wax dispenser to form tissue blocks in squared metallic plates block moulds. The blocks were then labeled, and placed in a refrigerator until sectioned (Yahya *et al*., 2013; Adjene and Igbigbi, 2012; Essawy *et al.*, 2012)

3.10.4. Sectioning

Rotary microtome (Leica RM 2125, Leica microsystem Nussloh GmbH, Germany) was used for sectioning of tissue blocks manually at a thickness of 5µm. The paraffin block having tissue was put in the rotary microtome. The ribbon of sections was carefully picked from the knife by a blunt forceps to float in a water bath of 40^0C to remove folds in the sections. Unfolded sections were picked by clean microscope glass slides and were placed in an oven maintained at a temperature of 56^0C for 15 minutes for proper drying and better adhesion. The tissue sections were then cooled, dried and stained (Yahya *et al*., 2013; Essawy *et al.*, 2012)

3.10.5. Staining

The paraffin wax was removed from the tissue sections using xylene. The sections were then immersed in a series of descending alcohol concentration to remove xylene after which distilled water was used to hydrate the tissue. The hydrated sections were immersed in hematoxylin for 3-5 minutes with an eosin counterstained and agitated with acid alcohol to prevent over staining. Sections were immersed in a mixture of sodium

bicarbonate, ethanol and distilled water to give blue colour to the nucleus. Then it was immersed in 95% alcohol and eosin to give pink color to the cytoplasm (Yahya *et al*., 2013; Essawy *et al.*, 2012)

Finally, tissue sections were dehydrated in 95% alcohol, cleared in xylene and mounted by adding a drop of DPX (Dibutyl Phthalate in Xylene) mounting medium on the section to cover the microscopic glass with cover glass and to increase the refractive index of the tissue under light microscope (Adjene and Igbigbi, 2012).

3.10.6. Photomicrography

Using light microscope, sections of the liver were examined at different magnifications (x25, x40, and x100) objectives using MC 80 DX Microscope Camera (Carl Zeiss, Germany). Photomicrographs of the selected samples of the liver were taken with Fuji color C200 film under microscope camera (Adjene and Igbigbi, 2012).

3.11. Statistical Analysis

The data (expressed as mean ± SEM) were analyzed by one way ANOVA followed by Tukey–Kramer post hoc test using SPSS software version16.0 program. P values less than 0.05 were considered to be statistically significant.

4. RESULTS

4.1. Percentage Yield from plant material

Percentage yield (%Yield) of the crude extract of *N. sativa* was calculated by the following formula.

$$\%\text{Yield} = \frac{\text{weight of the aqueous extract obtained}}{\text{Weight of the powder measured for extraction}} \times 100$$

$$= \frac{29\text{g}}{200\text{g}} \times 100\%$$

= 14.5% (w/w) of crude extract was obtained from dried powder.

4.2. Phytochemical screening Test

The result of phytochemical screening of powdered seed of *N. sativa* showed the presence of many secondary metabolites (**Table 4.1**). The result revealed the presence of saponins, flavonoids, alkaloids, tannins, phenols and glycosides.

Table 4.1: Phytochemical screening of the seed of *N. sativa*

Test	**Color observed**	**Result**
Alkaloids (Dragendroff's)	Yellow orange ppt*	Positive
Alkaloids (mayer's reagent)	White ppt*	Positive
Cardiac glycosides	Reddish brown	Positive
Anthranides	Yellow	Negative
Polyphenols	Green blue	Positive
Tannins	Blue black	Positive
Chromophores	Brown	Negative
Flavonoids	Yellow ppt*	Positive
Carotenoids	White	Negative
Saponins	Honey comb forth (foam)	Positive

Positive: secondary metabolite present; **Negative**: secondary metabolite absent; *ppt: precipitate

4.3. Pilot Experiment

A pilot experiment was conducted to determine the ranges of the median lethal doses of aqueous seed extract of *N. sativa*. Intragastric administration of extracts at doses of 100, 200, 400, 800 and 1200 mg/kg did not produce any death in male and female rats during 24hrs of the experiment. There are no significant signs of toxicity observed during this period. But erection of hair and dizziness were observed in the 1200 mg/kg extract administered rats. This indicated that aqueous extract of *N. sativa* seed is safe up to the dose of 1200 mg/kg and the medium lethal dose (LD_{50}) may be higher than 1200 mg/kg.

4.4. Effects of *Nigella sativa* seed extract on the Biochemical parameters

The mean values of liver biomarkers (ALP, ALT, AST and GGT) increased significantly ($P < 0.05$) in HAART administered rats (**group II**) compared to rats in normal control group (**Table 4.2**). On the other hand, mean values of albumin concentration decreased significantly ($P < 0.05$) in rats treated with HAART when compared to the rats in normal control group (**Table 4.3**). Administration of *N. sativa* plus HAART (**groups III – VI**) significantly decreased ($P < 0.05$) the mean values of ALP, ALT, AST and GGT and significantly increased ($P < 0.05$) the mean value of albumin concentration when compared to positive control group (**group II**). Mean values of total Bilirubin has shown non-significant increase in HAART administered rats and decreased in *N. sativa* extract plus HAART administered rats (**Table 4.3**). Though there were no statistically significant differences among different doses of *N. sativa*, slight decrease in mean values of ALT, ALP, AST, and GGT was observed as the concentration increases from 100 to 800 mg/kg.

Table 4.2: Comparison of the Mean ± SEM of the Biochemical parameters (Liver Enzymes)

Dose	ALP (U/L)	ALT (U/L)	AST (U/L)	GGT (U/L)
Normal control	117.00±14.92[a]	84.75±13.42[a]	286.00±28.62[a]	4.20±0.48[a]
Positive control	265.75±6.52	166.75±13.92	472.50±22.40	12.35±2.53
100mg/kg *N. sativa* + HAART	164.00±8.54[*]	99.33±9.26[*]	311.67±35.59[*]	5.90±0.30[*]
200mg/kg *N. sativa* + HAART	161.67±23.15[*]	98.00±9.29[*]	298.20±37.24[*]	5.84±0.69[*]
400mg/kg *N. sativa* + HAART	147.60±16.60[*]	92.60±2.98[*]	262.00±12.10[*]	5.37±0.69[*]
800mg/kg *N. sativa* + HAART	143.00±12.49[*]	87.67±12.55[*]	226.67±8.45[*]	5.07±0.88[*]

****** The mean difference of treated groups is significant at the 0.05 level when compared to positive control.*
[a] *The mean difference of normal control group is significant at the 0.05 level when compared to positive control.*

Table 4.3: Comparison of the Mean ± SEM of the Biochemical parameters (Albumin, Bilirubin)

Dose	Albumin (g/dl)	Total Bilirubin (mg/dl)
Normal control	4.68±0.08[a]	0.03±0.00
Positive control	3.32±0.10	0.27±0.14
100mg/kg *N. sativa* + HAART	4.53±0.03[*]	0.08±0.02
200mg/kg *N. sativa* + HAART	4.50±0.07[*]	0.06±0.00
400mg/kg *N. sativa* + HAART	4.33±0.03[*]	0.04±0.01
800mg/kg *N. sativa* + HAART	4.44±0.15[*]	0.08±0.01

****** The mean difference of treated groups is significant at the 0.05 level when compared to positive control.*
[a] *The mean difference of normal control group is significant at the 0.05 level when compared to positive control.*

4.6. Histopathological examination of Liver

Histopathological examination of the liver sections under the light microscope revealed that liver sections from normal control rats (**group I**) showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus, nucleolus, central vein and compact arrangement of hepatocytes (**Figure 4.1**).

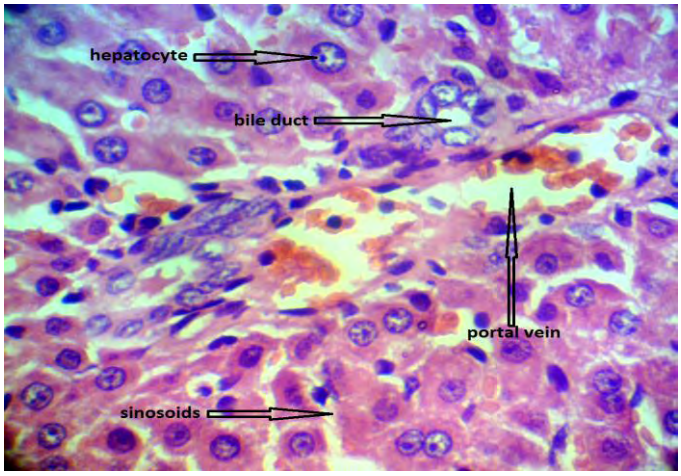

Figure 4.1: *Photomicrograph of liver sections of the rats in normal control group: exclusively normal architecture and normal morphology.*

However; the histology of the liver in HAART treated rats (**group II**) showed wide spread inflammation, vascular congestion, dilated sinusoidal spaces and focal necrosis (**Figure 4.2**).

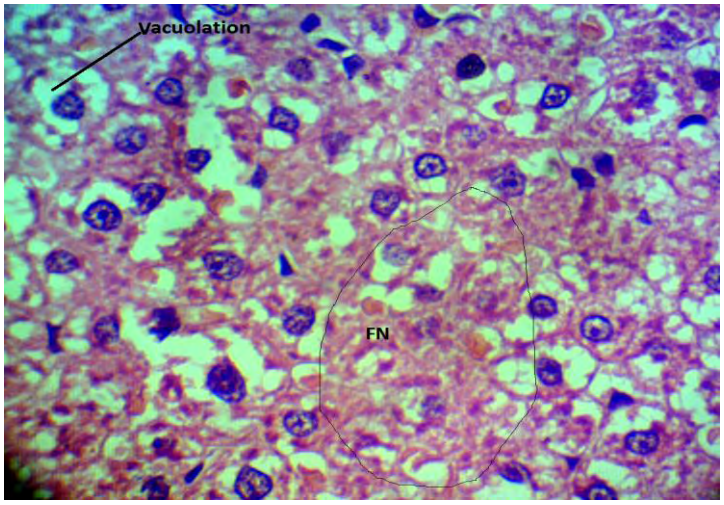

Figure 4.2**:** *Photomicrograph of liver sections of the rats in group II (HAART treated group). Normal architecture is distorted; sinusoidal space is not clear. Focal necrosis is observed.*

The histological examination of the liver tissues in rats treated with *N. sativa* aqueous extract (**groups III**) showed improvement in the liver tissue. Minor vacoulation in the cytoplasm of the hepatocytes and minor focal hepatocellular necrosis was observed (**Figure 4.3)**.

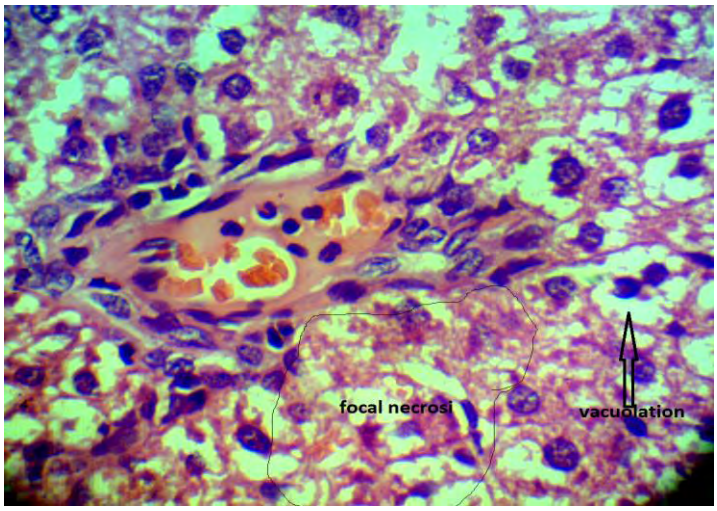

Figure 4.3**:** *Photomicrograph of liver sections of the rats in group III (100mg/kg N. sativa extract + HAART treated)*

The histological examination of the liver tissues in rats treated with *N. sativa* aqueous extract (**groups IV**) showed significant improvement in the liver tissue. Only minor distortion in architecture and vacuolation was observed (**Figure 4.4)**.

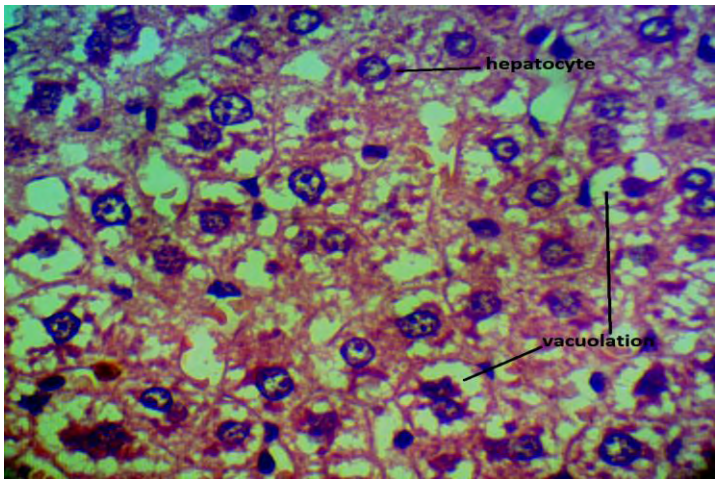

Figure 4.4**:** Photomicrograph of liver sections of the rats in group IV (200mg/kg *N.sativa* extract + HAART treated).

The histological examination of the liver tissues in rats treated with *N. sativa* aqueous extract (**groups V**) showed very minor change in cytoplasm of hepatocytes and small clear spaces in few hepatocytes were observed (**Figure 4.5)**.

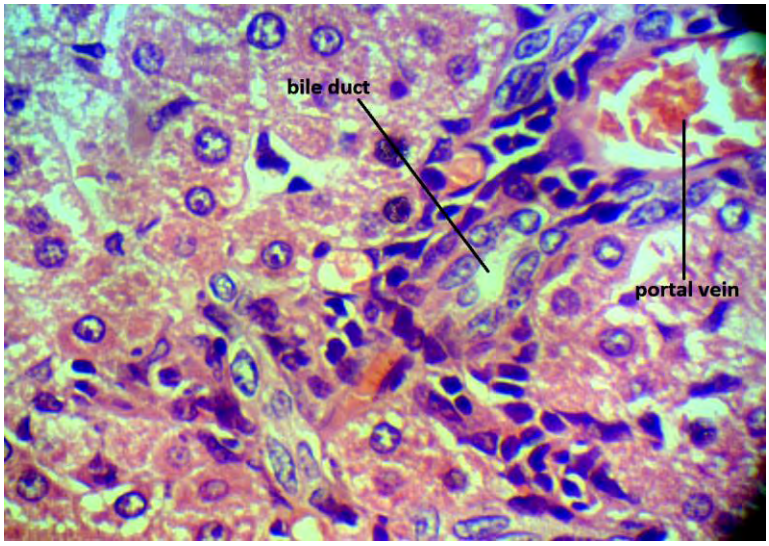

Figure 4.5: *Photomicrograph of liver sections of the rats in group V (400mg/kg N. sativa extract + HAART treated).*

The histological examination of the liver tissues in rats treated with *N. sativa* aqueous extract (**groups VI**) showed very minor change in cytoplasm of hepatocytes; Sinusoids begin to appear; Normal architecture of the liver begins to regenerate (**Figure 4.6**).

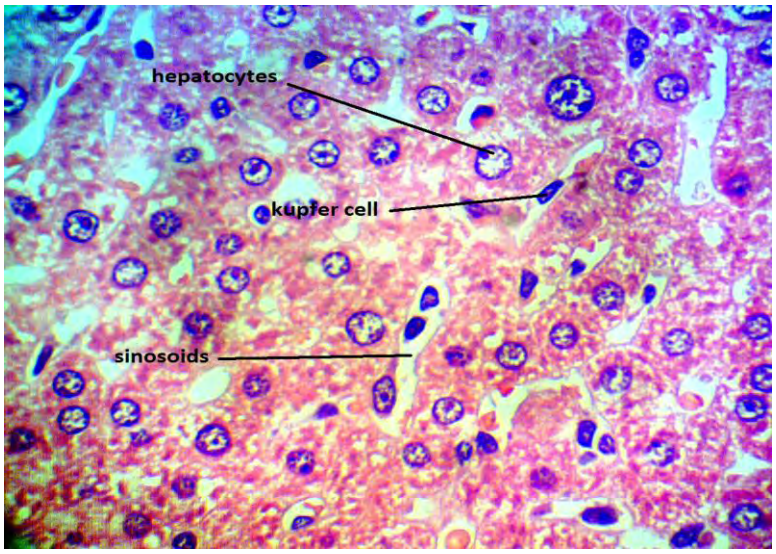

Figure 4.6*: *Photomicrograph of liver sections of the rats in group VI (800mg/kg N. sativa extract + HAART treated).

5. DISCUSSION

Liver diseases have become one of the major causes of morbidity and mortality all over the world. From among, drug induced liver injury is one of the most common causative factor that poses a major clinical and regulatory challenges (Parmar *et al.,* 2010).

Hepatotoxicity is a significant problem in patients on highly active antiretroviral therapy (HAART). In approximately 6% to 30% of treated patients, HAART is associated with significantly increased serum liver enzyme levels, which can lead to interruption of therapy, hepatitis, and death (Ugiagbe and Ugiagbe, 2012; Gil *et al*., 2007).

The widely used medicinal plants have formed the basis of health care throughout the world since the earlier days of humanity (Ebong *et al.*, 2008). Medicinal plants are part of Africa's heritages and a very significant part of present day ailment remedies worldwide. The majority of Ethiopians depend on medicinal plants as their source of health care especially in rural areas. The present study was undertaken to evaluate the hepatoprotective activity of aqueous extract of *N. sativa* seeds against liver damage induced by HAART in albino rats.

The pilot experiment reveals that the aqueous extract of *N. sativa* seed did not show signs of visual toxicities like changes in skin and fur, tremors, convulsions, salivations, diarrhea, lethargy, sleep and coma up to the dose of 1200 mg/kg. This result supports the work of Dollah *et al*., (2013) that concluded the supplementation of *N. sativa* up to the dose of 1 g/kg for a period of 28 days resulted no changes in liver enzymes level and did not cause any toxicity effect in rats.

Because of its functional roles in the body, liver is the major target organ of toxicity. Injury to the liver may affect the integrity of hepatocytes leading to the release of liver enzymes such as ALP, ALT, AST, and GGT since these enzymes are confined to hepatocytes and released into the blood following liver injury. Hence, these enzymes are commonly used as markers of hepatic injuries (Gaskill *et al.,* 2005; Panda *et al.,* 2009).

In the current study, damage of the liver caused by HAART was evident by the alteration in serum marker enzymes and albumin concentrations. Administration of HAART

(**Group II**) significantly increased the serum levels of liver enzymes (AST, ALT, ALP, and GGT) and significantly decreased albumin concentration. This result indicates liver cell damage; leakage of enzymes from cells and loss of functional integrity of cell. This is in consistent with the work done by Kayode *et al*. (2011) that indicated oral administration of rats with antiretroviral drugs (Efavirenz, Abacavir and Lamivudine) caused significant liver damage.

AST and ALT are elevated in nearly all liver diseases, but are particularly high in conditions that cause extensive cell necrosis, such as severe viral hepatitis, toxic injury, and prolonged circulatory collapse. ALT is a metabolic enzyme expressed primarily in the liver. Increase in serum ALT activity is typically associated with hepatocellular membrane damage and leakage of enzyme from hepatocytes (Gaskill *et al.,* 2005). Damage to the liver causes the release of ALT enzyme into the blood. Elevation of ALT levels is an indication of liver damage and has been associated with liver injury. The increase in serum ALP activity is also associated with a pathological damage occurrence in the liver (Haldar *et al*., 2007).

When damage to heart or liver cells occurs, intracellular enzymes, such as AST, are released into the peripheral blood. Since AST is located in the parenchymal hepatic cells and heart muscle, this enzyme is used to assess damage to these areas. Increases in AST can be seen in hepatitis, liver necrosis, cirrhosis, and liver metastasis (Parmar *et al*., 2010). Gamma Glutamyl Transferase (GGT) is also widely used to assess liver function. Some drugs, and also alcohol, induce the liver to produce more of this enzyme (Sule *et al*., 2012).

Total bilirubin results are comprised of the conjugated and unconjugated forms of bilirubin. Hyperbilirubinemia can occur in three areas as bilirubin is addressed by the body. In the prehepatic phase, increased bilirubin levels are caused by an increase in heme degradation and hemolysis. In the hepatic phase, increase levels are due to defective transport to the liver or defective conjugation by the liver. In the post hepatic phase, increase levels are due to defects transporting the conjugated bilirubin and bile out

of the liver. Therefore, total bilirubin measurements are used to diagnose and treat liver, hemolytic, hematological, and gallbladder obstructive disorders (Tietz, 2001).

Albumin is a small protein which accounts for nearly 50% of the total plasma protein. Albumin is primarily synthesized by the hepatic parenchymal cells. Albumin's primary function is the maintenance of colloidal osmotic pressure in the vascular and extravascular areas of the body and preventing edema. Additionally, albumin is a carrier transport protein. Hypoalbuminemia occurs in GI malabsorption, glomerulonephritis, nephritic syndrome, cirrhosis, severe burns, neoplasms, and autoimmune diseases. Low albumin levels indicate poor liver function and contribute to peripheral edema and ascites sometimes seen in very late stage liver disease. Albumin levels are usually normal in chronic liver disease until significant liver damage is present (Rosalki and Mcintyre, 1999).

In this study, results of histopathological studies also provided supportive evidence for biochemical analysis. Examination of liver sections of rats received HAART revealed disruption of the normal structural organization of the hepatic lobules and loss of the characteristic cord-like arrangement of the normal liver cells. Many hepatic cells were damaged and lost their characteristic appearance while others showed marked cytoplasmic vacuolization. The architecture of the liver elicited severe hepatic injury as evidenced by the observation of pathological changes in the architecture of the liver viz. focal necrosis, and degenerative changes in the hepatocytes. These pathological changes correlated well with the altered enzyme activities. This is in agreement with the observations noted by previous work of Gani and John, (2013). According to this previous study a significant increase in the activities of serum enzymes with in eighteen hours of exposure of the rats to single dose of D-Galactosamine Lipopolysaccharide (D-GalN/LPS) induced hepatotoxicity in rats, indicating the severity of hepatocellular injury. Rats given D- GalN/LPS elicited severe hepatic injury as confirmed by the observation of pathological changes like: infiltration of inflammatory cells, kupffer cell hyperplasia, neutrophil accumulation and focal necrosis.

Liver cells or hepatocytes are grouped in interconnected plates and constitute two-thirds of the mass of the liver (Janqueira and Carneiro, 2005). Hepatocytes, with their high degree of metabolic activities, are readily disturbed by toxins, especially drugs or alcohol, and may demonstrate the histological cell responses known as cloudy swelling, fatty changes and necrosis. With more severe metabolic disruption, the hepatocytes undergo hydropic degeneration and become swollen and vacuolated. The fatty changes due to metabolic injury to hepatocytes are also manifested by large cytoplasmic vacuoles within some hepatocytes, which usually displace nucleus to one side (Burkitt *et al.*, 1996).

Viral hepatitis, toxins, drugs, and systemic infections are the most important groups of conditions causing acute liver inflammation. Acute inflammation of the liver parenchyma is usually marked by focal accumulations of inflammatory cells in the site of necrotic hepatocytes. Liver sections may appear with vacuolated hepatocytes, dilated sinusoids and increased number of kupffer cells (Ebaid *et al*., 2007). Certain liver diseases cause obliteration of the normal sinusoidal arrangement that causes impairment of the liver function (Young *et al.,* 2006).

Many folk remedies from plant origin were tested for their potential antioxidant and hepatoprotective liver damages (Ahsan *et al*., 2009). Many active plant extracts are frequently utilized to treat a wide variety of clinical diseases including liver disease (Parmar *et al.,* 2010).

In this study, treatment with aqueous extract of *N. sativa* seeds significantly lowered the values of liver enzymes elevated by HAART and restored the damaged hepatocellular architecture. The decreased level of liver biomarkers and restoration of hepatocytes in *N. sativa* treated group leads to the inference that *N. sativa* seed aqueous extract counteracts the abnormal increase in serum enzymes and repair the hepatic tissue damage induced by HAART. These findings are in accordance with the finding of the previous study by Essawy *et al*., (2012) where the aqueous extract of *N. sativa* seed reported as an effective protector against CCl_4 induced liver damage which was evidenced by decreasing elevated levels of liver enzymes and restoration of hepatocellular architecture. Similar works were also reported by Michel *et al*., (2010); Turkdogan *et al*., (2003) and Yesmin *et al.*, (2013).

The mean values of ALT, ALP, AST and GGT decreases as the concentration of *N. sativa* extract increases (**Figures 5.1-5.4** respectively) which was supported by histopathological investigation (**Figures 4.1-4.6** respectively) provided that hepatoprotective activity of aqueous extract of *N. sativa* seed was enhanced with concentration and best activity was achieved at the dose of 800mg/kg.

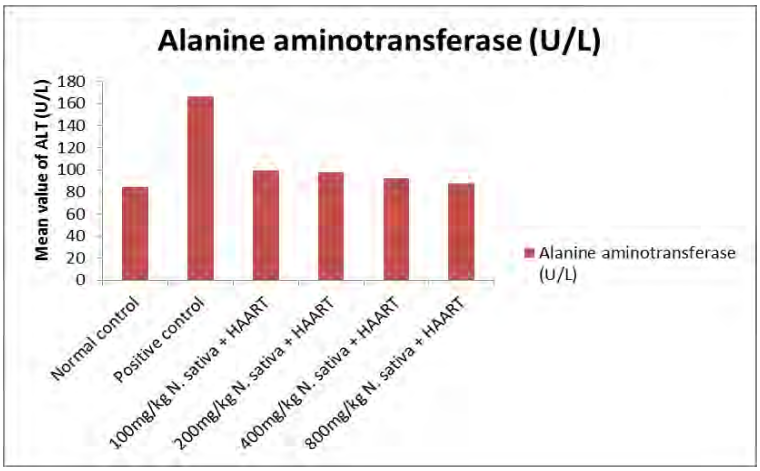

Figure 5.1: *Effect of N. sativa seed extract on the ALT value of HAART induced rats*

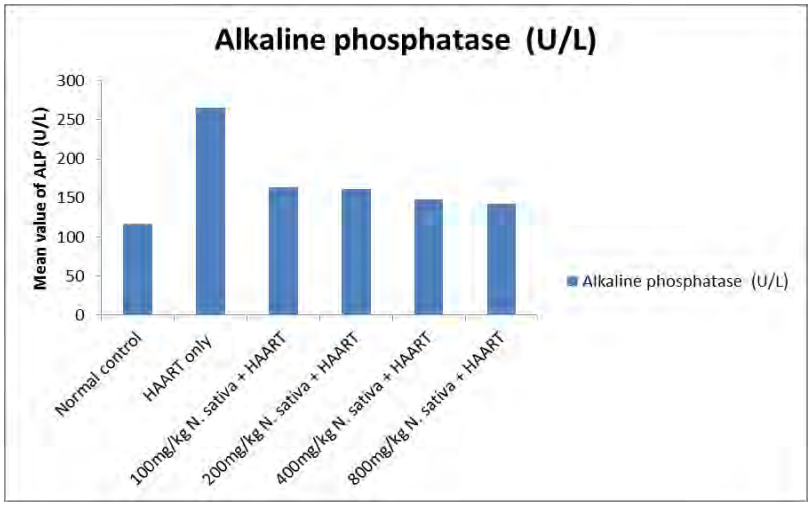

Figure 5.2: *Effect of N. sativa seed extract on the ALP value of HAART induced rats*

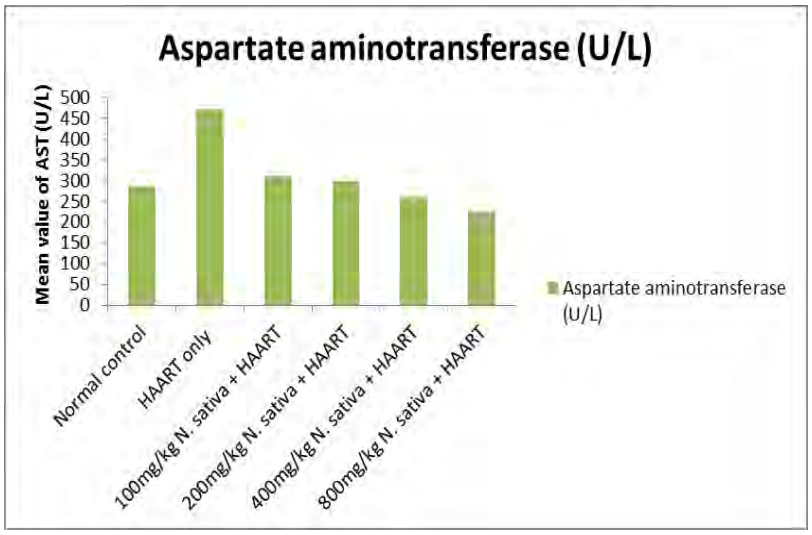

Figure 5.3: *Effect of N. sativa seed extract on the AST value of HAART induced rats*

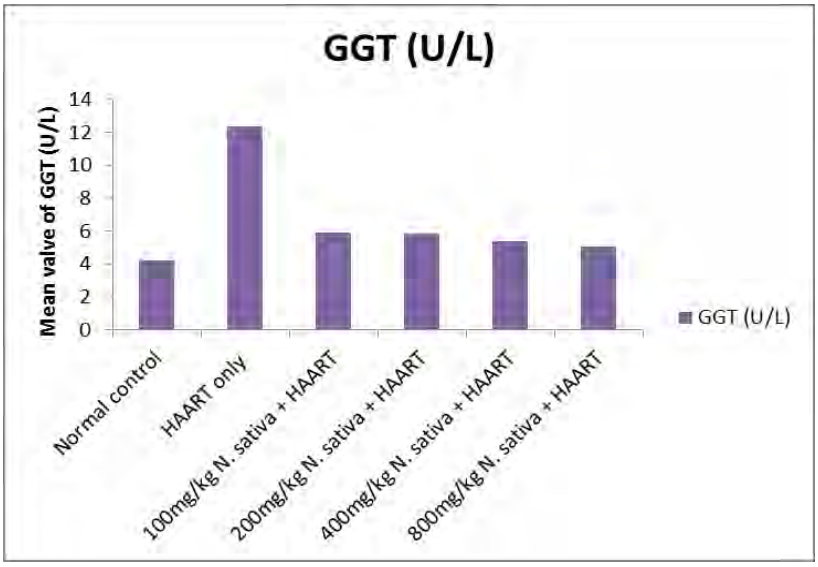

Figure 5.4: *Effect of N. sativa seed extract on the GGT value of HAART induced rats*

It is chiefly the plant based preparations which are employed for the treatment of liver disorders. A number of scientific reports indicated that certain flavonoids, triterpenoids and steroids have protective effect on liver due to its antioxidant properties (Parmar *et al.*, 2010).

According to Yahya *et al.*, (2013), flavonoids have been reported to exhibit antioxidant, anti-inflammatory, and hepatoprotective activities. Furthermore, condensed tannins have been suggested to possess free radical scavenging, antioxidant, anti-inflammatory, and

hepatoprotective activities, while saponins have been reported also to exhibit hepatoprotective activity via modulation of its antioxidant and anti-inflammatory activities. Hepatoprotective activity of medicinal plants may also be due to synergistic action of flavonoids, condensed tannins, and saponins.

Therefore; the hepatoprotective activity exhibited by *N. sativa* extract might be due to the anti-oxidative nature of the plant. The preliminary phytochemical analysis of the extracts in this study has also confirmed the presence of components such as flavonoids, tannins, saponins and phenolic compounds, which have been known for its antioxidant and hepatoprotective activities.

6. CONCLUSION

This study demonstrated that aqueous extract of *N. sativa* seed can protect against HAART induced acute hepatotoxicity through restoration of the anti-oxidative defense system and down-regulation of the pro-inflammatory pathway. Possible mechanism of hepatoprotective activity of *N. sativa* may be due to its free radical scavenging and antioxidant activity as the result of the presence of flavonoids, tannins and phenolic compounds in the extracts.

7. RECOMMENDATION

The present study demonstrated that aqueous extract of *N. sativa* seed has shown hepatoprotective activity against HAART administered rats. However;

- Hepatoprotective activity of *N. sativa* against chronic administration of HAART should be investigated.
- Characterization, purification and quantification of phytochemicals responsible for antioxidant activity of *N. sativa* should be conducted.
- Liver tests should be monitored closely in all patients commencing ART; throughout the course of ART therapy, liver chemistry tests should be monitored regularly.
- Preclinical and clinical trials should be conducted on HIV patients taking HAART

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