PROTECTIVE ROLE OF PROTOCATECHUIC ACID IN NEVIRAPINE-INDUCED HEPATOTOXICITY AND APOPTOSIS IN MALE WISTAR RATS

BY

ASEJEJE, FOLAKE OLUBUKOLA

B.Sc. Biochemistry (Ibadan), M.Sc. Pharmacology (Ibadan)

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ABSTRACT

Nevirapine is a Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) used in HIV-1 treatment. Although efficacious, it produces toxic responses such as hepatotoxicity which is characterised by generation of free radicals. Protocatechuic acid (3,4-dihydroxybenzoic acid, PCA), a phenolic antioxidant compound from edible plants is known to be hepatoprotective by mechanisms of action associated with inhibition of free radical generation, regulation of inflammation, and apoptosis. This study was designed to determine the protective role of PCA against nevirapineinduced hepatotoxicity.

Seventy-two male Wistar rats (150-170 g) were randomly assigned into six groups of twelve animals each. The animals were treated orally with distilled water alone (control), PCA (50.0 mg/kg), PCA (100.0 mg/kg), nevirapine alone (5.7 mg/kg), nevirapine (5.7 mg/kg) with PCA (50.0 mg/kg) and nevirapine (5.7 mg/kg) with PCA (100.0 mg/kg) daily for three weeks, respectively. Enzyme activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and hepatic levels of reduced glutathione (GSH) and malondialdehyde (MDA) were determined spectrophotometrically. The serum concentrations of tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), prostaglandin E_2 (PGE_2), caspase 3, caspase 9 and cytochrome C were assessed using ELISA. The expressions of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were measured by immunohistochemistry. Histology of liver was determined by microscopy and apoptosis by TUNEL assay. Data were analysed using Student t test and ANOVA at $p=0.05$.

Treatment with nevirapine alone caused significant elevations of serum activities of ALT (8.1±0.5 U/L) and AST (10.3±0.2 U/L) compared with control (3.2±0.7 and 8.1±0.8 U/L respectively). However, treatment with PCA (50.0 mg/kg and

100.0 mg/kg respectively) significantly lowered serum activities of ALT (7.5±0.7 and 6.4±1.3 U/L) and AST (9.0±2.9 and 9.5±1.7 U/L). Nevirapine lowered GSH level (0.4±0.1 mg/mL) compared with control (1.6±0.4 mg/mL) and significantly increased MDA level (366.0±39.0 nmol/g) compared with control (188.0±8.7 nmol/g). Treatment with PCA (50.0 mg/kg and 100.0 mg/kg respectively) however, caused significant increase in GSH (1.4±0.7 and 0.7±0.12 mg/mL) and decrease in MDA (245.0±20.2 and 262.9±9.0 nmol/g). Nevirapine elevated serum PGE_2 (56.0±5.2 ng/mL), TNF-α (20.3±1.4 ng/mL) and IL-1β (68.2±1.2 ng/mL) compared to control (37.9±4.6, 15.9±0.7 and 53.8±1.4 ng/mL respectively). Protocatechuic acid (50.0 mg/kg and 100.0 mg/kg respectively) significantly reduced serum PGE_2 (54.4±2.4 and 42.8±4.1 ng/mL), TNF-α (15.6±0.4 and 16.0±0.3 ng/mL) and IL-1β (59.5±2.0 and 55.3±1.0 ng/mL). Nevirapine induced COX-2 and iNOS expressions, increased serum caspase 3 (2.0±0.8 ng/mL) relative to control (1.5±0.3 ng/mL), and significantly elevated serum caspase 9 (159.0±3.0 ng/mL) and cytochrome C (215.0±51.2 ng/mL) compared to control (127.3±19.8 and 116.6±27.8 ng/L respectively). Protocatechuic acid (50.0 mg/kg and 100.0 mg/kg respectively) reversed the increased COX-2 and iNOS expressions, significantly lowered serum caspase 9 (108.0±37.0 and 112.3±18.5 ng/mL), cytochrome C (107.4±6.9 and 106.0±7.2 ng/mL) and caspase 3 (1.3±0.1 and 1.4±0.6 ng/mL). Nevirapine also induced hepatic apoptosis while protocatechuic acid at both concentrations reduced the apoptosis. Histology showed severe hepatic necrosis in nevirapine-treated group which was reduced with PCA treatment.

Protocatechuic acid ameliorated nevirapine-induced hepatotoxicity by its antioxidant, anti-inflammatory and anti-apoptotic properties.

Keywords: Nevirapine, Serum, Hepatotoxicity, Protocatechuic acid, Apoptosis

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CERTIFICATION

I certify that this work was carried out by ASEJEJE, FOLAKE OLUBUKOLA under my supervision in the Drug and Metabolism Unit of the Department of Biochemistry, University of Ibadan, Nigeria.

30/09/15

SUPERVISOR

EBENEZER OLATUNDE FAROMBI

Ph.D (Ibadan), MNYAS (USA), FRSC (UK), FATS (USA), FAS

Professor of Biochemistry and Molecular Toxicology,

Director, Drug Metabolism and Toxicology Unit,

Department of Biochemistry,

University of Ibadan

Date

DEDICATION

TO:

God Almighty who made all things, gave the dream and made it come true.

And

My beloved parents, Mr. and Mrs. E.L Olojede for encouraging me to pursue this dream.

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ALT	Alanine Aminotransferase
ALP	Alkaline Phosphatase
ART	Antiretroviral Therapy
AST	Aspartate Aminotransferase
CAT	Catalase
COX-2	Cyclooxygenase -2
DILI	Drug Induce Liver Injury
GCDC	Glycodeoxcholic Acid
GGT	Gamma Glutamyl Transferase
GPx	Glutathione Peroxidase
GSH	Reduced Glutathione
HIV-1	Human Immunodeficiency Virus Type-1
IL-1β	Interleukin 1-Beta
iNOS	Inducible Nitric Oxide Synthase
MPO	Myeloperoxidase
NVP	Nevirapine
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NO	Nitric Oxide
PCA	Protocatechuic Acid
PG -E2	Prostaglandin E-2
PC	Protein Carbonyl
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
SOD	Superoxide Dismutase
TA	Total Antioxidant Capacity
tBHP	Tert-butyl hydroperoxide
TPA	12-O- tetradecanoylphorbol-13- acetate
TNF-α	Tumor Necrosis Factor Alpha

CHAPTER ONE

INTRODUCTION

1.0 INTRODUCTION

Drug-induced liver toxicity is one of the most frequent reasons cited for the withdrawal of a previously approved drug from the market (European Medicines Agency, 2006). Drug toxicity has been a major limitation to drug development and therapy making it important to consider not only the therapeutic value of drugs but also their adverse effects or reactions.

A drug can cause liver toxicity *via* several mechanisms. For instance, it can be directly or indirectly acting through reactive metabolites. The drug or its metabolites may cause liver toxicity after specific receptor binding, or reacting with hepatic macromolecules leading to direct cytotoxicity. On the other hand, liver toxicity can be mediated *via* an immunological cascade (Blomhoff, 2005).

Increases in the serum levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in combination with increased bilirubin levels are usually considered to be the most relevant sign of liver toxicity (Akande *et al.*, 2007). Macroscopic and in particular histopathological observations allow confirmation of the occurrence of liver toxicity and provide further evidence of the type of liver damage. The absence of histological findings however, does not exclude liver toxicity (Akande *et al.*, 2007).

Antiretroviral therapy (ART) has proven to be highly effective in the treatment of human immunodeficiency virus (HIV) infection in industrialized countries (Palella *et al.*, 1998; Mocroft *et al.*, 2003) as well as in countries with limited resources (Zhou *et al.*, 2005; Wester *et al.*, 2005). While the benefits are well documented, ART also has significant toxicity that requires monitoring (French *et al.*, 2002; Colette *et al.*, 2005)

Hepatic toxicity is a common complication of anti-retroviral treatment in HIV patients, usually indicated by the elevation of liver transaminases measured in the serum (Akande *et al*., 2007; Anthony, 2001).

The toxicity of ART treatment is now widely accepted (Mae-Wan Ho, 2007). Many authors with different conclusions have evaluated the risk factors for hepatic toxicity associated with antiretroviral regimens and have demonstrated the toxicity of ARTs exemplified by elevated serum levels of liver enzymes (Carton *et al*., 1999; Ching-Lung *et al*., 1998; Akande *et al*., 2007) and elevated serum levels of bilirubin, which may either occur early or later in the course of therapy (Bellini *et al*., 2003).

Nevirapine, one member of the class of drugs known as non-nucleoside reverse transcriptase inhibitors (NNRTIs), prevents the non-nucleoside reverse transcriptase enzyme from functioning. This results in a reduced amount of the virus in the body and an increase in the CD4 cell (T cell) count, improving the host's immune function thereby reducing the risk of new and opportunistic infections, and death (Haroon, 2002).

Nevirapine has been widely used in adults as one of a combination of drugs to treat established HIV infection. It has a special role in the prevention of mother to child transmission of HIV, as it is effective when given alone as a single dose to the mother at the beginning of labour and one dose administered to the baby within 72 hours of birth. Nevirapine given to HIV-positive pregnant women rapidly crosses the placenta into the fetus with its effects lasting through the first week of life (Haroon, 2002).

Nevirapine was the first of the NNRTIs to be approved for clinical use. Early clinical trials found that its use as a monotherapy resulted in the rapid onset of resistance and hypersensitivity reactions (Carr and Cooper, 1996; Hammer, 2005). The use of Nevirapine has also been observed as a risk factor for transaminase elevation

under highly active antiretroviral therapy (Marina *et al*., 2003). Its use is associated with a relatively high incidence of skin rash. as well as life-threatening, liver toxicity, and the incidence of drug induced hypersensitivity reactions is higher when the drug is used for prophylaxis (Bersoff-Matcha *et al*., 2001).

Similar adverse events have been reported in health care workers taking Nevirapine in combination with other antiretroviral drugs for post-exposure prophylaxis after occupational exposure to HIV (Haroon, 2002). Umar *et al*., in 2008 also reported adverse hepatic effects associated with administration of Nevirapine, to albino rats.

Thus, Nevirapine is known to be associated with life-threatening liver toxicity. Although this hepatotoxic effect has been established, little or no information is available concerning the mechanisms underlying its hepatotoxicity and its effect on oxidative stress.

Among the mechanisms involved in hepatotoxicity induced by several xenobiotics is the oxidative damage due to free radical generation (Uma and Rao, 2005). Oxidative damage can accumulate in animal cells when the critical balance between generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and antioxidant defence is unfavourable. It has been established that such oxidative damage is involved in the pathogenesis of diseases and adverse reactions associated with drug usage (Blomhoff, 2005). This oxidative damage can be kept at manageable levels with the aid of antioxidants.

The study of numerous compounds that could be useful as antioxidants, ranging from α-tocopherol and β-carotene to other plant antioxidants such as flavones and tannins, has gained increasing interest in the fields of food and medicine. Protocatechuic acid (3, 4-dihydroxybenzoic acid, PCA), a simple phenolic antioxidant

compound, have been isolated from the dried flowers of *Hibiscus sabdariffa L.* (Malvaceae), is an ingredient of a local beverage and a Chinese herbal medicine used to treat hypertension, pyrexia and liver damage (Chen-Lan *et al.*, 2002).

Protocatechuic acid is a natural phenolic compound found in many edible and medicinal plants (Justyna, 2005). PCA has also been shown to have in vivo protective effect against tert-butyl hydroperoxide-induced rat hepatotoxicity (Chen-Lan *et al.*, 2002). In view of the hepatoprotective potential of PCA, its modulatory effect on the Nevirapine- induced liver toxicity was investigated in this study.

CHAPTER TWO

LITERATURE REVIEW

2.1 Drug-induced Hepatotoxicity

Liver damage caused by drug administration is known as drug induced hepatotoxicity or liver injury. The liver, an organ located between the absorptive surfaces of the gastrointestinal tract is central to the metabolism of virtually every foreign substance (Lee, 2003).

Most drugs are lipophlic in nature and this property enables them to cross the membranes of intestinal cells. However for these drugs to be metabolised and excreted from the system they must undergo hepatic biotransformation which renders them hydrophilic. This hepatic biotransformation involves oxidative pathways, primarily by way of the cytochrome P-450 enzyme system (Gucngerich, 2001). After further metabolic steps, which usually include conjugation to a glucuronide or a sulphate or glutathione, the hydrophilic product is exported into the blood or bile by transport proteins located on the hepatocyte membrane, and it is subsequently excreted by the kidney or the gastrointestinal tract (Lee, 2003).

This process of drug biotransformation leads to the generation chemically reactive metabolities which interact with cellular macromolecules such as proteins, lipids, and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress. Additionally, these reactive metabolites may induce disruption of ionic gradients and intracellular calcium stores, resulting in mitochondrial dysfunction and loss of energy production. This impairment of cellular function can culminate in cell death and possible liver failure (Holt and Ju, 2006).

2.1.1 Forms of drug induced hepatotoxicity

The predominant forms of drug-induced liver injury include acute hepatitis, cholestasis, and a mixed pattern (Gunawan and Kaplowitz, 2004). Acute hepatitis is defined as a marked increase in aminotransferases coinciding with hepatocellular necrosis. Cholestasis is characterized by jaundice with a concurrent elevation in alkaline phosphatase, conjugated bilirubin, and γ-glutamyl transferase. Mixed-pattern drug-induced liver injury includes clinical manifestations of both hepatocellular and cholestatic injury (Holt and Ju, 2006).

2.1.2 Key Biomarkers of drug induced hepatotoxicity

Aminotransferases

Aminotransferases are enzymes that catalyse reaction between an amino acid and a keto acid leading to the the production of aminocid. There are two types of aminotransferases; aspartate aminotransferase (AST) and alanine aminotransferase (ALT). AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) are widely distributed throughout the body. AST is found primarily in heart, liver, skeletal muscle, and kidney, whereas ALT is found primarily in liver and kidney, with lesser amounts in heart and skeletal muscle (Adolph *et al*., 1982; Jung *et al*., 1981; Wrobewski *et al*., 1958).

The AST and ALT activities in liver are 7000 - and 3000-fold higher than in the serum respectively (Ikui *et al*., 1986). ALT is exclusively cytoplasmic while both mitochondrial and cytoplasmic forms of AST are found in all cells (Rej, 1984). Liver disease is the most important cause of increased ALT and AST concentration in the serum. In most types of liver disease, ALT activity is higher than that of AST. Increased serum levels of ALT and AST directly reflect a major permeability problem or cell rupture (Benjamin, 1978; Umar et al., 2008).

Gamma-glutamyltransferase (GGT)

GGT (EC 2.3.2.2), a membrane-bound enzyme, is present (in decreasing order of abundance) in proximal renal tubule, liver, pancreas (ductules and acinar cells), and intestine (Miura *et al.*, 1981; Tate and Meiste.,1981; Jung and Wischke,1984) anchored to the cell surface through a small N-terminal transmembrane domain. It is one of the most widely used clinical indicators of liver damage and GGT assays form part of the routine screening procedures on blood or plasma (Nora *et al.*, 2008). GGT is an important enzyme in the metabolism of extracellular glutathione It is able to cleave the -glutamyl peptide bond in glutathione and other proteins and transfer the -glutamyl moiety to acceptors. GGT is also key to glutathione homeostasis because it provides the substrates for glutathione synthesis. GGT and glutathione are important to several organ systems such as the fetal liver, the kidney and the intestines (Ikeda and Taniguchi, 2005). GGT activity in serum comes primarily from liver and excessive increase of serum GGT concentration, is associated with liver damage.

Alkaline phosphatase

Alkaline phosphatase (ALP; EC 3.1.3.1) encompasses a group of heterogeneous enzymes that catalyze the hydrolysis of monophosphate esters at alkaline pH (Syakalima *et al.*, 1998). Serum ALP activity is used primarily as an indicator of hepatic disease. However, there are numerous non hepatic factors that result in an increase in serum ALP activity. Bone disease, endocrine disease, neoplasia and other disorders can result in increased alkaline phosphatase activity. In addition, alkaline phosphatase activity may be increased due to induction by certain drugs such as glucocorticoids and anticonvulsants (Nicole and Kidney, 2007).

Nuclear factor kappa-B (NF-kB)

The NF-kB family is one of the important dimeric transcription factor families (Trautwein et al., 1998). It consists of NF-kB1 (P50 and its precursor P105), NF-kB2 (P52 and its pre-cursor P100), RelA (P65), c-Rel (Rel) and RelB. RelA–NF-kB1 (P65–P50) is the most common dimer formed (Bacuerle and Batlimore, 1996).

Nuclear factor kappa-B (NF-kB) is a transcription factor involved in immune responses as well as inflammatory and cellular defence mechanisms (Siebenlist *et al.*, 1994). Downstream products of NF-kB activation include inflammatory cytokines such as tumor necrosis factor (TNF α), interleukin 1- beta, inducible nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX-2). Latest research has demonstrated relationship between reactive oxygen species (ROS) and NF-KB. *In vitro* studies have also shown a rapid activation of NF-kB after exposure of certain cell types to ROS (Cominacini *et al.*, 2004). Farombi *et al.*, (2009) also reported that the nuclear factor kappa B (NF-kB) plays a major role in dimethyl- nirosamine induced liver injury.

Several lines of evidence indicate that activation; translocation and binding of NF-kB are pivotal steps in the regulation of immune and pro-inflammatory cytokine genes (Bohrer *et al.*, 1997). Under resting conditions, NF-kB is sequestered in the cytoplasm through interaction with its inhibitor, IkB. When the cell is activated, the IkB protein is phosphorylated and degraded rapidly. NF-kB then undergoes rapid nuclear translocation and participates in the induction of numerous cellular genes (Baldwin, 1996).

NF-kB activation can be used as biomarker for assessing liver toxicity. Xue-Lian Ma and co-workers measured activation of NF-kB/degradation of inhibitor of kappa B alpha (IkBα), by western blot to demonstrate the role of (NF-kB) in concanavalin A-induced hepatitis (Xue-Lian Ma *et al.*, 2008).

Tumor necrosis factor alpha (TNF-α)

Tumor necrosis factor alpha (TNF-α) is a pleiotropic cytokine critically involved in inflammation and immunity. TNF-α, in conjunction with interleukin (IL)-6, regulates the acute-phase response, adhesion molecule activation, and antioxidant gene expression. TNF-α is perhaps the most critical and powerful mediator of inflammation, cellular injury, cell death/apoptosis, and wound healing (Edwards *et al*, 1994).

There appears to be a critical role for TNF-α in various models of hepatotoxicity. For instance, carbon tetrachloride (CCl_4)-induced hepatotoxicity is blocked by the administration of soluble TNF-α receptors (TNFRs) (Czaja *et al*., 1995). Liver repair following CCl_4 is mediated by TNF-α (Bruccoleri *et al*., 1997; Yamada and Fausto, 1998). In cadmium-induced hepatotoxicity, pretreatment with anti-TNF-α antibodies prevented focal inflammation as well as secretion of the acute-phase reactant, serum amyloid A (Kayama *et al*., 1995), indicating that these processes are cytokine-dependent.

Similarly, neutralizing antibodies to TNF-α delayed increases in serum levels of IL-1α and liver enzymes as well as shortened the recovery time following acetaminophen treatment (Blazka *et al*. 1995). Acute inflammatory responses to 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin were mimicked by the administration of exogenous IL-1β and TNF-α (Moos *et al*., 1994). Taken together, these data suggest that TNF-α contributes to pathological manifestations of chemical-induced liver damage.

Inducible nitric oxide synthase iNOS and nitic oxide (Nitric oxide)

Inducible nitric oxide synthase is an inducible member of the three nitric oxide synthase isoforms (endothelial nitric oxide synthase [eNOS], neural nitric oxide synthase [nNOS] and iNOS). They catalyze the oxidation-reduction reaction of L-arginine in the presence of oxygen to form nitric oxide (NO) and L-citrulline.

Nitric oxide is a highly reactive oxidant produced by parenchymal and non-parenchymal liver cells (Geller *et al.*, 1993; Laskin *et al.*, 1994). Under normal conditions, only the constitutive eNOS is present in the liver, and low levels of NO regulate hepatic perfusion (Li *et al.*, 1999). Under pathological conditions, however, iNOS is strongly up regulated and large amounts of NO are generated in the liver. It has been demonstrated that both NFkB and inducible nitric oxide synthase (iNOS) expression increase during liver injury.

There are also NF-kB binding sites in the iNOS promoter (Ganster *et al.*, 2001). NF-kB is therefore important in the regulation of human-inducible nitric oxide synthase (iNOS) transcription in A549 human lung epithelial cells (Chu *et al.*, 1998).

Cyclooxygenase- 2 (COX-2)

Cyclooxygenase -2 is an enzyme involved in inflammatory processes and the rate limiting enzyme in prostaglandin biosynthesis from arachidonic acid. COX-2 has been implicated in liver inflammation and fibrosis (Raquel *et al.*, 2008). Inappropriate up regulation of COX-2 has been frequently observed in various premalignant and malignant tissues (Mohan and Epstein, 2003). Like other early response gene products, COX-2 can be induced rapidly and transiently by pro-inflammatory mediators, endotoxins as well as carcinogens (Kim *et al.*, 2005).

COX-2 inhibitors have been shown to possess hepatoprotective effect. Farombi *et al.*, (2009) demonstrated that kolaviron inhibits dimethyl nitrosamine- induced liver injury by suppressing COX-2 expression.

2.1.3 Mechanisms of drug-induced hepatotoxicity

A review article (Lee. 2003) has outlined 6 basic mechanisms of drug induced liver injury. These include:

1. Free radical induced oxidative stress and metabolic bio-activation of chemicals
2. Autoimmunity and inflammation
3. Apoptosis
4. Mitochondrial injury.
5. Disruption of calcium homeostasis leading to cell surface blebbing and lysis
6. Canalicular injury.

2.1.3.1 Free radical and drug metabolite induced injury

The cytochrome p450 system comprise of a family of enzymes located in the smooth endoplasmic reticulum (SER). Although they are also found in many other cells of the body they are majorly found in the liver cells. A major role of this enzyme system is to metabolize lipid soluble chemicals (drugs and other xenobiotics) into more water soluble forms to enhance their excretion from the body via bile or urine (Weinshilboum. 2003).

This biotransformation process is typically in two steps; namely phases 1 and 2. In the phase 1 reaction xenobiotics are bioactivated to reactive intermediates, in preparation for the phase 2 reaction (Guengerich. 2001). The phase 2 involves majorly conjugation of phase1 products to polar molecules (such as glucuronic acid) via covalent bond formation forming a more soluble metabolite which is easily excreted.

The production of injurious metabolites by cytochromic p450 system is perhaps the most frequent mechanism of hepatocellular injury due to the generation of reactive metabolite and free radicals. For instance, carbon tetrachloride is metabolized by the cytochrome P450 system to trichlormethyl radical (CCl_3), a free radical that

induces cell membrane injury. Lesions induced by carbon telachloride are most severe in the periacinar (centrilobular) areas. because this is the area where the smooth endoplasmic reticulum is most abundant, and, therefore, where the active form of the chemical is present in greatest concentration. Consequently. the centrilobular region of the hepatic lobule is by far the most common site of acute toxic injury.

Acetaminophen toxicity is another and more commonly encountered example of this mechanism of liver injury (Zhang *et al.*, 2002). Adducts can also form between bioactivated compounds and nucleic acids. Adducts formed with DNA are more likely to lead to long-term consequences such as neoplasia, but adducts formed with RNA can interfere with protein synthesis and lead to acute hepatic toxicity (John, 2005).

Cytochrome P450 2E1 (CYP 2E1), the ethanol-inducible form, metabolizes and activates many toxicologically important substrates, including ethanol, carbon tetrachloride, acetaminophen, and N-nitrosodimethylamine, to more toxic products (Guengerich *et al.*, 1990; Koop, 1992). CYP 2E1-dependent ethanol metabolism produces oxidative stress through generation of reactive oxygen species (ROS), a possible mechanism by which ethanol is hepatotoxic (Bondy, 1992; Dianzani, 1985).

2.1.3.2. Autoimmunity and inflammation

In addition to direct damage of cellular protein and nucleic acid, adduct formation i.e. formation of drugs covalently linked to enzymes can lead to immune-mediated liver injury. Adducts that are large enough to serve as immune target may migrate to the surface of the hepatocyte, where they can induce the formation of antibodies (antibody-mediated cytotoxicity) or induce direct cytolytic T-cell responses (Robin *et al.*, 1997).

The cytokine response is also evoked and this may cause inflammation and additional neutrophil-mediated hepatotoxicity (Jaeschke et al., 2002). This process has

been demonstrated with several drugs that form adducts with the cytochrome P450 isoforms that are involved in their metabolism, including tienilic acid and dihydralazine (Lewis, 2000). There is growing evidence that the initial NAPQI (N –acetyl-parabenzoquinone imine)-induced hepatocyte damage may lead to activation of innate immune cells within the liver, thereby stimulating hepatic infiltration of inflammatory cells (Ishida *et al.*, 2002).

It has been demonstrated that various inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-1β, (Blazka *et al.*, 1995; Blazka *et al.*, 1996 Ishida *et al.*, 2002) and proinflammatory enzymes like cyclooxygenase -2 and inducible nitric oxide synthase produced during drug-induced liver damage are involved in promoting tissue damage.

2.1.3.3 Apoptosis

Apoptosis, a form of organized cell death (Rust and Gores, 2000), is characterized by cell organelles and cell membrane fragmentation into small membrane bound bodies resulting in cell death. Classically apoptosis can be initiated through two basic mechanisms in the hepatocyte interactions between death ligands (Fas-ligand, Tumor Necrosis Factor (TNF)) and death receptors (Fas and Tumor Necrosis Factor Receptor (TNFR-1)) that trigger caspase 8 activation or damage to mitochondrial inner membranes releasing cytochrome c that binds to Apaf-1 activating it, leading to downstream activation of caspase 9. These pathways are not completely separate as a protein named Bid that is activated by caspase 8 can cause mitochondria to release cytochrome c (John, 2005).

Programmed cell death (apoptosis) can occur in concert with immune-mediated injury, destroying hepatocytes via the TNF and the Fas pathways, with cell shrinkage and fragmentation of nuclear chromatin (Reed, 2001). Certain chemicals

may be able to trigger apoptosis by direct stimulation of the pro-apoptotic pathways in hepatocytes. On the other hand, other pathways including immune-mediated events can lead to release of TNF or activate the Fas pathways, and cholestasis is also known to stimulate apoptosis through action of pro-apoptotic bile acids such as glycodeoxcholic acid (GCDC). Similarly, chemicals that damage mitochondria can also initiate apoptosis via release of cytochrome c (Bissel *et al.*, 2001; Jaeschke *et al.*, 2002).

In addition, bioactivation by the cytochrome P450 system can produce reactive molecules that engender oxidative stress which can then be a stimulus to induce synthesis of Fas ligand and increase the susceptibility of hepatocytes to apoptosis (Lee, 2003).

2.1.3.4 Mitochondrial injury

Damage to the mitochondria can be very inimical to the health of any cell because the mitochondrion is the energy or power house of the cell. Chemicals that damage mitochondrial structure, enzymes or DNA synthesis can disrupt β-oxidation of lipids and oxidative energy production within the hepatocytes (Fromenty and Pessayre, 1995; Pessayre *et al.*, 1999; Bissel *et al.*, 2001).

Some drugs may inhibit β-oxidation (asprin, valproic acid, tetracyclines) and others may disrupt oxidative phosphorylation alone or in addition (bile acids, amiodarone) depleting the hepatocytes of energy. Certain antiviral dideoxynucleoside analogues can disrupt mitochondrial DNA synthesis through inhibition of DNA polymerase gamma, leading to depletion of mitochondrial DNA and mitochondria leading to hepatocyte death (John, 2005). Some other drugs induce liver injury by causing the opening of the permeability pore (PT).

2.1.3.5 Disruption of calcium homeostasis.

In healthy cells the intracellular free calcium in maintained at lower concentration than the extracellular concentration and most of the intracellular calcium is sequestered within the endoplasmic reticulum or mitochondria. The integrity of membranes within and surrounding the cell as well as the balance of intracellular ions are maintained by a variety of energy consuming processes involving the Ca^{2+} and Mg^{2+}- ATPases.

Drug-induced damage to these cellular proteins that are involved in ion balance can lead to an influx of calcium that disrupts, among other processes, normal actin filament assembly and disturbs ATP production. The resulting dispersal of the cytoskeletoo leads to blebbing of the cell membranes. Also, irreversible cell injury and cell lysis can occur if cell the membrane disruption is of sufficient magnitude (John, 2005).

It has been demonstrated that bioactivation of certain drugs (or chemical compounds) by the cytochrome p450 system produce reactive intermediates that can bind to various cellular proteins (including those involved in ion balance) leading to cellular dysfunction. (Watanabe and Phillips, 1986)

2.1.3.6. Canalicular and cholestatic injury

Any chemical that can damage the structure and function of the bile canaliculi will produce cholestasis (i.e. arrest of bile formation). Several drugs have been identified that can lead to cholestasis (Lewis, 2000).

Bile secretion depend majorly on the function of a series of ATP-dependent export pumps that moves bile salts; and other transporters that export other bile constituents from the hepatocyte cytoplasm to the lumen of the canaliculus. It is known that some drugs (or there bioactivation products) bind these transporters and thus

impede or arrest bile formation or movement within the canalicular system (Trauner *et al*., 1998) resulting in cholestasis. It is worthy of mention that secondary injury can also result from the detergent action of bile salts on cell membranes and biliary epithelium or hepatocytes during cholestasis.

2.2 Antioxidants

According to Halliwell and Gutteridge (1989), an antioxidant is any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or inhibits oxidation of that substrate. When there is an imbalance between the antioxidant system and reactive oxygen species generation, and this imbalance does not favour the antioxidant defense system, a condition known as oxidative stress sets in.

2.2.1 Classification of antioxidants

Synthetic and/or natural compounds with antioxidant functions have been used commercially, to minimize the negative effects of oxidative damage. Two broad groups of antioxidants exist:

(a) Synthetic Antioxidants

(b) Biological Antioxidants

(a) Synthetic Antioxidants

These are artificial or synthetic, non-natural antioxidants. Examples of these synthetic antioxidants include propyl gallate, butylated hydroxytoluene (BHT), and butylated hydroxyl anisole (BHA). Often times these compounds are used as food additives. However, a lot of these compounds were found to be carcinogenic or toxic, which excluded them from use in the food industry (Krasowka *et al*., 2001).

The formerly used phenolic antioxidant BHT has been withdrawn from use because in cells it is modified to carcinogenic chinone derivatives. New, non-toxic

antioxidants are very extensively sought after, on account of their potential application in the food industry or human health management. For example two antioxidants which are quaternary ammonium salts have been synthesized and studied (Krasowka *et al.* 2001) -quaternary ammonium salt pyrrolidinethyl esters of 3,5-di-t-butyl-4hydroxy-dihydrocinnamic acid (PYA-n) and quaternary ammonium salt of dimethyl aminoethyl esters of 3,5-di-t-butyl-4-hydroxy-dihydrocinnamic acid (PPA-n), with varying number of carbons (n) in the alkyl chain.

(b) Biological Antioxidants

Antioxidants that fall in this group could either be enzymic or naturally occuring. In healthy individuals, the antioxidant system defends tissues against free radical attack. Three classes of biological antioxidants have been identified:

(i) Primary Antioxidants include: Catalase, Superoxide dismutase (SOD), Glutathione peroxidase, Ceruloplasmin, Transferrin, Ferritin. These prevent the formation of new free radical species.

(ii) Secondary Antioxidants include: Vitamin E, β-carotene, Uric acid, Bilirubin, Albumin. These remove newly formed radicals before they can initiate chain reactions that can lead to cell damage and further free radical formation.

(ni) Tertiary Antioxidants include DNA repair enzymes, Methionine, Sulphoxide reductase. These repair cell structures damaged by free radical attack.

Plant materials have been explored severally as source of antioxidants Extracts from different types of teas especially those obtained from natural sources; plants to be specific have been subjected to a number of antioxidant studies (Yen *et al*., 1995; Von Gadow *et al*., 1997; Lanping et al, 2000). Polyphenols from different plant sources have been characterized and quantified in antioxidant studies.

2.2.2 Mode of Action of antioxidants

Mode of action of antioxidants can be via any of these four routes:

(i) Chain-breaking reactions e.g. α-tocopherol which acts in lipid phase to trap free radicals.

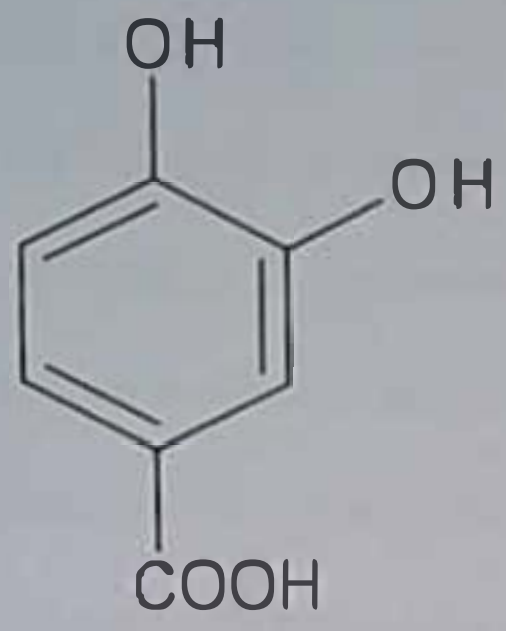
(ii) Reducing the concentration of reactive oxygen species (ROS) e.g. glutathione.

(iii) Scavenging initiating free radicals e.g. superoxide dismutase which acts in the aqueous phase to trap superoxide free radicals.

(iii) Chelation of transition metal catalysts. A group of compounds elicits antioxidant function by sequesteration of transition metals that are well-established pro-oxidants. In this way, tranferrin, lactoferrin, and ferritin function to keep iron-induced oxidant stress in check and ceruloplasmin and albumin act as copper sequestrants.

2.3 Protocatechuic acid

The study of numerous compounds that could be useful as antioxidants, ranging from α-tocopherol and β-carotene to plant antioxidants such as flavones and tannins, has gained increasing interest in the fields of food and medicine. Protocatechuic acid (3, 4-dihydroxybenzoic acid, PCA) is a natural phenolic compound found in many edible and medicinal plants (Justyna, 2005). PCA, a simple phenolic antioxidant compound, has been isolated from the dried flowers of Hibiscus subdariffa L. (Malvaceae), an ingredient of a local beverage and a Chinese herbal medicine used to treat hypertension, pyrexia and liver damage (Chuen-Lan *et al*., 2002).



Protocatechuic Acid (PCA)

Figure 1A

Studies indicate that it could be used as a protective agent against cardiovascular diseases and neoplasms, PCA has been shown to have strong antioxidant and antitumor promoting effects (Tseng *et al*., 1996, 1998) and induces apoptosis in HL-60 human leukemia. Besides that, PCA has been shown to possess chemopreventive activity by inhibiting the carcinogenic action of various chemicals in different tissues, such as diethylnitrosamine in liver (Tanaka *et al*., 1993), 4-nitroquinoline-1-oxide in the oral cavity (Tanaka *et al*., 1994), azoxymethane in the colon, N-methyl-N-nitrosourea in the glandular stomach tissue (Tanaka *et al*., 1995) and N-butyl-N- (4-hydroxybutyl) nitrosamine in the bladder. PCA has also been shown to have in vivo protective effect on tert-butyl hydroperoxide-induced rat hepatotoxicity (Chuen-Lan, *et al*, 2002).

2.3.1 Mechanisms of chemoprevention of PCA

Chemopreventive action of protocatechuic acid is primarily due to of its antioxidant properties. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) may damage or modify macromolecule which may lead to mutation and disrupuon in the signaling pathways in the cell with consequently the development of cancer (Klaunig and Kamendulis, 2004).

Several studies using in vitro cellular system and ROS generation have shown that protocatechuic acid inhibits both the formation (including the highly reactive hydroxylradical), and the scavenging of free radicals (Yen *et al*., 2000; Yan *et al*., 2004). Inhibition of the formation of free radicals is associated with the ability of protocatechuic acid to form complexes with transition metal ions; Cu (II) and Fe (II), or lowering the activity of enzymes, such as xanthine oxidase catalyzing reactions in the course of which these radicals are formed (Panoutsopoulos *et al*., 2005; Schmeda-Hirschmann *et al*., 2004).

The neutralization of free radicals is the result of their reaction with hydroxyl groups of protocatechuic acid. In vitro models showed that protocatechuic acid prevents oxidative DNA damage and lipid peroxidation (Yoshino and Murakami, 1998; Valentova *et al*., 2003). It has also been shown to affect different oxidative stress biomarkers like glutathione, glutathione peroxidase and glutathione reductase by restoring their levels near to control levels in several in vitro models (Masella *et al*., 2004).

Protocatechuic acid at concentrations 0.02-0.1 mg/ml, and 50 and100 mg/kg prevented the undesirable consequences of oxidative stress in the primary culture of rat hepatocytes and in liver of rats exposed to tert-butyl hydroperoxide (tBHP) (Chen-lan *et al*., 2002; Tsuda et al., 1999). These findings demonstrated that protocatechuic acid reduced the cytotoxicity of t-BHP. Protocatechuic acid has been shown to reduced the inflammation caused by the administration of 12-O- tetradecanoylphorbol-13- acetate (TPA), inhibited the production of hydrogen peroxide (H_2O_2), and decreased the activity of myeloperoxidase in the skin (Tseng *et al*., 1998). However, protocatechuic acid, like many other well-known antioxidants, may exhibit pro-oxidant action under certain conditions (Sroka et al., 2003). Protocatechuic acid works as an antioxidant at

low concentrations, whereas at high concentrations, it exhibits pro-oxidant properties (Nakamura *et al.*, 2000).

The chemopreventive action of protocatechuic acid is also linked to its effects on the metabolism of carcinogens. The process involves two groups of enzymes: Phase I and Phase II of drug metabolism enzymes (Baer-Dubowska *et al.*, 1998; Ignatowicz *et al.*, 2003; Szaefer *et al.*, 2003). Protocatechuic acid not only affects the activities of enzymes involved in the metabolism of carcinogens, but also neutralizes reactive intermediate metabolites, thereby preventing their binding to DNA. Blocking the DNA binding site of carcinogens by protocatechuic acid is likely to prevent DNA mutations and tumor initiation (Ignatowicz *et al.*, 2003).

Research have shown that protocatechuic acid possesses antiproliferative action on several human cell lines, including immortalized breast cells HBL 100, breast cancer cells T47D, gastric adenocarcinoma cells MKN45, lung cancer cells PC14, and promyelocytic leukemia cells HL-60 (Kampa *et al* 2004; Tseng *et al* , 2000).

Interestingly, protocatechuic acid suppresses the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) (Cichocki *et al*, 2010) and tumor necrosis factor (TNF) (Zhou-Stache *et al*. 2002) which are involved in carcinogenesis and/or inflammation.

Protocatechuic acid also affects apoptosis to eliminate damaged and neoplastic cells (Lin *et al* , 2007; Yin *et al*., 2009). Protocatechuic acid also inhibited hepatocyte apoptosis induced by TNF- α in rodent *in vivo* study (Yen and Hsieh, 2000, Lin *et al*., 2007, Morikawa *et al* , 1999).

Therefore, the mechanisms of chemoprevention of PCA is associated with its antioxidant activity which includes: inhibition of free radicals generation, scavenging free radicals, upregulation of antioxidant enzymes, influence on Phases I and II

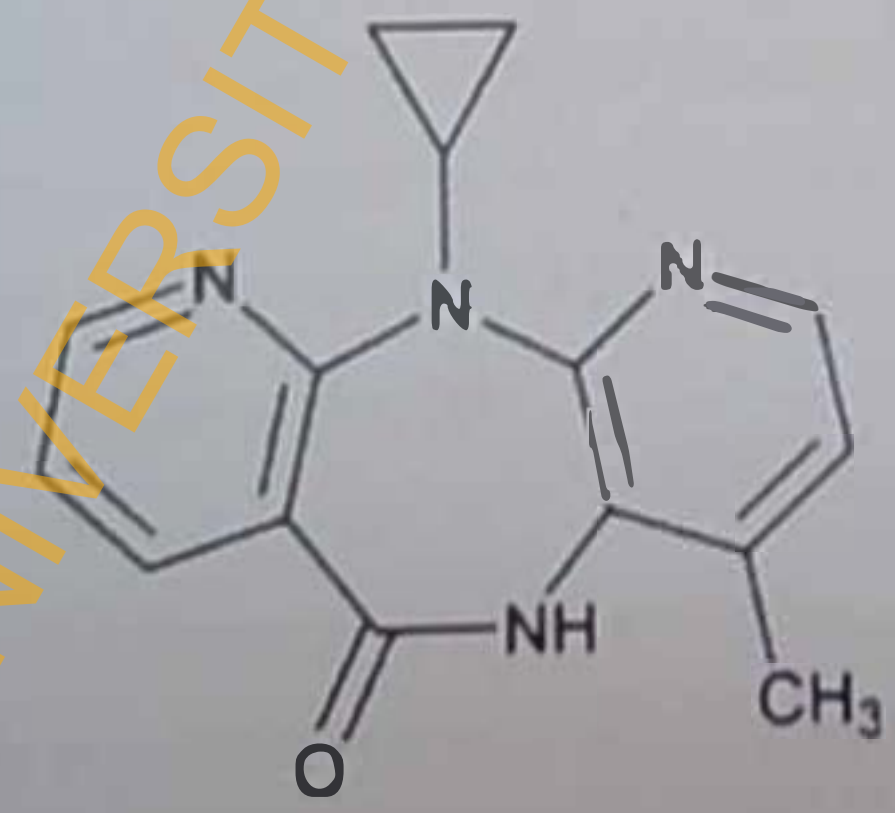
xenobiotic metabolising enzymes and suppression of the expression of proinflammatory mediators like cyclooxygenase- 2 inducible nitric oxide synthase, tumor necrosis factor, and interleukin 1- beta (Tanaka *et al*., 2011).

2.4 Nevirapine

Nevirapine (NVP) belongs to a class of drugs known as non-nucleoside reverse transcriptase inhibitors (NNRTIs). These drugs prevent the enzyme reverse transcriptase (RT) from functioning by binding directly to it and blocking its RNA- and DNA-dependent DNA polymerase activities thereby causing a disruption of the catalytic site of the enzyme (Merluzzi *et al*., 1990; Richman *et al*., 1991).

Nevirapine does not compete with template or nucleoside triphosphates. HIV-2 RT and eukaryotic DNA polymerases (such as human DNA polymerases α, β, γ, or δ) but it is specific for HIV -1.

Nevirapine is structurally a member of the dipyridodiazepinone chemical class of compounds. It is a white to off-white crystalline powder with the molecular weight of 266.30 and molecular formula $C_{15}H_{14}N_4O$.



Structural Formula of Nevirapine

NVP was the first NNRTI widely introduced in clinical practice and is regarded to be a potent (Murphy *et al*., 1999) and an effective component of highly active antiretroviral therapy (HAART) used in the treatment of HIV - 1 (Carr *et al*., 1996; D'Aquila *et al*.,1996; Harris *et al*., 1998; Montaner *et al*., 1998). Animal studies have shown that nevirapine is widely distributed to nearly all tissues, and readily crosses the blood-brain barrier. Its good penetrating ability into the central nervous system (CNS) makes it an attractive option for patients with HIV-associated CNS disease (Yazdanian *et al*., 1999).

2.4.1 Pharmacology

2.4.1.1 Dosing

Due to its long half-life, NVP can be given as part of a twice-daily dosing regimen making a total of 400mg/day (Cheeseman *et al*., 1995; Miller *et al*., 1997). The drug is available as tablets and oral suspension.

2.4.1.2 Adverse effects

The most common adverse effect of NVP is the development of mild or moderate rash in 32 to 48 % of patients (Havlir *et al*., 1995; Carr *et al*., 1996). Severe or life-threatening skin reactions have been observed in patients, including Stevens-Johnson syndrome, which is a toxic epidermal necrolysis and hypersensitivity reaction (Murphy and Montaner, 1996). NVP has been shown to cause severe or life-threatening liver toxicity (González de Requena *et al*., 2002).

2.4.1.3 Drug Interactions

In vivo studies in humans and in vitro studies with human liver microsomes have shown that Nevirapine induces the cytochrome P-450 enzymatic system (Sahai *et al*., 1997, Murphy *et al*., 1999). Evidence has shown that rifampicin decreases serum NVP concentrations (Burman *et al*., 1999; Pozniak *et al*., 1999).

2.4.1.4 Pharmacokinetics

Absorption and distribution

Nevirapine is well absorbed orally with > 90 % bioavailability, distributes well to all tissues, and is approximately 60 % bound to plasma proteins (Murphy and Montaner, 1996).

Metabolism

NVP is extensively biotransformed via cytochrome P450 through oxidative metabolism to several hydroxylated metabolites namely 2- and 3-hydroxyNVP glucuronide (Riska *et al.*, 1996; Riska *et al.*, 1999). In vitro studies with human liver microsomes suggest that oxidative metabolism of Nevirapine is mediated primarily by cytochrome P450 (CYP) isozymes from the CYP3A4 and CYP2B6 families, although other isozymes may have a secondary role.

Excretion

Renal excretion is the primary mode of elimination, accounting for 81.3 ± 11.1 % of the radio-labeled dose compared with faeces (10.1 ± 1.5 %). Excretion of the NVP parent compound in urine represented approximately 2.7 % of the dose (Riska *et al.*, 1999).

2.4.2 Nevirapine and genotoxicity

Nevirapine (NVP) has been shown to cause hepatocellular adenomas and carcinomas (Physicians' Desk Reference USA, 2009). While the reasons for the adverse effects of NVP are still unclear, several in vitro and in vivo data are consistent with the involvement of NVP bioactivation, via Phase I oxidation to 12-hydroxy-NVP and subsequent Phase II sulfonation to 12-sulfoxy-NVP in the onset of toxic events elicited by the parent drug (Popovic *et al*., 2006; Wen *et al*., 2009; Chen *et al*., 2008; Srivastava *et al*., 2010). Thus Phase II metabolite is a reactive electrophile, and

therefore expected to react directly with bionucleophiles (e.g., DNA) yielding covalent adducts. Antunes and co- workers (2013) showed that nevirapine metabolite has a potential of forming DNA adduct and could be genotoxic. There is a dearth of peer review article concerning the genotoxic potential of nevirapine.

2.5 Rationale, Aims and Objectives

Nevirapine is an important antiretroviral drug and vital in the management of HIV patients especially in the developing world owing to the fact that it is mostly prescribed. The high efficacy of the drug, favourable lipid profile (Ruiz *et al*., 2001) and suitability for use during pregnancy (Haroon, 2002) together with low cost (Marseille *et al*., 1999) have granted NVP-based regimens a significant role in HIV-1 treatment strategies.

In spite of the obvious clinical benefits of this drug, induction of life-threatening liver toxicity, drug induced hypersensitivity, (Marina, 2003; Bersoff-Matcha *et al*, 2001) and increased incidences of hepatoneoplasias in rodents (Physicians' Desk Reference USA, 2009) are major factors that put patients receiving Nevirapine therapy at risk.

Despite substantial evidence that the use of the drug is associated with adverse reactions, very little information is available concerning the mechanisms underlying these reactions. Among the little information available, is the current evidence that shows the involvement of metabolic activation to reactive electrophiles in NVP toxicity (Antunes *et al*., 2013). The generation of reactive metabolite could lead to oxidative damage and it has been established that such oxidative damage is involved in the pathogenesis of diseases and adverse reactions associated with drug usage (Ilhamhoff, 2005). This oxidative damage can be kept at manageable levels with the aid of antioxidants.

Studies indicate that a natural phenolic compound, protocatechuic acid (PCA - 3, 4 -dihydroxybenzoic acid), present in many edible and medicinal plants could be protective against the development of epithelial malignancy in different tissues as well cardiovascular diseases (Tanaka *et al.*, 2011). PCA has also been shown to have in vivo protective effect against tertbutyl hydroperoxide-induced rat hepatotoxicity (Chen-Lan *et al.*, 2002).The mechanism of its action is mostly associated with antioxidant activity, including inhibition of generation as well as scavenging of free radicals, upregulation of antioxidant enzymes, influence on Phases I and II xenobiotic metabolising enzymes and suppression of the expression of proinflammatory mediators like cyclooxygenase- 2, inducible nitric oxide synthase, tumor necrosis factor, and interleukin 1- beta (Tanaka *et al.*, 2011).

In view of the aforementioned, this study seeks to:

- Study the relationship between nevirapine -induced liver damage and duration of exposure to the drug.
- Investigate the relationship between nevirapine-induced liver toxicity and oxidative stress.
- Investigate whether the stimulation of inflammatory response is involved in the mechanism of nevirapine- induced hepatotoxicity.
- Investigate whether the stimulation of apoptotic response is involved in the mechanism of nevirapine- induced hepatotoxicity.
- Investigate the modulatory effect of protocatechuic acid (PCA) on nevirapine -induced hepatotoxicity and oxidative stress.
- Investigate the genotoxicity of nevirapine using micronuclei assay.
- Investigate the modulatory effect of protocatechuic acid (PCA) on nevirapine induced genotoxicity.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals

Nevirapine(R) manufactured by Aurobindo Pharma Limited India, protocatechuic acid, 1-chloro-2,4-dinitrobenzene (CDNB), 5',5'-dithiobis-2-nitrobenzoic (DTNB), reduced glutathione (GSH), adrenaline, hydrogen peroxide, sodium acetate, trichloroacetic (TCA), ferrous sulphate, potassium dichromate, glacial acetic acid, folin- Ciocalteau reagent were all obtained from Sigma Aldrich incorporated, USA.

Tumor Necrosis Factor Alpha (TNFα), interleukin 1 β, prostaglandin E-2, caspase 3, caspase 9, p53, cytrochrome C protein ELISA kits were obtained from Cusabio Biotech Company, China.

Goat polyclonal anti COX-2 antibody and rabbit polyclonal anti-iNOS antibody from Santa Cruz Biotechnology Inc. USA. Conjugated secondary antibodies obtained from Vector Labs, USA.

Alanine amino transferase, asparatate amino transferase, bilirubin, alkaline phosphate and gamma glutamyl transferase assay kits obtained from Randox incorporated, UK.

Sodium chloride, sodium hydroxide, sorbitol, amino ferrous sulphate, potassium dihyrogen phosphate, dipotassium hydrogen phosphate, ethanol, and hydrogen chloride used were of analytical grade.

3.2 Methods

3.2.1 Preparation of Serum and Post Mitochondrial Fraction (PMF)

The rats were sacrificed by cervical dislocation: blood was collected by cardiac puncture technique with the aid of clean needle and syringe into clean dry centrifuge tubes and allowed to coagulate by standing for 30 mins. The blood samples were then centrifuged for 10 mins at 3000 g using a bench centrifuge. The clear supernatant (serum) was collected and stored in the refrigerator 4° C.

Liver was quickly removed, rinsed in ice-cold 1.15% KCl, blotted and weighed. Then minced with scissors in 4 volumes/weight of ice-cold 0.1M phosphate buffer pH 7.4 and homogenized using Potter-Elvehgen homogenizer. The homogenate was centrifuged at 10,000g for 15 minutes at 4°C and the supernatant termed post mitochondrial fraction (PMF) were aliquoted and used for the enzymes assays.

3.2.2 Preparation of Samples used for Immunohistochemistry

Briefly, liver sections were immersed in 4% phosphate buffer formalin, dehydrated in graded alcohol and embedded in paraffin. Fine sections were obtained, mounted on salinized glass slides.

3.2.3 Preparation of Samples used for Histology

Liver samples were fixed in 10% formaldehyde, dehydrated in graded alcohol and embedded in paraffin. Fine sections were obtained, mounted on glass slides and stained with haematoxylin and eosin for light microscopic analyses.

3.2.4 Preparation of Samples used for Tunel Assay

Briefly, liver sections were immersed in 4% phosphate buffer formalin, dehydrated in graded alcohol and embedded in paraffin. Fine sections were obtained, and mounted on albuminized glass slides

3.2.5 Preparation of Samples used for Micronuclei Assay

The method of Schmidt (1975) was employed. Bone marrow was flushed out of the femur of each rat and spread onto slides. Slides were then air-dried, fixed and stained with maygrunward stain. Bone marrow cells were then examined microscopically and scored per animal for micro-nucleated polychromatic erythrocytes (PCE).

3.2.6 Determination of Protein Concentration

Protein concentration of the various samples was determined using the Biuret method as described by Gornal *et al.*, (1949) with slight modifications. Potassium iodide was added to the Biuret reagent to prevent precipitation of Cu^{2+} ions.

Principle

The assay is based on the reaction of Cu^{2+} and protein under alkaline condition to form a blue coloured complex with maximum absorbance at 540nm. This procedure is usually calibrated with a bovine serum albumin BSA standard curve.

Reagents

- **0.9% NaCl (Normal Saline)**

2.7 g of NaCl was dissolved in distilled water and made up to 300 ml.

- **0.2M Sodium Hydroxide (NaOH)**

8 g of NaOH was dissolved in distilled water and made up to 1 litre.

- **Stock Bovine Serum Albumin Standard**

0.1g of BSA dissolved in 100ml of normal saline to give a final concentration of 1mg/ml.

- **Biuret Reagent**

3 g of $CuSO_4.5H_2O$ and 9 g of sodium potassium tartarate dissolved in 500 ml of 0.2 M NaOH. 5 g of potassium iodide was dissolved in and the solution made up to a litre with 0.2 M NaOH.

Preparation of Standard Curve

Several dilutions of stock solution of BSA containing 0.05 -0.5 mg protein/ml were made using normal saline. 4 ml of Biuret reagent was added to 1 ml of each protein standard solution and the mixture allowed standing at room temperature for 30 mins. The absorbance of the solutions was then read at 540 nm and a graph of absorbance against mg BSA was then plotted.

Table 3.0 Protocol for Protein Determination by the Method of Gornal *et al.*, (1949).

Tube no.	1	2	3	4	5	6	7
BSA (ml)	0	0.05	0.1	0.2	0.3	0.4	0.5
Normal saline (ml)	1.0	0.95	0.9	0.8	0.7	0.6	0.5
Biuret reagent (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Amount of BSA (mg)	0	0.05	0.1	0.2	0.3	0.4	0.5
Absorbance (540nm)	0	0.007	0.013	0.016	0.023	0.027	0.037

Estimation of Protein in Test Samples

Procedures identical to those described above for the standard curve were used except that suitable dilutions of the test samples (liver supernatant and serum) were made i.e 1:100 with distilled water. This was done to reduce the level of protein in the samples to the sensitivity range of the Biuret method. 1ml of diluted sample was taken and the process for protein determination repeated. Protein content of sample was

obtained from the standard curve and multiplied by 100 to get the actual amount of protein in the sample.

3.2.7 Estimation of Reduced Glutathione (GSH) Level

The method of Beutler *et al.*, (1963) was used in estimating the level of reduced glutathione in liver supernatants.

Principle

The reduced form of glutathione comprises in most instances the bulk of cellular non-protein sulphydryl groups. As such deproteinization of samples with sulphosalicylic acid is necessary and ensures that no protein cysteine thiol groups can react with the colour reagent. This method is therefore based upon the development of a relatively stable (yellow) colour when 5', 5'-dithiobis- (2-nitrobenzoic acid), (Ellman's reagent) is added to sulphydryl compounds.

The chromophoric product resulting from the reaction of Ellman's reagent with the reduced glutathione, 2-nitro-5-benzoic acid possesses a characteristic absorbance at 412nm and the amount of reduced glutathione in the sample is proportional to the absorbance at this wavelength.

Reagents

- **Ellman's Reagent (DTNB)**

40 mg of Ellman's reagent was dissolved in little amount of 0.1M phosphate buffer, pH 7.4 and made up to 100 mL. It was stored at 4°C.

- **0.1M Phosphate Buffer (pH 7.4)**

(a) 11.87 g of Na_2HPO_4 was dissolved in 1000 ml. of distilled water.

(b) 2.72 g of KH_2PO_4 was dissolved in 100 ml. distilled water.

4 volumes of solution (a) i.e. 825 ml. was then mixed with 1 volume of solution (b) i.e. 175ml and the pH was adjusted to 7.4.

- **4% Sulphosalicylic Acid (Precipitating Agent)**

4 g of sulphosalicylic acid was dissolved in 100mL of distilled water, and then stored at 4°C.

- **Glutathione Working Standard (0.04g/ml)**

40 mg of glutathione was dissolved in 100ml of 0.1M phosphate buffer pH 7.4 at 4°C.

Preparation of GSH Standard Curve

Serial dilutions of stock GSH solution containing 20-200µg of reduced glutathione were prepared in different test tubes and made up to 0.5 mL with 0.1M phosphate buffer, pH 7.4. 4.5mL of Ellman's reagent was then added to each test tube. Readings were taken immediately as there is a loss of 1-2% of colour 5-10 mins after addition of Ellman's reagent. The blank was prepared by mixing 0.5 mL of phosphate buffer with 4.5 mL of Ellman's reagent. GSH concentration in each test tube was determined and the absorbance was read at 412nm. A graph of concentration against absorbance was plotted.

Table 3.1 Protocol for the Preparation of GSH Standard Curve

Tube No.	1	2	3	4	5	6	7	8	9
GSH (mL)	0.00	0.02	0.05	0.10	0.15	0.20	0.25	0.30	0.40
Phosphate buffer (mL)	0.50	0.48	0.45	0.40	0.35	0.30	0.25	0.20	0.10
Ellman's reagent (mL)	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50
GSH conc. (mg/mL)	0	8.0	20.0	40.0	60.0	80.0	100.0	120.0	160.0
Absorbance (412 nm)	0.000	0.035	0.109	0.179	0.273	0.321	0.472	0.580	0.745

- **4% Sulphosalicylic Acid (Precipitating Agent)**

4 g of sulphosalicylic acid was dissolved in 100mL of distilled water, and then stored at 4°C.

- **Glutathione Working Standard (0.0-1g/ml)**

40 mg of glutathione was dissolved in 100ml of 0.1M phosphate buffer pH 7.4 at 4°C.

Preparation of GSH Standard Curve

Serial dilutions of stock GSH solution containing 20-200µg of reduced glutathione were prepared in different test tubes and made up to 0.5 mL with 0.1M phosphate buffer, pH 7.4. 4.5mL of Ellman's reagent was then added to each test tube. Readings were taken immediately as there is a loss of 1-2% of colour 5-10 mins after addition of Ellman's reagent. The blank was prepared by mixing 0.5 mL of phosphate buffer with 4.5 mL of Ellman's reagent. GSH concentration in each test tube was determined and the absorbance was read at 412nm. A graph of concentration against absorbance was plotted.

Table 3.1 Protocol for the Preparation of GSH Standard Curve

Tube No.	1	2	3	4	5	6	7	8	9
GSH (mL)	0.00	0.02	0.05	0.10	0.15	0.20	0.25	0.30	0.40
Phosphate buffer (mL)	0.50	0.48	0.45	0.40	0.35	0.30	0.25	0.20	0.10
Ellman's reagent (mL)	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50
GSH conc. (mg/mL)	0	8.0	20.0	40.0	60.0	80.0	100.0	120.0	160.0
Absorbance (412 nm)	0.000	0.035	0.109	0.179	0.273	0.321	0.472	0.580	0.745

Procedure for GSH Determination in Samples

0.2mL of sample was mixed with 1.8ml of distilled water to give a 1 in 10 dilution. 3 mL of the precipitating reagent was added to the diluted sample and then allowed to stand for 10 minutes. The mixture was centrifuged for 4 minutes at 3000 g. and 0.5mL of the supernatant was added to 4 ml of phosphate buffer pH 7.4. Finally, 0.5 mL of Ellman's reagent was added. The absorbance was read at 412 nm within 30 minutes of the colour development.

3.2.8 Assessment of Lipid Peroxidation

This was carried out by the method of Varshney and Kale (1990).

Principle

Small amounts of malondialdehyde (MDA) are produced during lipid peroxidation and these are able to react with thiobarbituric acid (TBA) to generate a pink coloured complex which in an acidic solution absorbs light at 532 nm.

Reagents

- **30% Trichloroacetic Acid (TCA) Solution**

9 g of TCA was dissolved in distilled H_2O and made up to 30 ml.

- **0.75% Thiobarbituric Acid (TBA) Solution**

0.225 g of TBA was dissolved in 30 mL 0.1M HCl, shaking in hot water bath.

- **0.1 M Hydrochloric acid**

26 µl of concentrated HCl was added to distilled water and volume made up to 30 ml with the same

- **0.15 M Tris KCl buffer (pH 7.4)**

1.12 g of KCl and 2.36 g of Tris base were dissolved in 100 ml of distilled water separately, and the two solutions were mixed together and pH adjusted to 7.4.

Procedure

0.4 mL of sample was added to 1.6 mL of Trsi-KCl then 0.5mL of 30% TCA was added. Addition of TBA and incubation for 45 mins at 80°C produced pink coloured reaction mixtures was centrifuged at 14000 g for 15mins. The absorbance of the clear pink supernatant was then read at 532nm.

Calculation

Malondialdehyde (MDA) Concentration (units/mg protein)

$$= \frac{\text{absorbance x volume of mixture}}{E_{532} \text{ x volume of sample x mg protein}}$$

Where E_{532} is molar absorbtivity at 532nm = 1.56×10^{-5}

3.2.9 Determination of Catalase Activity

Catalase activity was determined according to the method of Sinha (1972).

Principle

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured colometrically at 570-610 nm. Since dichromate has no absorbency in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate.

The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorometrically after heating the reaction mixture

Reagents

- **5% $K_2Cr_2O_7$ (Dichromate Solution)**

5 g of $K_2Cr_2O_7$ was dissolved in 80 ml of distilled water and made up to 100ml.

- **0.2 M H_2O_2**

0.67 ml of 30% (w/w) aqueous H_2O_2 solution was mixed with distilled water and the solution made up to 300 ml.

- **Dichromate/acetic acid**

This reagent was prepared by mixing 5% solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3 by volume) and could be used indefinitely.

- **Phosphate buffer (0.01M, pH 7.0)**

3.5814 g of $Na_2HPO_4.12\ H_20$ and 1.19g $NaH_2PO_4.2\ H_20$ was dissolved in litre of distilled water. The pH was adjusted to 7.0.

Procedure for Colorimetric determination of H_2O_2

Different amounts of H_2O_2, ranging from 10 to 100 µmoles was taken in small test tubes and 2 mL of dichromate/acetic acid was added to each. Addition of the reagent instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10 mins in a boiling water bath changed the color of the solution to stable green due to formation of chromic acetate.

After cooling at room temperature, the volume of the reaction mixture was made up to 3 mL and the optical density measured at 570 nm. The concentrations of the standards were plotted against the absorbance

Table 3.2 Catalase Standard Curve

Tube no.	1	2	3	4	5	6	7
H_2O_2 (mL)	0.00	0.10	0.20	0.30	0.40	0.50	0.60
Dichromate/ acetic acid (mL)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Distilled water (mL)	1.00	0.90	0.80	0.70	0.60	0.50	0.40
H_2O_2 conc.(mg/mL)	0.00	0.67	1.34	2.01	2.68	3.35	4.02
Absorbance (570nm)	0.00	0.157	0.291	0.441	0.518	0.704	0.826

Determination of catalase activity of samples

1m L of sample was mixed with 49 mL distilled water to give 1 in 50 dilution of the sample. The assay mixture contained 4 mL of H_2O_2 solution (800µmoles) and 5ml of phosphate buffer in a 10mL flat bottom flask. 1mL of properly diluted enzyme preparation was rapidly mixed with the reaction mixture by gentle swirling motion.

The reaction was run at room temperature. A 1mL aliquot of the reaction mixture was withdrawn and blown into 2 mL dichromate/acetic acid reagent at 60 seconds interval for three mins. The hydrogen peroxide contents of the withdrawn samples were determined by the method described above.

Calculation of results

The mononuclear velocity constant, K, for the decomposition of hydrogen peroxide by Catalase was determined by using the equation for a first order reaction:

$K = 1/t \log S_1/S$

Where S_0 is the initial concentration of hydrogen peroxide and S the concentration of the peroxide at time t mins. The values of the K are plotted against time in minutes and the velocity constant of Catalase K (o) at 0 min determined by extrapolation.

The Catalase contents of the enzyme preparations were expressed in term of Katalase feiahigkeit or 'Kat.f

$$\text{Kat.f} = \frac{\text{K (o)}}{\text{mg protein/ml}}$$

3.2.10 Assay for Serum Aspartate Aminotransferase (AST) Level

Serum AST level was determined following the method of Reitman and Frankel (1957).

Principle

The enzyme aspartate aminotransferase catalyze the transfer of amino group from L-aspartate to α-oxoglutarate to form L-glutamate and oxaloacetate. The oxaloacetate formed is unstable and is quantitatively decarboxylated to pyruvate which is then complexed with 2, 4-dinitrophenylhydrazine (DNPH) to produce an intensely cotoured hydrazone on the addition of NaOH. This coloured complex absorbs radiation at 530-550nm.

Thus aspartate aminotransferase is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dintrophenylhydrazine

Reagents

- **Solution 1**

Phosphate buffer (100 mmol/L, pH7.4). L-aspartate (100 mmol/L), and α-oxoglutarate (2mmol/L).

- **Solution 2**

2, 4-dinitrophenylhydrazine (2 mmol/L).

- **0.4mol/L NaOH**

Procedure

Briefly, 0.1 mL of diluted serum sample was mixed with solution 1 and the mixture was incubated for exactly 30 minutes at 37°C. 0.5mL of solution 2 was then added to the reaction mixture and allowed to stand for exactly 20 minutes at 25°C. Then 0.5 mL of NaOH was added and the absorbance was read against reagent blank after 5 minutes at 546 nm. Reagent blank was prepared as described above replacing sample with 0.1mL of distilled water.

Table 3.3 Calibration of AST Standard Curve

Absorbance	U/l
0.020	7
0.040	13
0.060	19
0.080	27
0.100	36
0.120	47
0.140	59
0.160	76

3.2.11 Assay for Serum Alanine Aminotransferase (ALT) Level.

Serum ALT activity was determined following the method of Reitman and Frankel (1957). The enzyme alanine aminotransferase catalyze the transfer of amino group from L-alanine to α-oxoglutarate to form L-glutamate and oxaloacetate.

The oxaloacetate formed is unstable and is quantitatively decarboxylated to pyruvate which is then complexed with 2, 4-dinitrophenylhydrazine (DNPH) to produce an intensely coloured hydrazone on the addition of NaOH. This coloured complex absorbs radiation at 530-550nm.

Thus alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dintrophenylhydrazine.

Reagents

- **Solution 1**

Phosphate buffer (100mmol/L, pH7.4). L-alanine (200mmol/L), and α-oxoglutarate (2mmol/L).Solution 2

2, 4-dinitrophenylhydrazine (2mmol/L).

- **0.4mol/L NaOH**

Procedure

Briefly, 0.1 mL of diluted sample was mixed with solution 1 and the mixture was incubated for exactly 30 minutes at 37°C. 0.5mL of solution 2 was then added to the reaction mixture and allowed to stand for exactly 20 minutes at 25°C. Then 0.5ml. of NaOH was added and the absorbance was read against reagent blank after 5 minutes at 546 nm. Reagent blank was prepared as described above replacing sample with 0.1ml. of distilled water.

Table 3.4 Calibration of ALT Standard Curve

Absorbance	U/l
0.025	4
0.075	12
0.125	21
0.175	29
0.225	39
0.275	48
0.325	57
0.375	67
0.425	77
0.475	88

3.2.12 Determination of Gamma Glutamyl Transferase activity (GGT)

Gamma gutamyl transferase activity was determined by the method of Szasz, 1969

Principle

Gamma- glutamyl transferase was determined using a colorimetric method which is based on the fact that the substrate L- γ-glutamyl -3-carboxy-4- nitroanilide in the presence of glcylglycine is converted by γ -GT in the sample to 5-amino -2-nitrobenzoate which can be measured at 405 nm.

Table 3.5 Reagent Compostion for Ganima- Glutamyl Transferase colorimetric assay

Reagent contents	Concentration in test
Tris buffer (Glycl glycine)	100 mmol/L, pH8.25 100 mmol/L
L- Y-glutamyl -3-carboxy-4- nitroanilide (substate)	2.9 mmol/L

Procedure

0.1mL of sample and 1mL of reagent was mixed together in the cuvene. The initial absorbance was read, and the reading was taken again at 1. 2 and 3minutes. To calculate the GGT activity, the following formula was used

U/L =1158 X ΔA405 nm.

3.2.13 Determination of Alkaline Phosphatise Activity (ALP)

Alkaline phoshatase (ALP) was determined by the method of Englehardt (1970).

Principle

Alkaline phosphatase was determined based on the principle that ALP catalyses the reaction between p-nitrophenyl phosphate and water to form p-nitrophenol which can be measured at 405 nm

Table 3.6 Reagent Compostion for Alkaline Phosphatase Colorimetric assay

Reagent contents	Concentration in test
Dichanlaminebuffer	1 mol/l, pH9.8
$MgCl_2$	0.5 mmol/l
(Substrate) p-nitrophenylphosphate	10 mmol/l

Procedure

0.01 mL of sample and to 0.5 mL of reagent was mixed together in the cuvette. The initial absorbance was read, and the reading was taken again at 1, 2 and 3minutes. To calculate the ALP activity, the following formula was used:

U/L =2760 X ΔA405 nm

3.2.14. Determination of Bilirubin Concentration

Bilirubin concentration was determined following the principle described by Jendrassik and Grof, (1938).

Principle

This is based on the fact that conjugated bilirubin reacts with diazotised sulphanilic acid in in alkaline medium to form a blue coloured complex that is measured colorimetrically at 546 nm. Total bilirubin is determined in the presence of caffeine which releases albumin bound bilirubin when reacted with diazotised sulphanilic acid at 578 nm.

Preparation of Reagents

1. Reagent kit

The commercial kit contains Reagents 1, 2, 3 and 4.

Reagent 1 contains sulphanilic acid (29 mmol/L) and hydrochloric acid (0.17 N)

Reagent 2 contains sodium nitrite (38.5mmol/L)

Reagent 3 contains caffeine (0.26 mol/L) and sodium benzoate (0.52 mol/L)

Reagent 4 contains tartrate (0.93mol/L) and sodium hydroxide (38.5mmol/L)

2. 0.9% NaCl

0.9 g of NaCl dissolved in 100 mL of distilled water.

Procedure

Bilirubin concentration was determined according to the manufacturer's instruction as shown below

Table 3.7 Assay for Total Bilirubin Concentration Determination.

	Sample Blank	Sample
Reagents 1	200 µL	200 µL
Reagents 2	----	50 µL
Reagents 3	1000 µL	1000µL
Reagents 4	200 µL	200 µL

Mixed and incubated for exactly 10 minutes at 20°C-25°C

Reagents 4	1000 µL	1000 µL

The reaction mixture was incubated for a further 5-30 minutes at 25°C and the absorbance of the sample read against the sample blank at 578 nm

Calculation

Total bilirubin concentration in the sample (µmol/L) = 158 X ABS_{TB}

Table 3.8 Assay for Direct (Conjugated) Bilirubin Concentration Determination

	Sample Blank	Sample
Reagents 1	200 µL	200 µL
Reagents 2	--------	50 µL
NaCl (0.9%)	2000 µL	2000 µL
Sample	200 µL	200 µL

Mixed and incubated for exactly 10 minutes at 20°C-25°C

The reaction mixture was mixed and the absorbance of the sample read against the sample blank at 546 nm.

Calculation

Direct bilirubin concentration in the sample (µmol/L)

= 246 X ABS_{DB}

3.2.15 Determination of Glutathione-S-Transferase Activity

Glutathione-S-transferase GST activity was determined according to the method of Habig *et al.*, (1974).

Principle

The assay is based on the fact that all GST demonstrate a relatively high activity with 1-chloro-2, 4-dinitrobenzene as the second substrate. Consequently, the conventional assay for GST activity utilizes 1-chloro-2, 4-dinitrobenzene as substrate. When this substance is conjugated with reduced glutathione its absorption maximum shifts to a longer wavelength. The absorption increase at the new wavelength of 340 nm provides a direct measurement of the enzymatic reaction

Reagents

- **1-Chloro-2, 4-Dinitrobenzene (20 mM)**

3.37mg of 1-chloro-2, 4-dinitrobenzene (CDNB) was dissolved in 1 mL of ethanol.

- **Reduced Glutathione (0.1 M)**

30.73 mg of reduced glutathione GSH was dissolved in 1 mL of 0.1M phosphate buffer (pH 6.5).

- **0.1 M Phosphate Buffer (pH 6.5)**

This was prepared by dissolving 4.96 g of dipotassium hydrogen phosphate and 9.73 g of potassium dihydrogen phosphate in little amount of distilled water and then made up to the mark in a 1 litre volumetric flask. The pH was adjusted to 6.5.

Procedure

The medium for the estimation was prepared as shown in the table below and the reaction was allowed to run for 60 seconds each time before the absorbance was read against blank at 340nm. The temperature was maintained at approximately 31°C.

Table 3.9 Glutathione-S-transferase Assay Medium

Reagent	Blank	Test
Reduced glutathione	30 µL	30 µl.
CDNB	150 µl.	150 µl.
0.1M phosphate buffer, pH 6.5	2.82 ml.	2.79 ml.
Cytosol	-	30 µl.

Calculations

The extinction coefficient of CDNB = 9.6 $mmol^{-1}$ cm^{-1}

GST activity = $\frac{\text{absorbance}}{9.6} \times \frac{1}{\text{mg/0.03 mL protein}}$

= µmole/min/mg protein.

3.2.16 Determination of Superoxide Dismutase (SOD) Activity

SOD activity was determined by the method of Misra and Fridovich, (1972).

Principle

The ability of superoxide dismutase to inhibit the auto oxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for dismutase.

Super oxide radical (O_2^-) generated by xanthine oxidase reaction cause the oxidation of epinephrine to adenochrome and the yield of adenochrome produced increase per O_2^- introduced with increasing pH and also increase with increasing concentration of epinephrine. These results led to the proposal that auto oxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving super oxide radical and hence inhibitable by SOD

Reagents

- **0.05 M Carbonate Buffer (pH10.2)**

14.3 g of $Na_2CO_3.10H_2O$ and 4.2 g of $NaHCO_3$ were dissolved in 900 ml of distilled water and then made up to 1 litre. The pH was the adjusted to 10.2.

- **0.3 mM Adrenaline**

0.0137 g of adrenaline (epinephrine) was dissolved in 200 mL of distilled water and made up to 250 mL. This solution was prepared fresh just before use for the experiment.

Procedure

1 mL of sample was diluted in 9 mL of distilled water to make a 1 in 10 dilution. An aliquot of the diluted sample was added to 2.5 mL of 0.05M carbonate buffer pH10.2 to equilibrate in the spectrophotometer and the reaction started by addition of 0.3 mL of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion.

The reference cuvette contained 2.5 mL buffer, 0.3 mL of substrate (adrenaline) and 0.2 mL of distilled water. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

Calculations

$$\text{Increase in absorbance per minute} = \frac{A_1 - A_0}{2.5}$$

Where A_0 = absorbance after 30 seconds

A_1 = absorbance after 150 seconds

$$\% \text{ Inhibition} = \frac{\text{increase in absorbance for substrate}}{\text{Increase in absorbance of blank}} \times 100$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adenochrome during 1 minute.

3.2.17 Determination of Glutathione Peroxidase Activity

Glutathione peroxidase (GPX) activity was measured according to the procedure of Rotruck *et al* (1973)

Principle

Glutathione peroxidase is allowed to congugate hydrogen peroxide and glutathione for a fixed period of time after which the reaction is quenched. The remaining glutathione is reacted with Ellman's reagent and GSH consumed is then used as a measure of enzyme activity.

Reagents

- **Sodium azide (NaN_3; 10 mM)**

0.0325 g of sodium nitrite was dissolved in small quantity of distilled water and made up to 50 mL.

- **Reduced glutathione (GSH 4 mM)**

0.0123 g of reduced GSH was dissolved in in small quantity of phosphate buffer and made up to 100 mL.

- **Hydrogen peroxide (H_2O_2; 2.5 mM)**

28 µL of hydrogen peroxide was dissolved in small quantity of distilled water and made up to in 100 mL.

- **Tricholoroacetic acid (TCA, 10%)**

2 g of TCA was dissolved in small quantity of distilled water and made up to 20 mL.

- **Di-potassium Hydrogen Orthophosphate (K_2HPO_4; 0.3 M)**

5.23 g of di-potassium hydrogen orthophosphate was dissolved in small quantity of distilled water and made up to in 100 mL.

- **5'-5'-dithiobis-(2-dinitrobenzoic acid) DTNB**

0.04 g of DTNB was dissolved in in small quantity of phosphate buffer and made up to 100mL.

- **Phosphate Buffer**

0.992 g of K_2HPO_4 and 1.946 g of KH_2PO_4 were dissolved in small quantity of distilled water and made up 200 mL and adjusted to pH of 7.4

Table 3.10 Glutatbione Peroxidase Assay Medium

Phosphate buffer	500 µL
NaN_3	100 µL
GSH	200 µL
H_2O_2	100 µl
Sample	500 µL
Distilled water	600 µL

The whole reaction mixture was incubated at 37°C for 3 minutes after which 0.5 ml. of TCA was added and thereafter centrifuged at 3000 rpm for 5 minutes. To 1 mL of each of the supernatants, 2 mL of K_2HPO_4 and 1 ml of DTNB was added and the absorbance was read at 412 nm against a blank.

Glutathione peroxidase activity was observed by plotting the standard curve and the concentration of the remaining GSH was extrapolated from the curve

GSH consumed = 245.34 – GSH remaining

Glutathione peroxidase activity = GSH Consumed/mg Protein

3.2.18 Determination of Hydrogen Peroxide Generation

Principle

The method utilized was based on the method of Wolff (1994).

Principle

In dilute acid hydrogen peroxide oxidizes Fe(II) to Fe(III) which then selectively forms a blue-purple complex with an absorption maximum at 560 nm. The addition of sorbitol initiates a chain reaction with the produced hydroxyl radical that yield Fe(III) and therefore greatly amplifies the response per hydrogen peroxide molecule present, thereby increasing the sensitivity of the method.

Reagents

- **100 µmol/l xylenol orange (molecular weight: 760.6)**

0.0152 g of xylenol orange was dissolved in small quantity of distilled water and made up to 200 mL.

- **250 µmol/l ammonium ferrous sulphate (mw 392.14)**

0.0196 g of ferrous sulfate was dissolved in in small quantity of distilled water and made up to 200 mL.

- **100 mmol/l Sorbitol (mw: 182.2)**

3.64 g of sorbitol was dissolved in small quantity of distilled water and made up 200 mL of distilled water.

- **25 mmol/L H_2SO_4**

1 mL of 1 M H_2SO_4 was made up to 40 mL with distilled water

- **0.1M Phosphate buffer, pH7.4** .0.496 g of di-potassium hydrogen orthophosphate, K_2HPO_4 (Hopkins and Williams, Ltd) and 0.973 g of potassium di-hydrogen orthophosphate, KH_2PO_4 (Hopkins and Williams Ltd) were dissolved in 9 ml of distilled water. The pH was adjusted to 7.4 and then made up to a 100 ml with distilled water

Table 3.11: Hydrogen Peroxide Assay Medium

Reagents	Volume
Buffer	2.5 ml
AFS	250 µL
Sorbitol	100 µL
Xylenol orange	100 ml
H_2SO_4	25 µL
Sample	50Ml

The assay mixture was thoroughly mixed by vortexing till it foamed. A pale pink colour complex is generated after incubation for 30 minutes at room temperature. The absorbance was read against blank (distilled water) at 560 nm wavelength.

The concentration of the hydrogen peroxide generated was extrapolated from the standard curve.

Table 3.12 Protocol for the Estimation of Hydrogen Peroxide

Test tube	1	2	3	4	5	6	7
H_2O_2 (mL)	0.05	0.10	0.15	0.20	0.30	0.40	0.50
Dichromate/acetic acid (mL)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Distilled water (mL)	0.95	0.90	0.85	0.80	0.70	0.60	0.50
H_2O_2 concentration (μ moles)	10	20	30	40	60	80	100
Absorbance (570 nm)	0.049	0.095	0.145	0.195	0.291	0.385	0.484

3.2.19 Determination of ascorbic acid (Vitamin C)

The ascorbic acid concentration was determined according to the method of Jakota and Dani (1982)

Principle

This method is based on the fact that, ascorbic acid present in biological samples react with Folin C reagent, an oxidizing agent, to give a blue colour which has maximum spectrophotometric absorption at 760 nm.

Reagents

- **10% Trichloroacetic acid (TCA)**

  10 g of TCA (BDH Chemicals Ltd., England) was dissolved with distilled water in a conical flask and made up to the 100 ml mark with same.

- **Folin-Ciocalteu reagent**

  Commercially prepared folin-Ciocalteu reagent of 2.0 M concentration was diluted 10-fold with double distilled water.

- **Ascorbic Acid Standard Solution (stock)**

  0.1 g of ascorbic acid (Sigma Chemical Co., London) was dissolved in distilled water and made up to the 1 litre flask in a round bottom flask such that the final concentration is 100 µg ascorbic acid/ mL.

Calibration of Ascorbic Acid Standard Curve

Procedure

A standard curve was prepared by taking varying concentrations of standard solutions of ascorbic acid in water, ranging from 0.05-0.7 mL. Then, 0.8 ml of 10% TCA was added to each tube. After vigorous shaking, the tubes were kept in an ice bath for 5 min and centrifuged at 3000 g for another 5 minutes.

Supernatant of the same range (i.e 0.05-0.7 ml.) were withdrawn and diluted to 2.0 ml using double-distilled, and after 0.2 ml of diluted folin's reagent was added, the tubes vigorously shaken. After 10 minutes, the absorbance of the blue colour developed was measured in a spectrophotometer at 760 nm.

Table 3.13 Preparation of Vitamin C Standard Curve

Content	1	2	3	4	5	6	7
Stock A.A.(mL)	0.00	0.03	0.1	0.2	0.3	0.4	0.5
TCA (mL)	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Supernatant (ml.)	-	0.05	0.1	0.2	0.3	0.4	0.5
Distilled water (mL)	2	1.95	1.9	1.8	1.7	1.6	1.5
Folin's reagent (mL)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
A.A. conc. (µg/ml.)	-	5	10	20	30	40	50
Absorbance(760 nm)	-	0.04	0.06	0.13	0.22	0.30	0.31

Procedure for Determination of Vitamin C in Tissue Samples

Procedures identical to those employed for the standard curves were used in the determination of ascorbic acid concentrations in the test samples.

3.2.20 Determination of Myeloperoxidase Activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined according to the method of Eiscrich *et al.* (1998).

Principle

Myeloperoxidase (MPO) is a lysosomal enzyme present in azurophilic granules of polymorphonuclear leukocytes (PMNs) and is unique to neutrophils and monocytes. However, monocytes contain only one-third of the MPO found in PMNs.

MPO utilizes H_2O_2 produced by the neutrophils to oxidize a variety of aromatic compounds to give substrate radicals for bacterial activity (Hampton *et al.*, 1998). This enzyme is unique in that it can oxidize chloride ions to produce a strong non-radical oxidant, hypochlorous acid (HOCl). HOCl is the most powerful bactericidal produced by neutrophils. Excessive production of these radicals can cause oxidative stress leading to oxidative tissue injury. In this assay, the ability of MPO to oxidize tetramethylbenzidine (TMB) in the presence of H_2O_2 to generate the oxidized product, which was then measured spectrophotometrically served as the basis for this assay.

$$2\text{TMB} + H_2O_2 \xrightarrow{\text{MPO}} 2\text{Oxidized TMB} + 2H_2O$$

(Tetramethylbenzidine) (Oxidized tetramethylbenzidine)

Preparation of reagents

- **NaH_2PO_4 buffer (43 mM, pH 5.4)**

  This was prepared by dissolving 515.9 mg of NaH_2PO_4 (MW. 119.98) in 80 mL of distilled water. The pH was adjusted to 5.4 with HCl or NaOH and made up to 100 mL with distilled water.

- **Tetramethylbenzidine (1.6 mM)**

  Tetramethylbenzidine (38.5 mg, MW. 240.3) was dissolved in DMSO and made up to 100 mL

- **H_2O_2 (100mM)**

  0.786 mL of 30% H_2O_2 was made up to 100 mL with the distilled water.

Procedure

An aliquot (0.4 mL) of tissue homogenate was allowed to react with n solution of tetramethylbenzidine (0.2 mL) and 100 mM H_2O_2 in 43mM NaH_2PO_4 (1.4 mL) in a final volume of 3mL. Absorbance kinetics was monitored spectrophotometrically at 450nm. MPO activity was defined as the quantity of enzyme degrading 1μmol peroxide min^{-1} and was expressed in units per mg protein.

3.2.21 Determination of Protein Carbonyl

Protein carbonyl contents were determined according to the methods of Uchida and Stadtman (1993).

Preparation of reagents

- **0.1% 2, 4-Dinitrophenyl hydrazine (DNPH)**

  0.1 g of DNPH was dissolved in a small amount of distilled water and made up to 100ml. with the same.

- **20% Trichloroacetic acid (TCA)**

20 g of TCA was dissolved in small amount of distilled water and made up to 100mL with the same.

- **2 N HCl**

8.36 ml of 37% (w/v) HCl was added to 50 ml of distilled water.

- **133 mM Tris (MW= 121.14)**

1.61 g of Tris was dissolved in a small amount of distilled water and made up to 100 mL with the same.

1) 13 mM Acid ethylenediamine tetraacetic (EDTA, MW= 292.25)

3.80 g of EDTA was dissolved in small amount of 133 mM Tris solution and made up to 100mL with the same.

2) 8 M Guanidine hydrochloride

76.42 g of guanidine hydrochloride was dissolved small amount of Tris-EDTA solution made up to 100mL with the same.

3) Ethanol/Ethyl acetate (EtOH/EtOAc) solution

150 mL each of ethanol and ethyl acetate solutions were mixed in a ratio of (1:1 v/v) to give a final volume of 300 mL.

Procedure

0.5 mL of sample was treated with an equal volume of 0.1% (w/v) 2, 4-DNPH in 2 N HCl and incubated for 1 h at room temperature with vortexing every 10 min. 0.5 mL of 20% TCA was added followed by centrifugation for 5 min. The precipitate was washed three times with 1mL of EtOH/EtOAc solution and dissolved in 1 mL guanidine hydrochloride solution for 15min at 37°C with mixing. The absorbance was recorded at 365 nm. The results were expressed as nmol of DNPH incorporated/mg protein based on the molar extinction coefficient of 22,000M^{-1} cm^{-1} for aliphatic hydrazones.

3.2.22 Determination of Total Antioxidant Activity

Total antioxidant activity in samples was determined by the method of Koracevic *et al*., (2001).

Principle

A standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton type reaction, leading to the formation of hydroxyl radicals (OH). These reactive oxygen species degrade benzoate, resulting in the release of thiobarbituric acid reactive substance (TBARS). Antioxidants from the added sample cause suppression of the production of TBARS. This reaction can be measured spectrophotometrically and the inhibition of colour development defined as the antioxidative activity or capacity (TAC).

Preparation of reagents

- **0.1M Phosphate buffer (pH 7.4)**

This was prepared by dissolving 0.496 g of dipotassium hydrogen phosphate (BDH Chemical Ltd., England) and 0.973 g of potassium dihydrogen phosphate (Hopkins and Williams Ltd., England) in a little amount of distilled water and then made up to 100 ml with the same. The pH was adjusted to 7.4.

- **10 mM Sodium benzoate**

0.144 g of sodium benzoate (MW=144.11) was dissolved in a little amount of distilled water and made up to 100 ml with the same.

- **50 mM NaOH**

0.3 g was dissolved with a little amount of distilled water and made up to 150 ml with the same.

- **2 mM Acid ethylenediamine tetraacetic (EDTA)**

0.015 g of EDTA (MW=292.25) was dissolved in small quantity of phosphate buffer (pH 7.4) and made up to 25mL with the same

2 mM Ammonium ferric sulphate

0.0241 g of ammonium ferric sulphate Fe $(NH_4)_2SO_4$; MW= 482.19) was dissolve in small quantity of distilled water and made up to 25mL of the same

- **Fe–EDTA complex**

This was prepared freshly by mixing equal volumes of solutions 4 and 5 and left to stand 60 minutes at room temperature.

- **10 mM H_2O_2**

0.04 ml (30%w/v) H_2O_2 was made up to 50 ml with distilled water.

- **20% Acetic acid**

30 ml acetic acid was added to 120 ml of distilled water.

- **0.8% Thiobarbituric acid (TBA)**

1.2 g TBA was dissolved in 150 ml of 50 mM NaOH

- **1 mM Uric acid**

0.00336 g of uric acid (MW= 168.1103) was dissolved in 20 ml of 5 mM NaOH. Solutions 4–9 were prepared immediately before use. Sodium benzoate can be kept at (0–4°C) and the Uric acid solution in a deep freeze (–20 to –30°C).

Procedure

The reaction mixture containing 0.4 mL of phosphate buffer (pH 7.4), 0.5 ml of sodium benzoate, 0.2 mL of Fe- EDTA (2mM EDTA + 2mM ferric ammonium sulphate), 0.2 mL of H_2O_2 and 0.1mL of sample was incubated for 60 minutes at 37°C. The reaction was then stopped by addition of 1 ml. of 20% acetic acid and 1ml. of TBA. The reaction mixture was further incubated for 10 minutes at 100°C (in a boiling water bath) then cooled on an ice. The absorbance was measures at 532 nm

against distilled water. 1mM uric acid was used as standard (i.e 0.1mL uric acid solution in place of sample).

Calculation

TAC of the sample was then calculated by the following formula:

TAC (mmol/litre) = $(C_{UA})(K - A)/(K - UA)$

Where:

K = absorbance of control

A = absorbance of sample

UA = absorbance of uric acid solution

C_{UA} = concentration of uric acid (in mmol/litre).

3.2.23 Nitrite determination/ Quantitation

Principle

Quantitation was based on the Griess reaction as described by Navarro-Gonzalvez *et al.* (1998).

Nitric oxide (NO) is a molecular mediator of many physiological processes, including vasodilation, inflammation, thrombosis, immunity and neurotransmission. A number of methods exist for measuring NO in biological systems.

One of these methods involves the use of the Griess diazotization reaction to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under physiological conditions. This method involves the use of the Griess diazotization reaction to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under physiological conditions.

Reagents

Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride; 1% sulfanilamide in 5% phosphoric acid.

Procedure

The amounts of nitrite in supernatants or in serum were measured following the Griess reaction by incubating a100 µLof samplewith100µL of Griess reagent (1:1) at room temperature for 20 min. The absorbance at 550 nm (OD 550) was measured spectrophotometically.

Nitrite concentration was calculated by comparison with the OD 550 of a standard solution of known sodium nitrite concentrations.

Calibration curve

Various concentrations were prepared by diluting stock 20 mmol/l solutions of $NaNO_2$ with distilled water. The nitrite calibrator was diluted with glycine buffer just as the serum samples were. Calibration curve was made over a linear range of nitrate between 0 and 100 µmol/L.

Table 3.14 Nitrite Calibration Table

$NaNO_2$Concentration	Absorbance
0	0
2.86	0.015
5.71	0.025
11.4	0.049
22.86	0.056

3.2.24 Determination of Tumor Necrosis Factor Alpha (Tnfα), Interleukin 1β, Prostaglanin E-2, Caspase 3, Caspase 9, P53, Cytrochrome C Protein Levels

Principle

These proteins present in a serum sample conjugates with an immobilized respective antibody when added into wells. Addition of a secondary antibody conjugated with horse radish peroxidase and TMB (tetra methyl benzidine) substrate to the well results in an antibody-antigen-antibody colored complex. The intensity of color developed at 450 nm is directly proportional to the concentration of protein in the samples

Reagents and Materials

ELISA KITS from Cusabio biotech co limited China was procured for each of these proteins

8 x 12 (96) well plate,

Sealed bags.

Standard (450 ng/L),

Standard diluent.

Sample diluent.

Chromogen solution A.

Chromogen solution B.

Stop solution,

Wash solution.

ELISA reader (DMM 9602 microplate reader)

Procedure

Serial dilution of standard solution (450 ng/L) was prepared with standard diluent to make 300, 200, 100 and 50 ng/L. 50 µl. of standard / sample were added

3.2.2.4 Determination of Tumor Necrosis Factor Alpha (Tnfα), Interleukin 1β, Prostaglanin E-2, Caspase 3, Caspase 9, P53, Cytrochrome C Protein Levels

Principle

These proteins present in a serum sample conjugates with an immobilized respective antibody when added into wells. Addition of a secondary antibody conjugated with horse radish peroxidase and TMB (tetra methyl benzidine) substrate to the well results in an antibody-antigen-antibody colored complex. The intensity of color developed at 450 nm is directly proportional to the concentration of protein in the samples

Reagents and Materials

ELISA KITS from Cusabio biotech co limited China was procured for each of these proteins

8 x 12 (96) well plate,

Sealed bags,

Standard (450 ng/L),

Standard diluent,

Sample diluent,

Chromogen solution A,

Chromogen solution B,

Stop solution,

Wash solution.

ELISA reader (DNM 9602 microplate reader)

Procedure

Serial dilution of standard solution (450 ng/L) was prepared with standard diluent to make 300, 200, 100 and 50 ng/L. 50 µL of standard / sample were added

into each well (except the first well : A1 for blank). The plate was mixed gently and incubated at 37°C for 30 minutes. The incubation mixture was removed by aspiration and each well was washed 3 times with 200 µl of wash solution. The microtitre plate was blot dry and 50 µL of HRP-conjugate reagent (2° antibody) into each well and the plate was covered and incubated for 30 minutes.

The incubation mixture was removed again by aspiration and each well was washed 3 times with 200 µL of wash solution. 50 µL of chromogen A and chromogen B was added to the plate and the plate was incubated in the dark at 37°C for 15 minutes; after which 50 µL of stop solution (1 M H_2SO_4) was added to each well.

The absorbance was read against the blank well (A1) at 450 nm using an ELISA reader.

3.2.25 Determination of Expression of COX-2 and iNOS using Immune-Histochemical Technique

Reagents

Goat polyclonal anti COX -2 antibodies (Santa Cruz biotechnology Inc. USA).

Rabbit polyclonal anti-iNOS antibody (Santa Cruz biotechnology Inc. USA.)

Xylene: This requires no dilution. The volume required is determined by the capacity of the copling jars.

Graded portions of Ethanol

100% ethanol

This is absolute ethanol it requires no dilution. The volume required is determined by the capacity of the copling jars.

95% ethanol

95% ethanol was prepared by making 95 ml of absolute ethanol to 100 ml with distilled water by adding 5 mL of distilled water

85%ethanol

85% ethanol was prepared by making 85 ml of absolute ethanol to 100 ml with distilled water by adding 15 mL of distilled water.

70% ethanol

70% ethanol was prepared by making 70 mL of absolute ethanol to 100 mL with distilled water by adding 30 mL of distilled water.

50% ethanol

50% ethanol was prepared by making 50 mL of absolute ethanol to 100 mL with distilled water by adding 50 mL of distilled water.

Phosphate buffer saline (PBS) pH7.4

Phosphate buffer saline pH 7.4 was prepared by dissolving 2 g of potassium chloride (KCl) 80 g of sodium Chloride (NaCl), 2 g Potassium dihydrogen phosphate and 11.6g disodium hyrogen phosphate were all dissolved in 1litres of distilled water pH 7.2-7.6.

10% Buffered formalin

10% buffered Formalin was made by making 125mL of 40% formalin to 500mls with PBS

0.3% Hydrogen peroxide

0.3% hydrogen peroxide was prepared by dissolving 10mls of 30% hydrogen peroxide in 1000 mL PBS

Skimmed milk

Citric acid

Methanol

Triton x

Horseradish Peroxidase-conjugated secondary antibodies in VECTASTAIN kit (Vector Labs, USA)

Diaminobenzidine (DAB)

Procedure

Antigen retrieval was done by immersing the slides in 10 mM citrate buffer for 25 minutes, with subsequent peroxidase quenching in 3% H_2O_2/methanol. All the sections were blocked in 2% skimmed milk overnight and probed with Goat polyclonal anti COX -2 antibody(Santa cruz biotechnology inc)and rabbit polyclonal anti-iNOS antibody (Santa cruz biotechnology inc.) for COX2 and iNOS expression respectively for 16 hours at 4°C.

Detection of bound antibody was done using appropriate HRP-conjugated secondary antibodies in VECTASTAIN kit (Vector Labs, USA) according to manufacturer's protocol. Reaction product was enhanced with diaminobenzidine (DAB) for 6-10 minutes, with subsequent dehydration in ethanol and mounting on salinized slides. Images were acquired with Sony® digital camera.

3.2.26. Detection of apoptosis

Principle

The DeadEnd™ Colorimetric TUNEL System is a non-radioactive system designed to provide simple, accurate and rapid detection of apoptotic cells insitu at the single-cell level. The system can be used to assay apoptotic cell death in both tissue sections and cultured cells by measuring nuclear DNAfragmentation, an important biochemical indicator of apoptosis in many cell types.

The DeadEnd™ Colorimetric TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL assay. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which

are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen. diaminobenzidine.

Reagents in kit

- 9.6 mlEquilibration Buffer
- 40 µl Biotinylated Nucleotide
- 40 µl TerminalDeoxynucleotidyl Transferase.Recombinant
- 70 ml SSC, 20X
- 10 mg Proteinase K

Prepared reagent

Phosphate buffer saline (PBS) pH7.4

Phosphate buffer saline pH 7.4 was prepared by dissolving 2 g of potassium chloride (KCl) 80g of sodium Chloride (NaCl). 2 g Potassium dihydrogen phosphate and 11.6 g disodium hyrogen phosphate were all dissolved in 10 litres of distilled water. pH 7.2-7.6

10% Buffered formalin

10%buffered formalin was made bymaking125 ml of 40% formalin to 500 mL with PBS

0.3%Hydrogen peroxide

0.3% hydrogen peroxide was preparedby dissolving10 ml of 30% hydrogen peroxide in 1000 ml. PBS

100% ethanol

This is absolute ethanol it requires no dilution. The volume required is determined by the capacity of the copping jars

95% ethanol

95% ethanol was prepared by making 95 mL of absulute ethanol to 100 ml with disulled water by adding 5 mL of distilled water

85%ethanol

85% ethanol was prepared by making 85 mL of absolute ethanol to 100 ml with distilled water by adding 15 ml of distilled water.

70%ethanol

70% ethanol was prepared by making 70 mL of absolute ethanol to 100 mL with distilled water by adding 30 mL of distilled water.

50%ethanol

50% ethanol was prepared by making 50 mL of absolute ethanol to 100 mL with distilled water by adding 50 mL of distilled water.

Xylene: This requires no dilution .The volume required is determined by the capacity of the copling jars.

Procedure

Briefly, the tissues section were deparaffinized by embedding in xylene, rehydrated in graded potions of ethanol at different time intervals and washed in PBS. The slides were later re- fixed in 10% buffered formalin solution. Proteinase- k (20ug/mL) was used to treat the slides to make the tissues more permeable. This was followed by another fixation in 10% buffered formalin. The sections were later incubated in equlbration buffer for 10 mins.

After this, the sections were allowed to react with rTDT reaction mixture (98ul equilibration buffer. 1µL biotinated nucleotides mix and 1 ul rTDT reaction mixture) for 1hr at 37 °C in humidified chamber. The reaction was terminated the by immersing the slides in 2X SSC in a Coplin jar for 15 minutes at room temperature. The endogenous peroxidases were blocked by inuncrsing the slides in 0.3% hydrogen peroxide in PBS for 3–5 minutes at room temperature.

Slides were later incubated with streptavidin-HRP for 3mins at room temperature. After this, slides were stained with DAB in the dark and then rinsed several times in distilled water. Slides were mounted in 100% glycerol. A light microscope was used to observe staining.

3.2.27 Micronuclei Assay

Principle

The principle of the micronuclei assay is based on the fact that polychromatic erythrocyte (PCE) cells have a staining property that is different from the normal natural erythrocyte. The polychromatic staining property results from the presence of ribosomal RNA 24 hours prior to the formation of the cell. As PCEs develop into mature erythrocytes, they lose the ribosomal RNA and the staining property. In mammals, mature erythrocytes expel their nuclei 8-12 hours after the last mitosis preceding the formation of an erythrocyte. The micronuclei for some reasons are not expelled completely. Micronuclei are not normally found in the circulating erythrocytes in blood because they are filtered out by the spleen.

Reagents

Bovine serum albumin

Absolute methanol

Xylene

0.4% May Grunwald Stain

5% Giemsa Stain

0.01 M Phosphate buffer pH 6.8

Depex (DPX) mountant

Preparation of bone marrow smears

The method of Schmidt (1975) was adopted in the preparation of bone marrow smears. After the rats were sacrificed by cervical dislocation and the femur of each rat was removed and stripped clean of muscle tissue. A pair of scissors was used to make an opening in the iliac region of the femur. A small pin was then introduced into the

marrow canal at the epiphyseal end. As the pin was pushed inside the canal, the marrow exuded through the hole at the iliac end.

The marrow was placed on a slide and a drop of bovine serum albumin was added to the smear using a Pasteur pipette. The whole was mixed to become homogenous by using a clean edge of another slide. The homogenous mixture was then spread on the slide as a smear and allowed to dry.

Fixing andStaining of Slides

Procedure for staining and fixing the slides involved the following steps:

- Slides were fixed in absolute methanol for 5 minutes.
- They were dried to allow for removal of the methanol.
- They were then stained with 0.4% May Grunwald stain 1 and 2 and air dried.
- The slides were again stained with 5% Giemsa for 30 minutes and rinsed in phosphate buffer for about 30 seconds.
- The slides were rinsed in distilled water and allowed to air dry.
- They were finally fixed in xylene for 20 minutes and air dried.
- The slides were mounted in DPX (a natural mountant) with cover slips so that they could be viewed under the microscope.

Scoring of the Slides

The fixed cells on the slides were viewed under a light microscope to detect the presence of micro-nucleated polychromatic erythrocytes (PCE). A tally counter was used for scoring. The slides were first screened at medium magnification to get suitable regions for scoring. PCEs and micronuclei stain blue while normal mature erythrocytes stain red.

3.3.0 Statistics

Statistics data are expressed as mean ± SD and analyzed with Microsoft Excel and SPSS statistical packages. Statistical analyses were performed by Student *t*-test and one way analyses of variance (ANOVA) *P* value of less than 0.05 was considered statistically significant.

CHAPTER FOUR

EXPERIMENTS AND RESULTS

4.1 EXPERIMENT 1: EFFECT OF NEVIRAPINE ON THE LIVER WITH RESPECT TO TIME OF EXPOSURE

INTRODUCTION

Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs), constitute a class of medications that have contributed significantly to the management of Human Immunodeficiency Virus (HIV) infection. Some approved drugs in this class include efavirenz, nevirapine, etravirine and rilpivirine (Schafer *et al.*, 2011). Although NNRTIs have contributed tremendously to the fight against HIV/AIDS, there are reservations on some of their side effects. Nevirapine - a drug of keen interest in this study has been associated with hepatotoxicity depicted by elevation of transaminases (AST and ALT) levels and hepatocytes necrosis (Elias and Brambaifa, 2013; Elias *et al.*, 2013).

Although hepatotoxicity of nevirapine is established, effect of nevirapine on the liver with respect to time has not been well-explored. It is important to know if extent of nevirapine induced liver damage is dependent on the duration of exposure to the drug; since the drug is used by the patients almost for a life time.

In the present study, therapeutic dose of nevirapine was administered for different durations (1-4 weeks) to assess the relationship between nevirapine-induced liver damage and duration of exposure to the drug. Various biomarkers of hepatic damage and oxidative stress were used to assess the extent of the damage.

PROCEDURE

Forty male albino rats of Wistar strain weighing between 125-200 g were used in this study. They were purchased from the animal house of the Faculty of Basic Medical Sciences, University of Ibadan and housed in cages in the animal house of Biochemistry Department, University of Ibadan. The animals were given pellet feed and water *ad libitum*.

They were randomly assigned to five groups with eight animals each. Group 1 (control) received only water orally. Rats in Group 2, 3, 4, and 5 received 5.71 mg/kg (therapeutic dose) of nevirapine orally for 1, 2, 3, and 4 weeks respectively. The rats were sacrificed by cervical dislocation 24 hours after the last day of drug administration; blood was collected by cardiac puncture technique with the aid of clean needle and syringe into clean dry centrifuge tubes and allowed to coagulate by standing for 30 minutes. The blood samples were then centrifuged for 10 mins at 3000g using a bench centrifuge. The clear supernatant (serum) was collected and stored in the refrigerator.

Liver was quickly removed, rinsed in ice-cold 1.15% KCl, blotted and weighed. A portion of the liver was prepared for histological examination as previously described in section 3.3 and the remainder was minced with scissors in 4 volumes of ice-cold 0.1M phosphate buffer pH 7.4 and homogenized using Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 g for 15 minutes at 4° C and the supernatant termed post mitochondrial fraction (PMF) were aliquoted and used for the enzymes assays. The serum samples were used to determine ALT and AST activities according the method of Reitman and Frankel (1957) and also bilirubin concentration according to the method of Lendrassik and Cinof (1938). PMF of liver was used to determine reduced glutathione level according to the method of Beutler et

PROCEDURE

Forty male albino rats of Wistar strain weighing between 125-200 g were used in this study. They were purchased from the animal house of the Faculty of Basic Medical Sciences. University of Ibadan and housed in cages in the animal house of Biochemistry Department, University of Ibadan. The animals were given pellet feed and water *ad libitum*.

They were randomly assigned to five groups with eight animals each. Group I (control) received only water orally. Rats in Group 2, 3, 4, and 5 received 5.71 mg/kg (therapeutic dose) of nevirapine orally for 1, 2, 3, and 4 weeks respectively. The rats were sacrificed by cervical dislocation 24 hours after the last day of drug administration; blood was collected by cardiac puncture technique with the aid of clean needle and syringe into clean dry centrifuge tubes and allowed to coagulate by standing for 30 minutes. The blood samples were then centrifuged for 10 mins at 3000g using a bench centrifuge. The clear supernatant (serum) was collected and stored in the refrigerator.

Liver was quickly removed, rinsed in ice-cold 1.15% KCl, blotted and weighed. A portion of the liver was prepared for histological examination as previously described in section 3.2 and the remainder was minced with scissors in 4 volumes of ice-cold 0.1M phosphate buffer pH 7.4 and homogenized using Potter-Elvehgen homogenizer. The homogenate was centrifuged at 10,000 g for 15 minutes at 4° C and the supernatant termed post mitochondrial fraction (PMF) were aliquoted and used for the enzymes assays. The serum samples were used to determine ALT and AST activities according the method of Reitman and Frankel (1957) and also bilirubin concentration according to the method of Lendrassik and Grof (1938). PMF of liver was used to determine reduced glutathione level according to the method of Beutler et

al., (1963), lipid peroxidation using the procedure of Vashney and Kale (1990), superoxide dismutase activity was determined as described by Misra and Fridovich (1972) and catalase activity according to the method of Sinha (1971) and protein concentration using biuret method as described by Gornal *et al.*, (1949). The different assay procedures are described under materials and method in section 3.2.6 to 3.2.14.

RESULTS

The result of the present study has showed that treatment with therapeutic dose of nevirapine orally caused elevation of serum levels of alanine (ALT) and aspartate (AST) aminotransferase compared with control throughout weeks 1, 2, 3, and 4. AST level was significantly elevated at the weeks 2, 3, and 4 ($p<0.05$; 11.2%, 33.2%, and 18.7% increase respectively compared to control); while ALT levels were significantly elevated throughout the period of treatment ($p<0.05$; 300%, 547%, 946%, and 660% increase respectively compared to control) (Table 1A). A similar increase was also observed in the levels of direct bilirubin ($p<0.05$; 15.5%, 29.9%, and 24.1% respectively at weeks 2, 3, and 4) (Table 1A).

Similarly, treatment with therapeutic dose of nevirapine increased oxidative stress of the liver by significantly decreasing reduced glutathione (GSH) level at weeks 2 and 3 ($p<0.05$; 62% and 55% decrease respectively compared to control); increasing malondialdehyde (MDA) concentration at weeks 1, 2, 3, and 4 ($p<0.05$; 231%, 62.7%, 83.0%, and 110.0% increase respectively compared to control). (Table 1B) and induced reduction (significant at weeks 1 and 4) in SOD activity throughout the period of treatment (59.3%, 11.6%, 9.3%, and 32.6% reduction respectively). A decrease in catalase activity was observed throughout weeks 1, 2, 3, and 4 compared with control. The observed decrease in catalase activity was significant at week 1, 3, and 4 ($p<0.05$; 53.3%, 53.3%, and 34.5% decrease respectively). (Table 1B)

A significant decrease in protein concentration was observed in all groups compared with control during the course of the study. (Table 1B)

Histopathological analysis showed increase in the severity of hepatic degeneration during the course of treatment. At the first two weeks of drug administration, there was moderate diffuse hepatic degeneration (Plates 1B and 1C respectively). Conversely, by the third and fourth weeks of administration there was more severe hepatic degeneration (Plates 1D and 1E respectively).

CONCLUSION

This study shows that nevirapine induces hepatotoxicity and oxidative stress and the duration of exposure affects the extent of the damage.

Table 1A. Effect of Nevirapine on Biomarkers of Hepatic Damage

Treatment	ALT(U/L)	AST (U/L)	DIRECT BILIRUBIN (µg/ml)
Control	1.3±0.3	33.0±1.00	34.8±0.5
Week 1	5.3±1.15*(300%) **	34±7.0(5.2%) **	40.2±7.9(15.5%) **
Week 2	8.8±2.7*(577%) **	36.7±2.3*(11.2%) **	45.2±6.4*(29.9%) **
Week 3	13.6±1.7*(946%) **	44.0±5.7*(33.3%) **	43.2±3.8*(24.1%) **
Week 4	9.9±1.2*(660%) **	94.7±4.2*(187%) **	55.8±4.5*(60.3%) **

* p<0.05 when compared with control
** Percentage change compared with control alone.
n=8

Table 1B. Effect of Nevirapine on Liver Protein Concentration, Markers of Oxidative Stress and Antioxidant Enzyme Activities.

Treatment	Protein concentration (mg/ml)	MDA (nmol/g liver)	Reduced glutathione concentration (mg/ml)	SOD activity (Unit SOD)	Catalase activity (μmoles H_2O_2/ min/mg protein)
Control	25.0±4.2	35.13±11.13	10.0±1.9	4.3±0.4	0.26±0.04
Week 1	16.4±4.3*(36.0%)**	116.3±12.2*(231%)**	9.0±1.9(10%)**	1.8±0.5*(59.3%)**	0.12±0.03*(53.3%)**
Week 2	8.7±2.1*(65.2%)**	57.1±7.3*(62.7%)**	3.8±1.4*(62%)**	3.8±0.9 (11.6%)**	0.2±0.04(16.4%)**
Week 3	5.2±1.1*(79.2%)**	64.3± 5.5*(83.0%**	4.5±1.0*(55%)**	3.9±0.4 (9.3%)**	0.12±0.03*(53.3%)**
Week 4	10.3±3.1*(58.8%)**	74.0±7.9*(110%)**	9.0± 2.2(10%)**	2.9±0.7(32.6%)**	0.09±0.01*(34.5%)**

*p<0.05 compared with control
** Percentage change compared with control alone
n=6

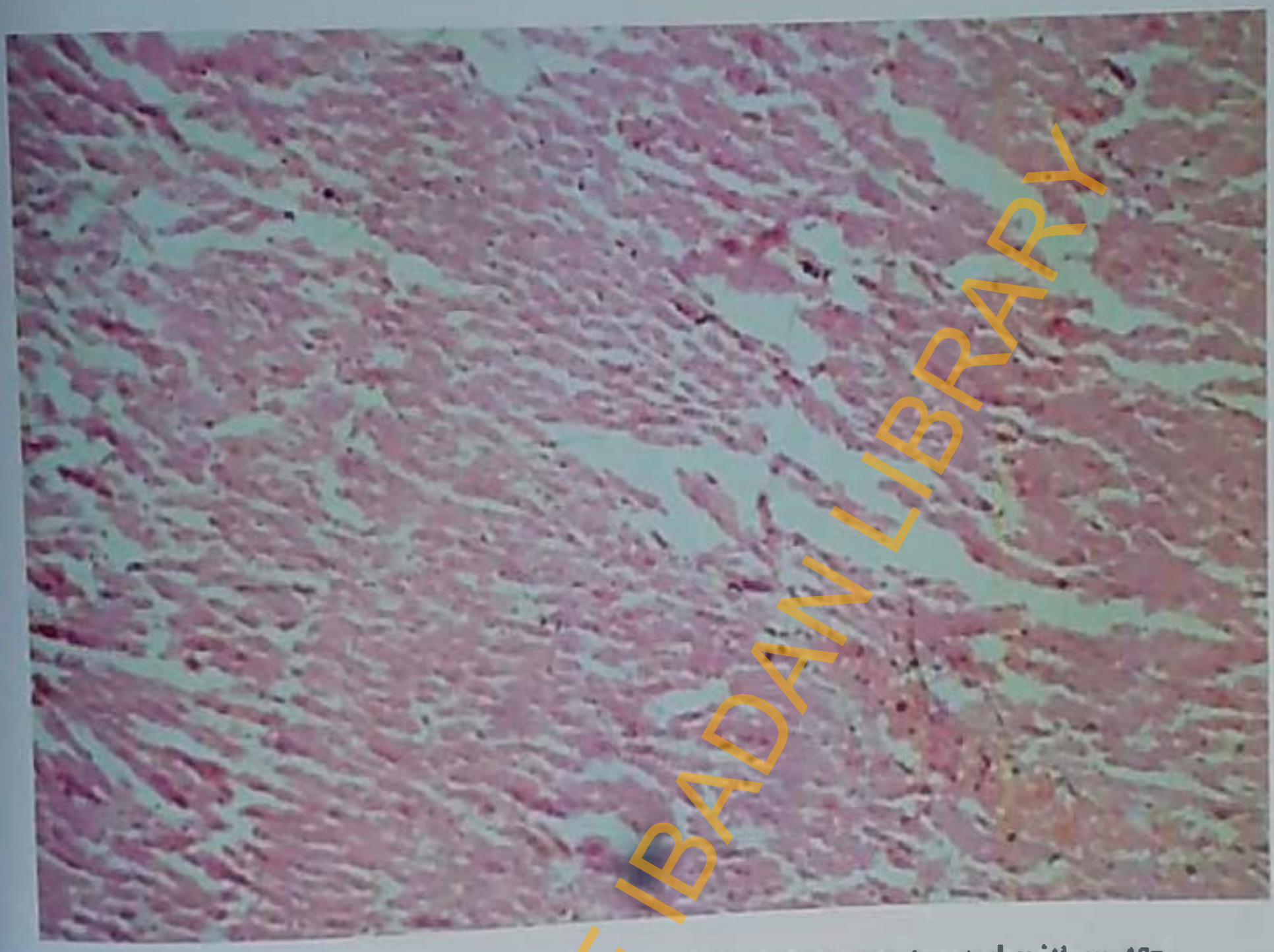


Plate I A. Histological sections (x400) of normal liver from rat treated with water only (Control) showing no visible lesions.

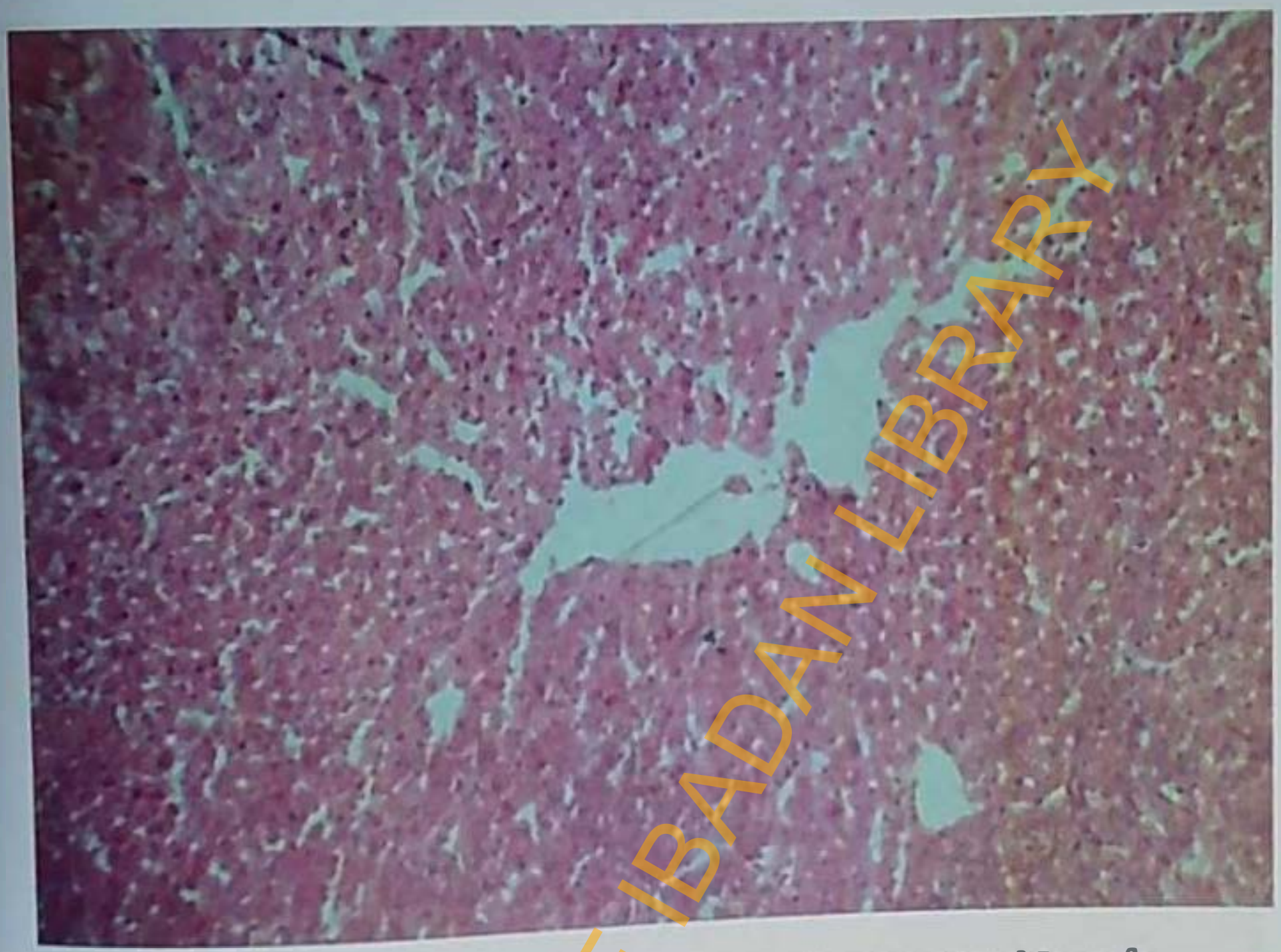


Plate 1B. Histological sections (x400) of liver from rat treated with 5.71 mg/kg (therapeutic dose) of nevirapine orally for 1 week. Section shows moderate diffuse hepatic degeneration.

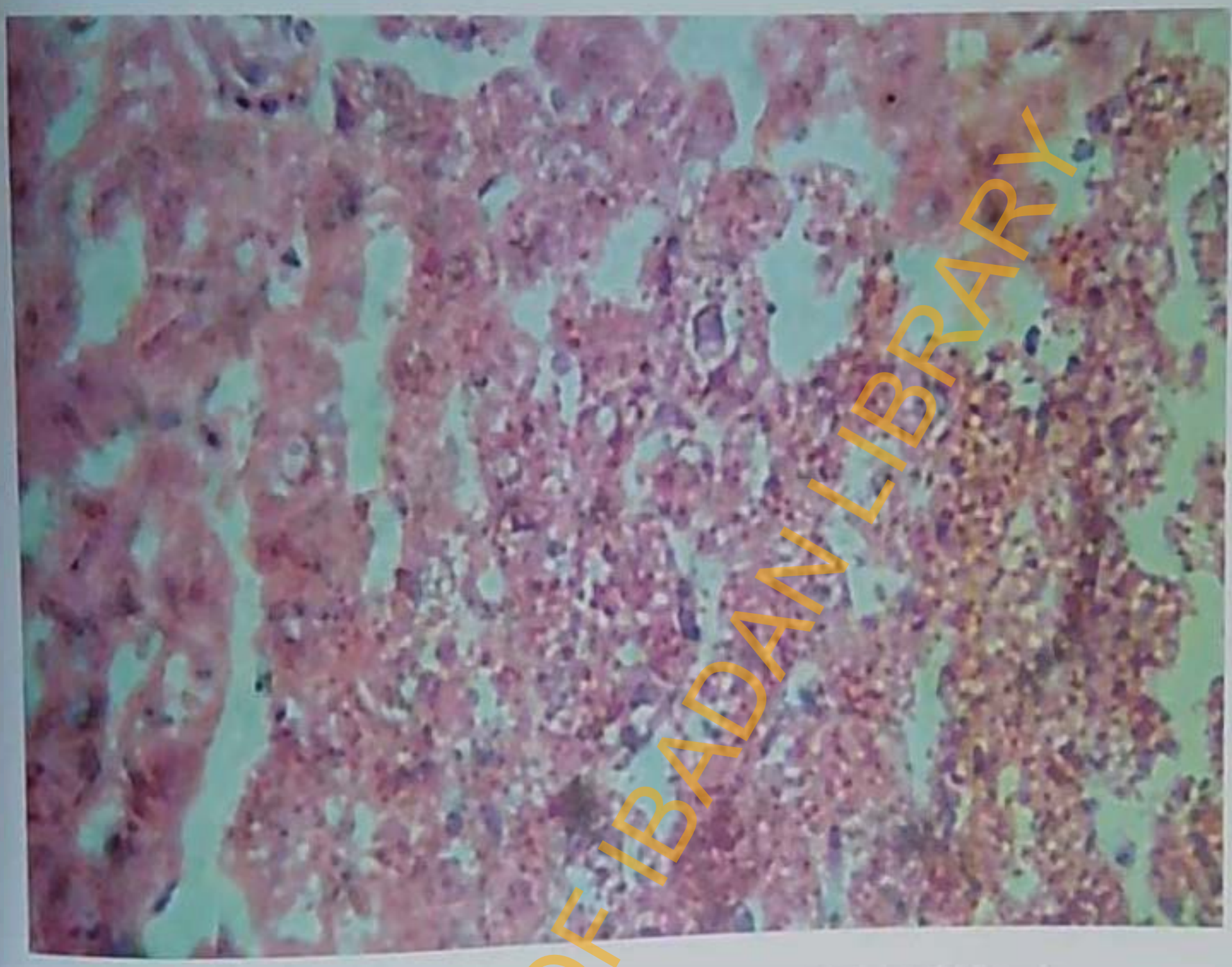


Plate 1C. Histological sections (x400) of liver from rat treated with 5.71 mg/kg (therapeutic dose) of nevirapine orally for 2 weeks. Section shows moderate diffuse hepatic degeneration.

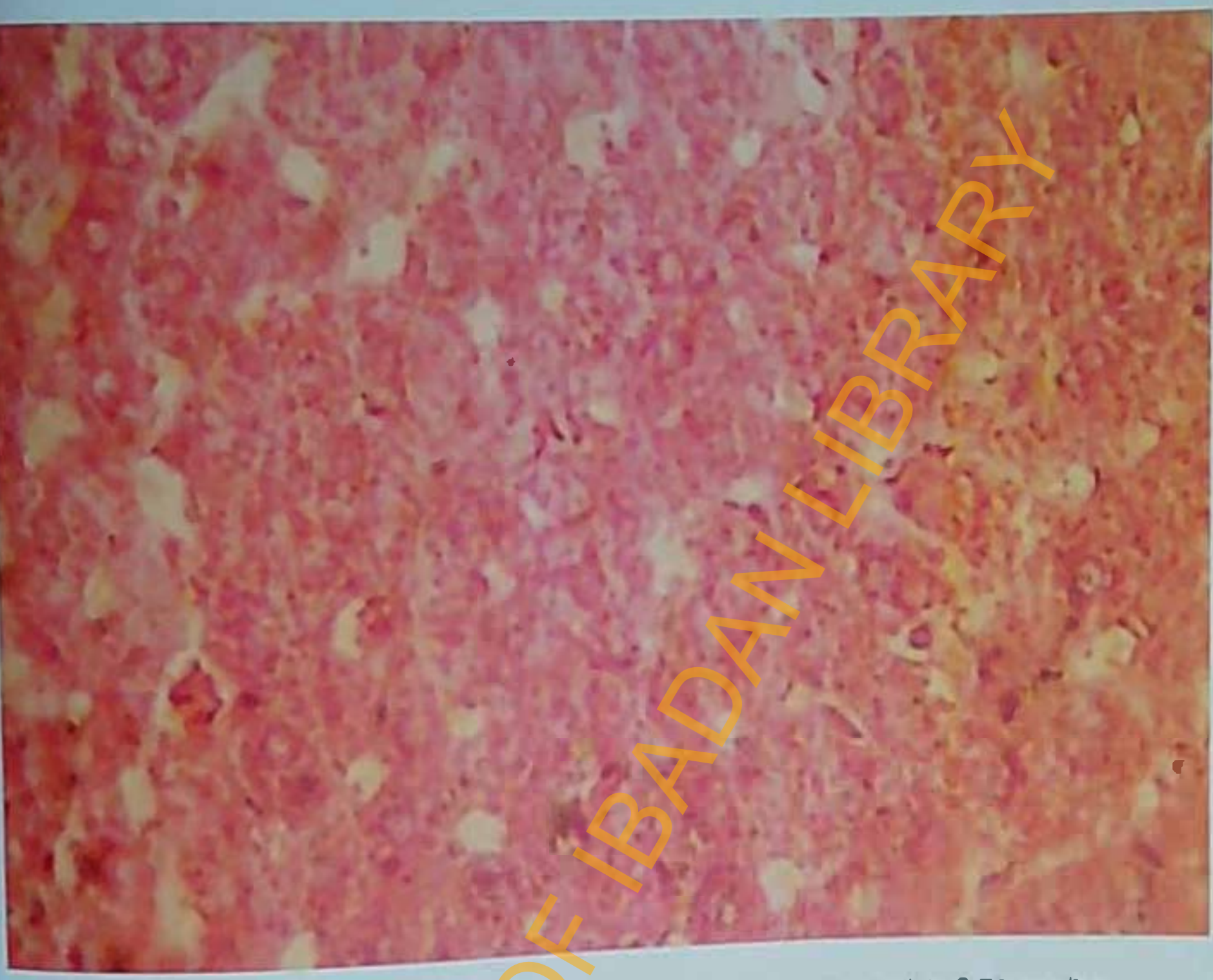


Plate 1D. Histological sections (x400) of liver from rat treated with 5.71 mg/kg (therapeutic dose) of nevirapine orally for 3 weeks. Section shows severe diffuse hepatic degeneration.

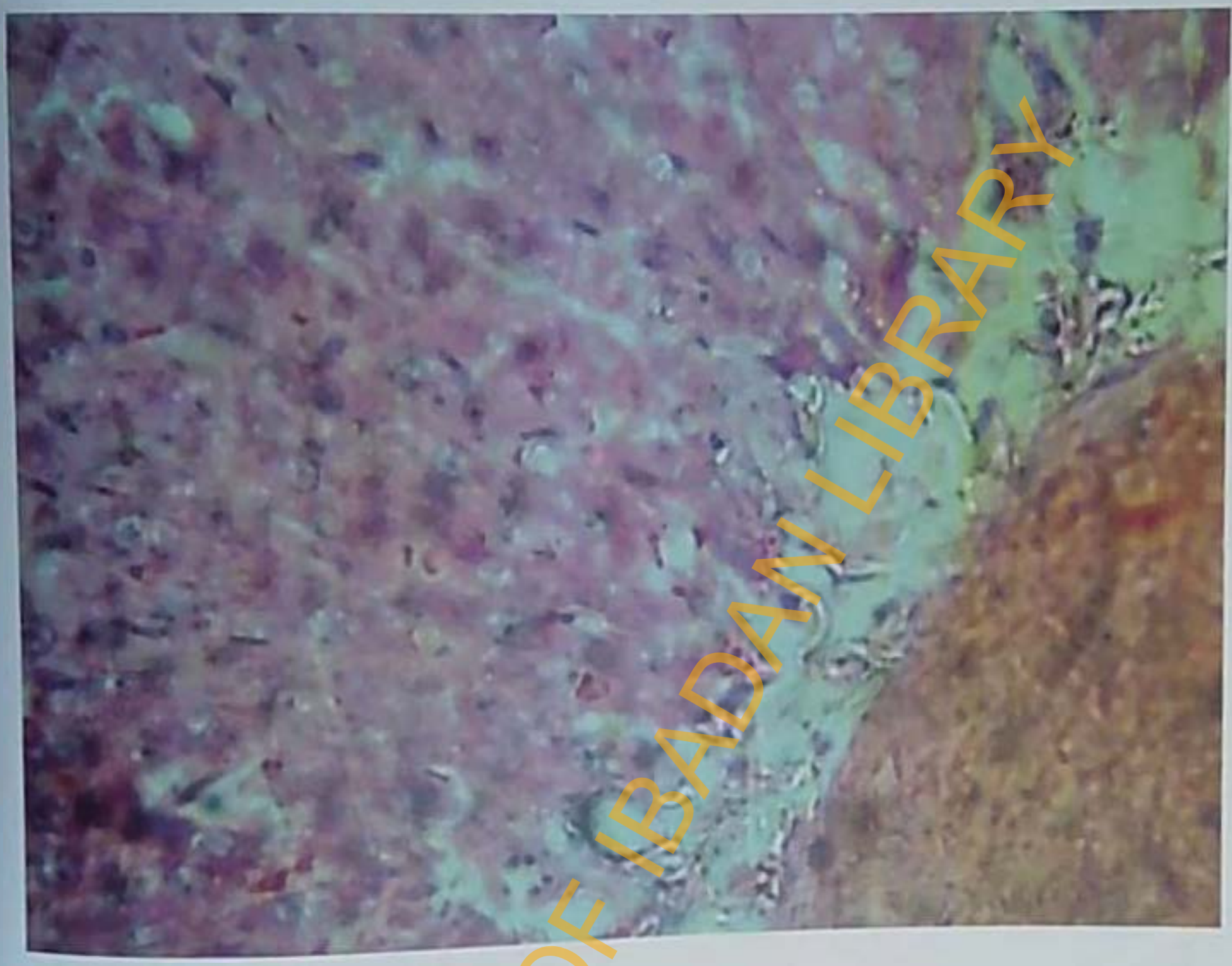
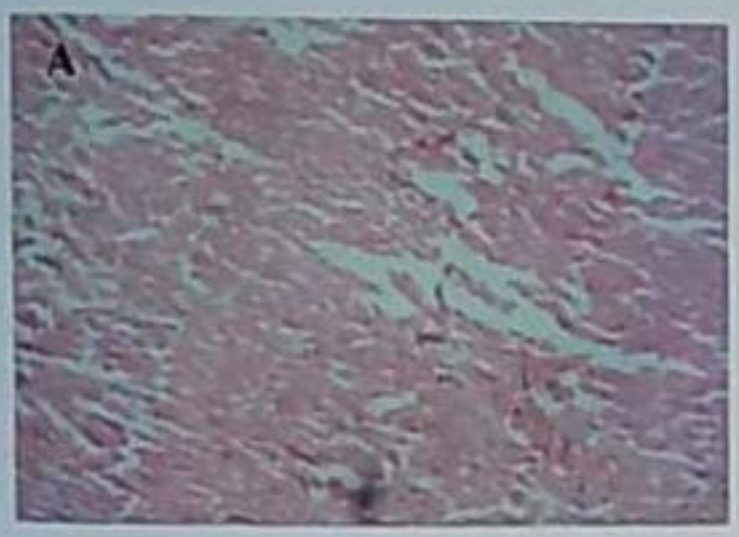
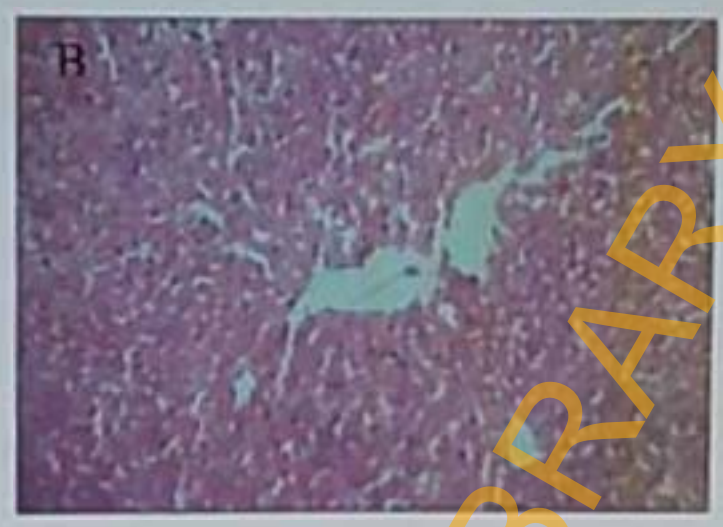
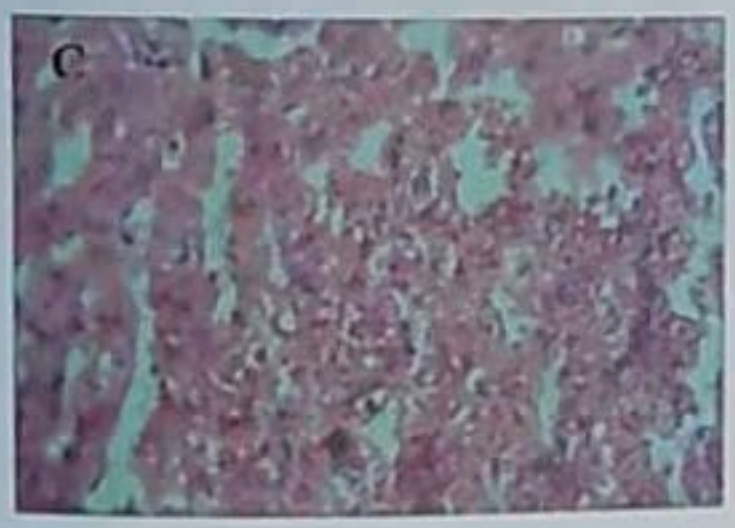


Plate 1E. Histological sections (x400) of liver from rat treated with 5.71 mg/kg (therapeutic dose) of nevirapine orally for 4 weeks. Section shows severe diffuse hepatic degeneration.







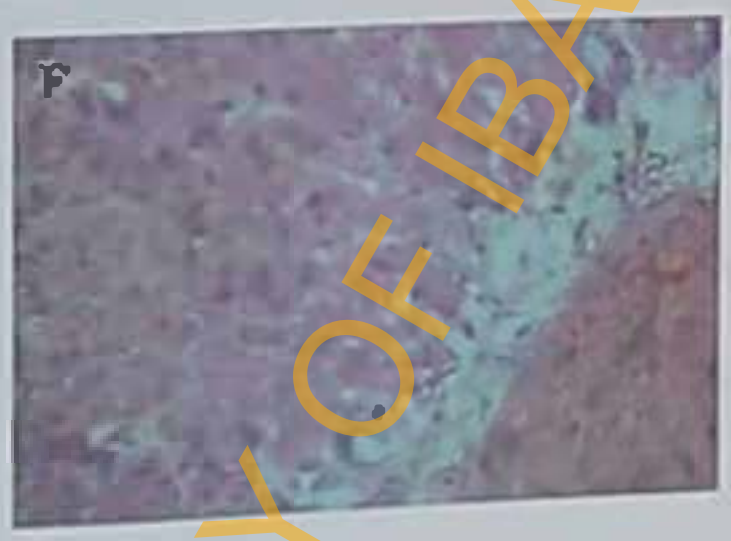


Plate IF. photomicrographs of rat liver sections (x400).
A- Control (water only) showing no visible lesions. B- Shows moderate diffuse hepatic degeneration at week 1. C- Shows moderate diffuse hepatic degeneration at week 2. D- Shows severe diffuse hepatic degeneration at week 3. E- Shows severe diffuse hepatic degeneration at week 4.

4.2 EXPERIMENT 2a: EFFECT OF NEVIRAPINE ON ANTIOXIDANT STATUS AND SOME INFLAMMATORY BIOMARKERS

INTRODUCTION

Nevirapine a NNRTI is known to induce life-threatening liver toxicity (Marina, *et al*., 2003; Bersoff-Matcha, *et al*., 2001) and for increased incidences of hepatoneoplasias in rodents (Physician Desk Reference USA, 2009). Inspite of substantial evidence that the use of the drug is associated with adverse reaction, the mechanisms for the adverse effects of Nevirapine are still unclear.

Hepatotoxicity is often caused by the direct action of a drug, or more often a reactive metabolite of a drug, against hepatocytes. In most instances of drug induced liver injury, it appears that hepatocyte damage triggers the activation of other cells which can initiate an inflammatory reaction and oxidative stress. These events may overwhelm the capacity of the liver for adaptive repair and regeneration thereby contributing to the pathogenesis of liver injury (Holt and Ju, 2006).

From the aforementioned, it will be reasonable to investigate whether these effects are involved in nevirapine-induced liver injury. Therefore, the present experiment was undertaken to investigate whether the stimulation of inflammatory responses and oxidative stress is involved in the mechanism of nevirapine-induced hepatotoxicity.

PROCEDURE

Fourteen male albino rats of Wistar strain weighing between 130-175 g were used for the assay. They were purchased from the animal house of the Faculty of Basic Medical Sciences, University of Ibadan and housed in cages in the animal house of Biochemistry Department, University of Ibadan. The animals were given pellet feed

They were randomly assigned to two groups with seven animals each. The groups were treated as follows: Group (control) received water only orally while Group 2 – received 5.71 mg/kg (therapeutic dose) of nevirapine orally for 3 weeks.

The rats were sacrificed by cervical dislocation on the last day of drug administration: blood was collected by cardiac puncture technique with the aid of clean needle and syringe into clean dry centrifuge tubes and allowed to coagulate by standing for 30 minutes. The blood samples were then centrifuged for 10mins at 3000 g using a bench centrifuge. The clear supernatant (serum) was collected and stored in the refrigerator.

Liver was quickly removed, rinsed in ice-cold 1.15% KCl, blotted and weighed. A portion of the liver was prepared for histological examination as described in section 3.2.3 and the remainder was then minced with scissors in 4 volumes of ice-cold 0.1M phosphate buffer pH 7.4 and homogenized using Potter-Elvehgen homogenizer. The homogenate was centrifuged at 10,000 g for 15 minutes at 4° C and the supernatant termed post mitochondrial fraction (PMF) were aliquoted and used for the enzymes assays.

The serum samples were used to determine ALT and AST activities according the method of Reitman and Frankel (1957). ALP by the method of Englehardt (1970). GGT using the method of Szasz (1967). TNFα and IL-1β concentrations were measured in rat serum by using an ELISA technique. PMF of liver was used to determine reduced glutathione level according to the method of Beutler *et al*. (1963). lipid peroxidation using the procedure of Vashney and Kale (1990). superoxide dismutase activity was determined as described by Misra and Fridovich (1972). catalase activity according to the method of Sinha (1971). protein concentration using biuret method as described by Gornal *et al*. (1949). HCl concentration

according to the method of Wolff (1994). GPx activity was determined by the method of Rotruck *et al.*, (1973). Vitamin C level was determined according to the method of Jakota and Dani (1982). The different assay procedures were previously described under materials and method in section 3.2.6 to 3.2.18.

RESULTS

The result of the present study showed that treatment orally with therapeutic dose of nevirapine caused elevation of serum levels of alanine (ALT) and aspartate (AST) aminotransferase compared with control at the third week of administration (Figure 2A). ALT levels was significantly elevated in the treatment group ($p<0.05$: 31.9% increase compared to control respectively) while AST level was elevated by 11.5% compared to control. There was also a significant elevation ($p<0.05$) in serum ALP and GGT levels during the course of administration (Figure 2A). (ALP increased by 189%, while GGT increased by 25.14% at the third week of administration when compared with control).

Also oral treatment with therapeutic dose of nevirapine increased oxidative stress of the liver by significantly decreasing reduced glutathione (GSH) level at week 3 ($p<0.05$; 53.1% decrease compared to control) (Table 2A); increasing malondialdehyde (MDA) concentration (29.0% increase compared to control) (Table 2A); and induced reduction in superoxide dismutase (SOD) activity (25.8%) (Table 2B). A decrease of 15.6% was observed in catalase (CAT) activity after weeks 3 compared with control respectively (Table 2B). The activity of the enzyme glutathione peroxidase (GPx) was also lowered by 16.7% when compared with control (Table 2B).

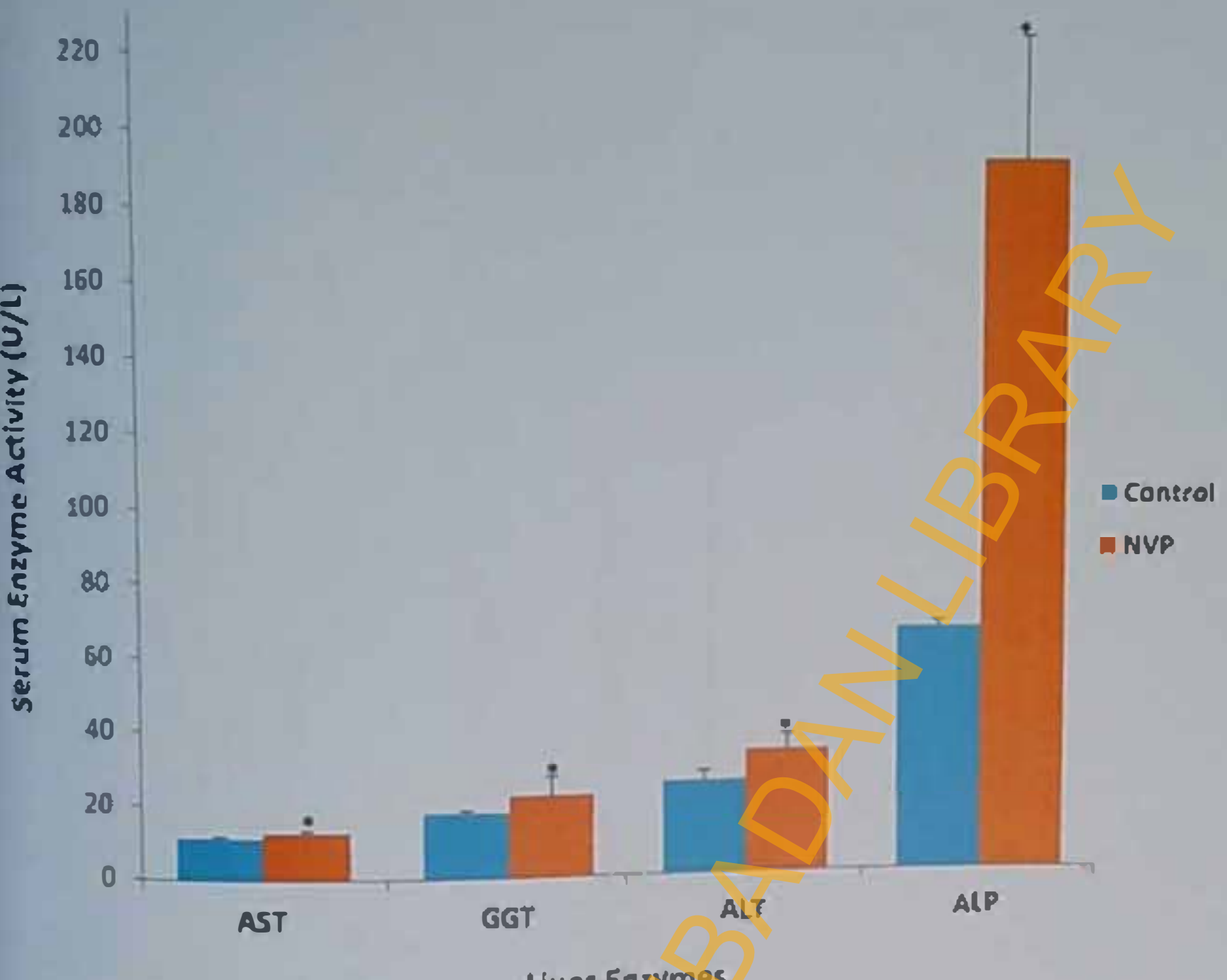
Nevirapine also caused an elevation of hydrogen peroxide concentration by 64.7% at the end of the third week when compared with control (Table 2A). Vitamin

C concentration was lowered by 33.3% at weeks 3 when compared with control (Table 2A). Furthermore, nevirapine also caused a significant elevation of tumor necrosis factor alpha (TNF α) and interleukin 1-beta (IL-1β). TNF α was increased by 22.5% at week 3 and IL-1β was increased by 25% when compared with control (Figure 2B).

Histopathological analysis showed severe hepatic degeneration, by the third week of administration of the drug when compared with control (Plates 2A and B respectively).

Conclusion

Nevirapine has shown the potential of inducing inflammation and oxidative stress.



* $p<0.05$ when compared with control
n=7

Figure 2A: Effect of Nevirapine on Biomarkers of Hepatic Damage.

Table 2A Effect of Nevirapine on Biomarkers of Hepatic Oxidative Stress

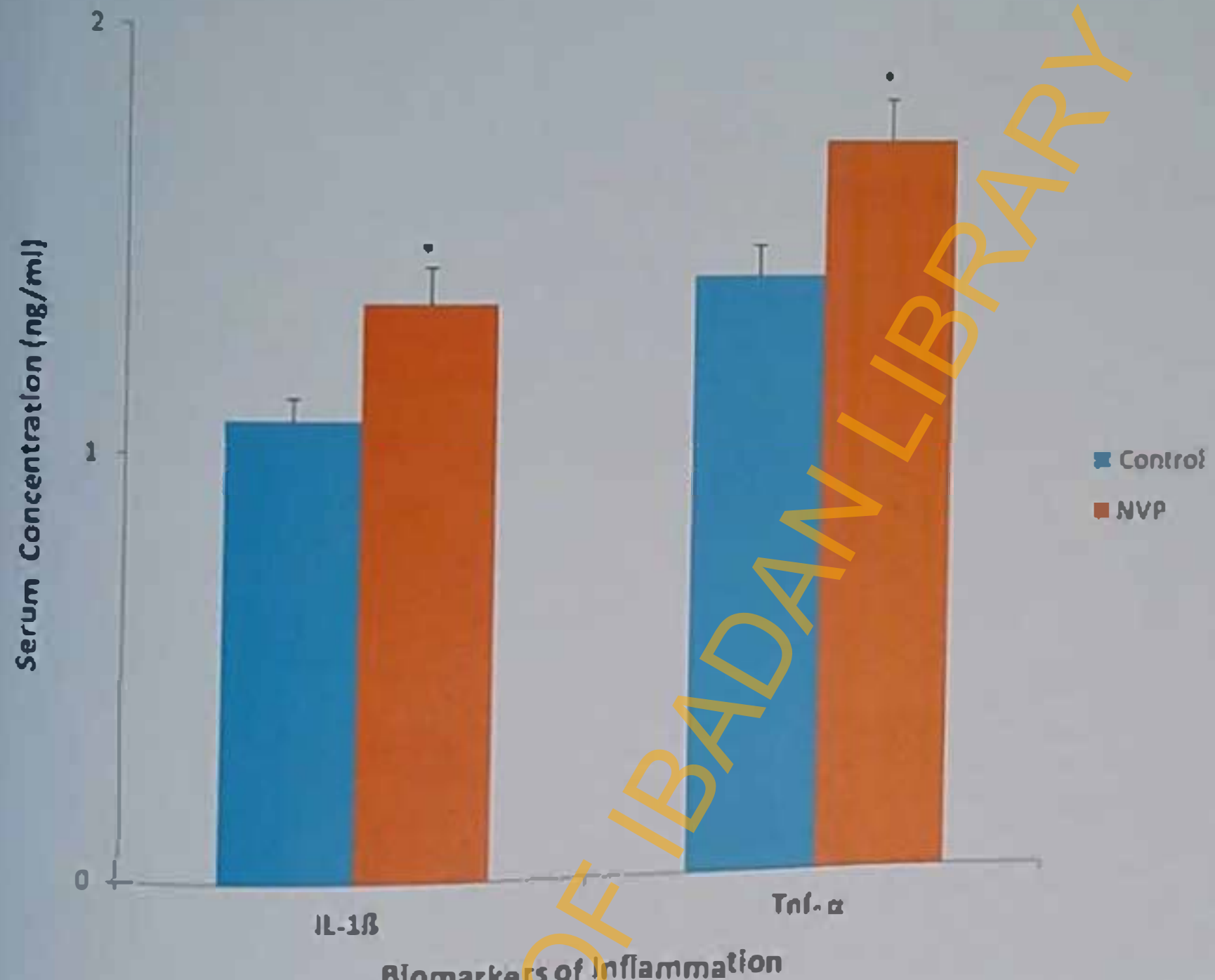
Treatment	MDA (nmol/g liver)	GSH (mg/ml)	H_2O_2 (μmolH_2O_2 consumed/min/ mgprotein)	VIT C (μg/ml)
Control	61.47±21.13	3.09±0.75	0.034±0.02	0.33±0.0067
Nevirapine	78.8±12.5(29.0%) **	1.45±0.38*(53.07%)**	0.056±0.019(64.7%) **	0.22±0.022(33.3)**

* $p<0.05$ when compared with control
** Percentage change compared with control alone.
n=7

Table 2B Effect of Nevirapine on Hepatic Antioxidant Enzyme Activities

Treatment	SOD (Unit SOD/mg protein)	CAT (μmol H_2O_2 consumed /min/mg protein)	GPx (unit/mg protein)
Control	0.062±0.022	0.045±0.02	11.5±4.18
Nevirapine	0.046±0.0052(25.8%)**	0.038±0.0043(15.6%) **	9.58±0.50(16.7%)**

* $p<0.05$ when compared with control
** Percentage change compared with control alone.
n=7



*p<0.05 when compared with control
n=7

Figure 2B: Effect of Nevirapine on Biomarkers of Inflammation.

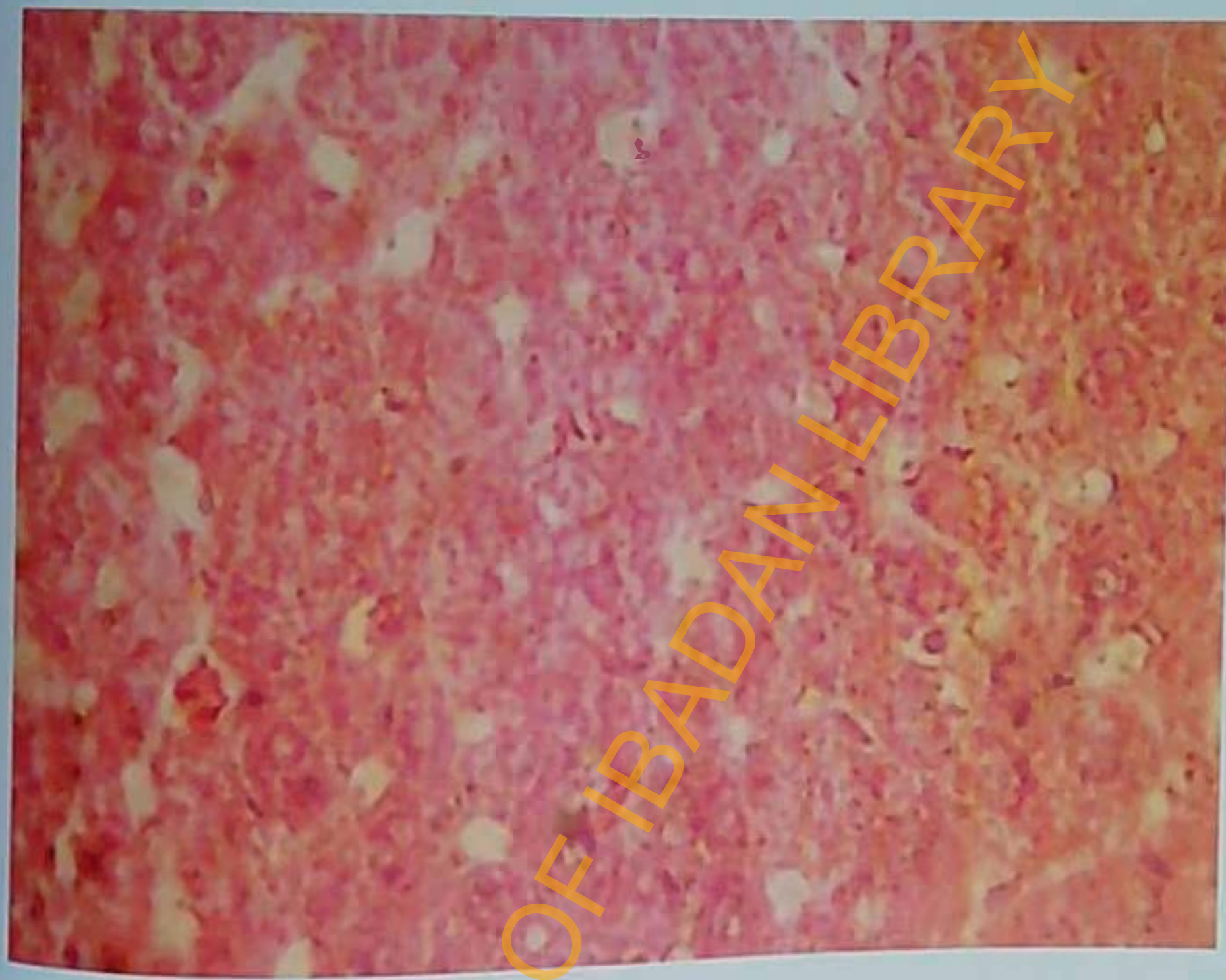


Plate 2A: Histological sections (x400) of liver from rat treated with 5.71 mg/kg (therapeutic dose) of nevirapine orally for 3 weeks. Section shows severe diffuse hepatic degeneration.

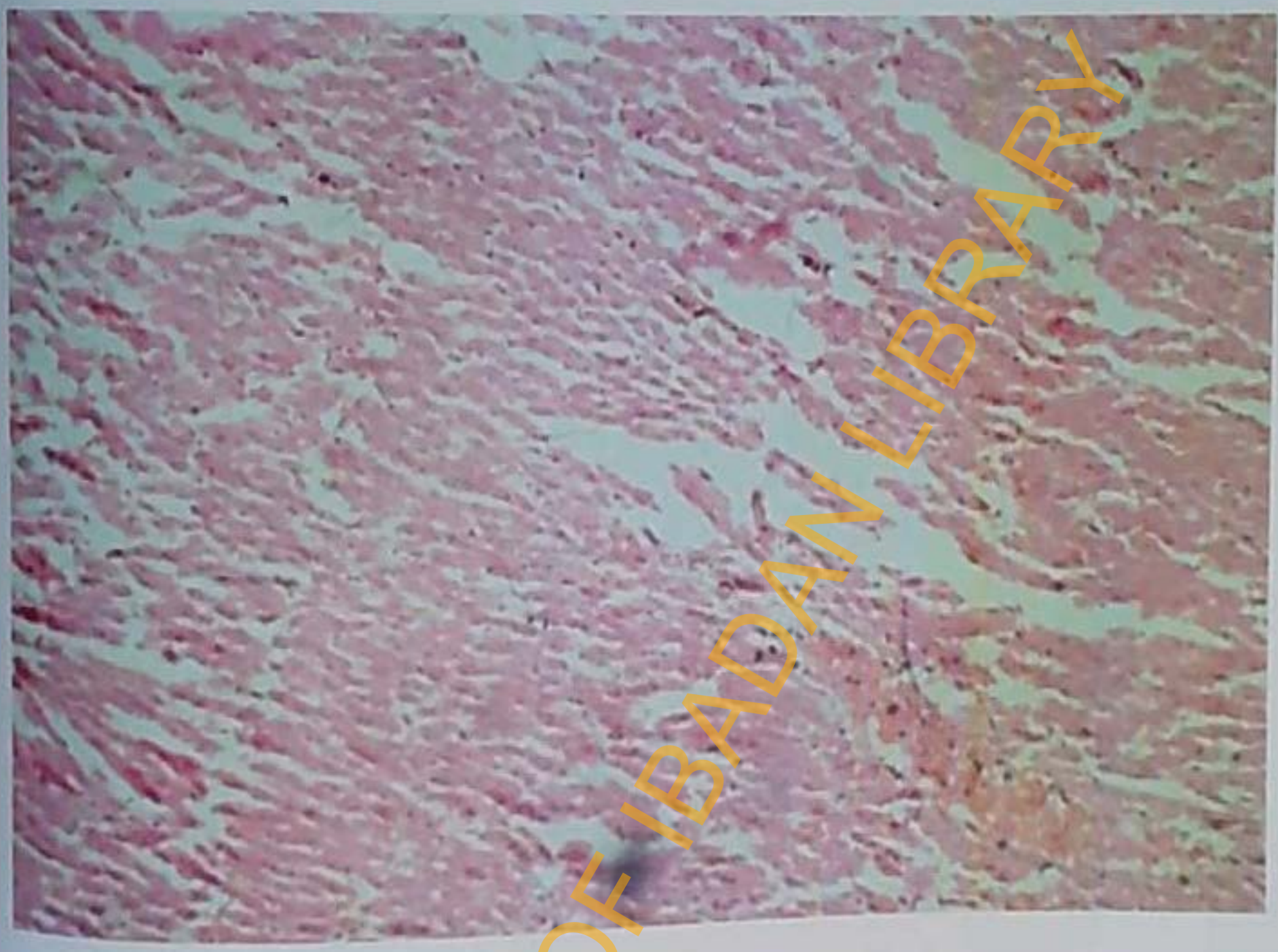


Plate 2B: Histological sections (x400) of normal liver from rat treated with water only (Control) showing no visible lesions.

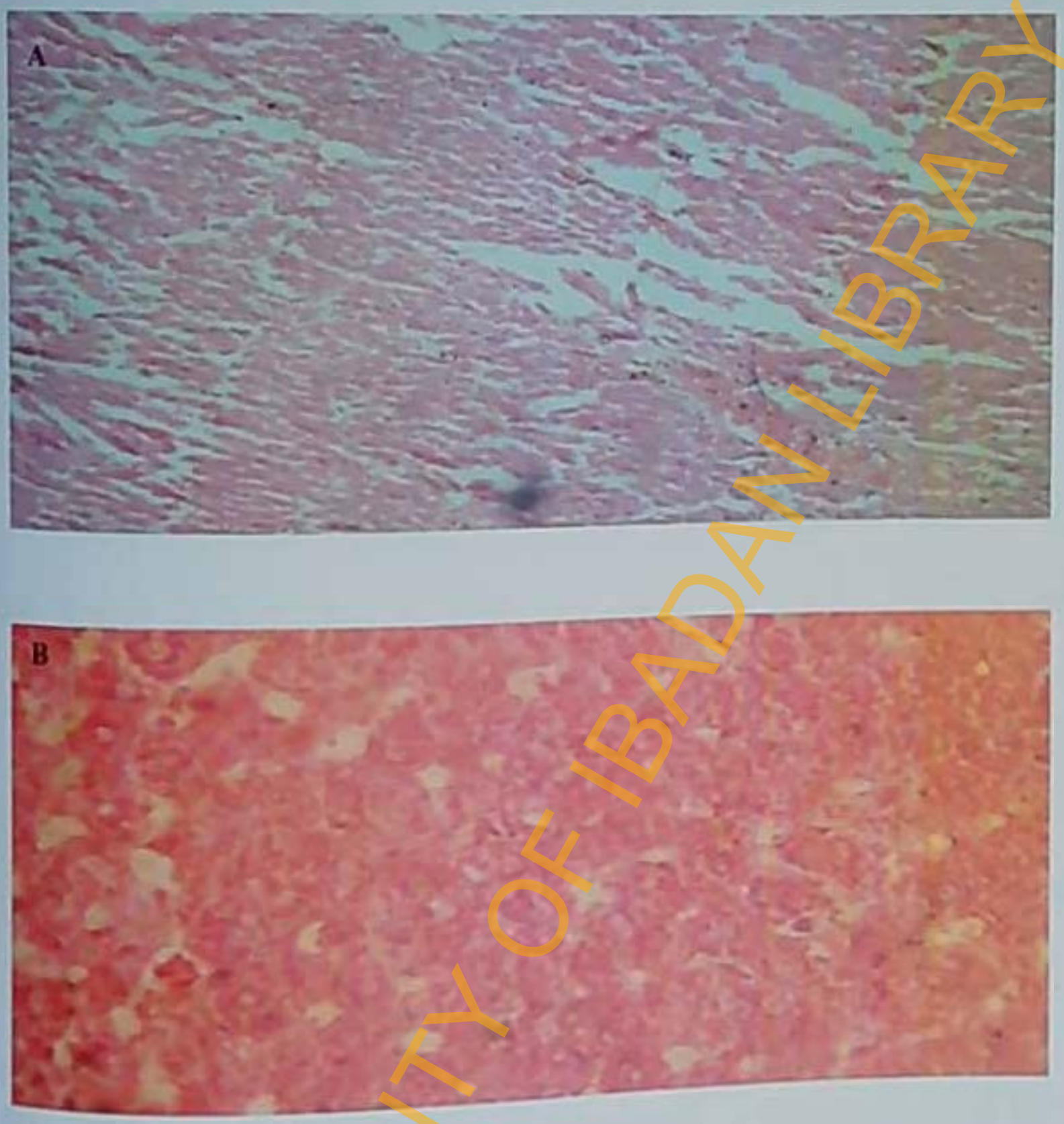


Plate 2C. Summary of Photomicrograph of rat liver sections (x400). A- Control (water only) showing no visible lesions. B- Shows severe hepatic degeneration at week 3.

EXPERIMENT 2b: INVESTIGATION OF THE GENOTOXIC POTENTIAL OF NEVIRAPINE USING MICRONUCLEI ASSAY

Introduction

The micronucleus test is one of the most widely applied short term test used in genetic toxicology and has become one of the most important tests implemented by the regulatory entities of different countries to evaluate mutagenicity of, and sensitivity to, xenobiotics (OECD, 1997; EPA, 1998).

Nevirapine (NVP) a non-nucleoside reverse transcriptase inhibitor (NNRTI), a drug of high efficacy has been shown to cause hepatocellular adenomas and carcinomas (Physicians' Desk Reference USA, 2009).

While the reasons for the adverse effects of NVP are still unclear, several in vitro and in vivo data are consistent with the involvement of NVP bioactivation, via Phase I oxidation to 12-hydroxy-NVP and subsequent Phase II sulfonation to 12-sulfoxy-NVP in the onset of toxic events elicited by the parent drug (Popovic *et al*., 2006; Wen et al., 2009; Chen *et al*., 2008; Srivastava *et al*, 2010).

This Phase II metabolite is a reactive electrophile, and therefore is expected to react directly with bionucleophiles (e.g., DNA) yielding covalent adducts. Antunes and coworkers showed that nevirapine metabolite has a potential of forming DNA adduct and could be genotoxic. This study therefore aims at investigating genotoxic potential of Nevirapine using Micronucleus Assay.

Procedure

Fourteen male albino rats, ages 6-8 weeks with body weight ranging from 40 - 60 g were used for the study. They were purchased from the animal house of the Faculty of Basic Medical Sciences, University of Ibadan and housed in cages in the animal house of Biochemistry Department, University of Ibadan. The animals were

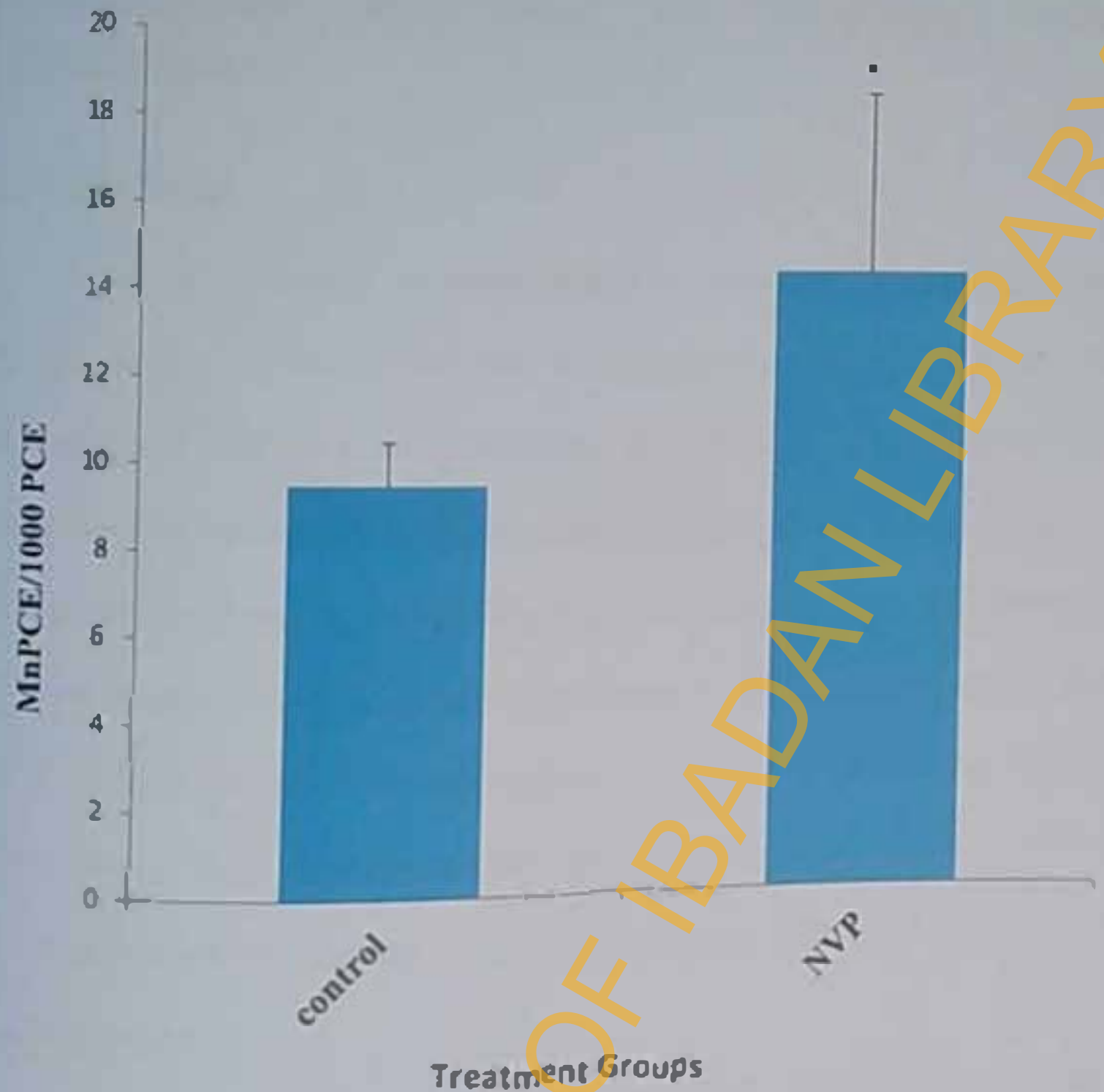
given pellet feed and water *ad libitum*. Rats were divided into two groups of 7 animals each. The groups were treated as follows: Group 1(control) received distilled water only while Group 2 (test group) received 5.71 mg/kg (therapeutic dose) of nevirapine orally for 3 weeks. Rats were sacrificed 24 hours after last administration through cervical dislocation and bone marrow was flushed from femur of each rat and spread onto slides. Slides were then air-dried, fixed and stained with maygrunward stain. Bone marrow cells were then examined microscopically and scored per animal for micro-nucleated polychromatic erythrocytes (PCE). The assay procedure was previously described under materials and method in section 3.2.27.

RESULTS

Result of our above study indicate that treatment with nevirapine caused a significant elevation ($p<0.05$; 52.6%) in the number of micronucleated polychromatic erythrocytes when compared with control.

CONCLUSION

This study suggests that nevirapine may be genotoxic.



$p \leq 0.05$ when compared with control

n=7

Figure 2C: Genotoxicity Test of Nevirapine using Micronuclei Assay

4.3 EXPERIMENT 3: THE MODULATORY EFFECT OF PROTOCATECHUIC ACID (PCA) ON NEVIRAPINE INDUCED HEPATOTOXICITY

INTRODUCTION

In our previous study We demonstrated the involvement of oxidative stress in nevirapine induced hepatotoxicity. This finding is also corroborated by the work of Adaramoye *et al*., (2012), and Adikwu *et al*., 2013. Among the mechanisms involved in hepatotoxicity induced by several xenobiotics is the oxidative damage due to free radical generation (Uma and Rao, 2005). Oxidative damage can accumulate in animal cells when the critical balance between generation of reactive oxygen species (ROS) / reactive nitrogen species (RNS) and antioxidant defense is unfavorable, a condition termed oxidative stress. It has been established that such oxidative damage is involved in the pathogenesis of diseases and adverse reactions associated with drug usage (Blomhoff, 2005).

Protocatechuic acid (3, 4-dihydroxybenzoic acid; PCA) is a natural phenolic compound found in many edible and medicinal plants (Justyna, 2005). PCA, a simple phenolic antioxidant compound, has been isolated from the dried flowers of *Hibiscus sabdariffa L.* (Malvaceae), and is an ingredient of a local beverage in Chinese herbal medicine used to treat hypertension, pyrexia and liver damage (Chen-Lan *et al*., 2002).

Studies have indicated that PCA could be used as a protective agent against cardiovascular diseases and neoplasms. It has been shown to have strong antioxidant and antitumor promotion effects (Tseng *et al*., 1996, 1998) and induced apoptosis in HL-60 human leukemia cells (Tseng *et al*., 2000). Besides that, PCA has been shown

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Studies have indicated that PCA could be used as a protective agent against cardiovascular diseases and neoplasms. It has been shown to have strong antioxidant and antitumor promotion effects (Tseng *et al*., 1996, 1998) and induced apoptosis in HL-60 human leukemia cells (Tseng *et al*., 2000). Besides that, PCA has been shown to possess chemopreventive activity by inhibiting the carcinogenic action of various

chemicals in different tissues. such as diethylnitrosamine in liver (Tanaka *et al*., 1993), 4-nitroquinoline-1-oxide in the oral cavity (Tanaka *et al*., 1994), azoxymethane in the colon), N-methyl-N-nitrosourea in glandular stomach tissue (Tanaka *et al*., 1995) and N-butyl-N- (4-hydroxybutyl) nitrosamine in the bladder (Hirose *et al*., 1995).

In view of the hepatoprotective and strong antioxidant potential of PCA, the present study was carried out to examine the protective influence of protocatechuic acid (PCA) on oxidative stress observed in nevirapine-induced hepatotoxicity in male Wistar albino rats.

PROCEDURE

Thirty six male albino rats of Wistar strain weighing between 150-170 g were used for this study. They were purchased from the animal house of the Faculty of Basic Medical Sciences, University of Ibadan and housed in cages in the animal house of Biochemistry Department, University of Ibadan. The animals were given pellet feed and water *ad libitum*. Rats were randomly divided into 6 groups. The groups were treated for 3 weeks as follows. Group 1 control received only the water orally. Group 2 received 50 mg/kg (PCA) only orally. Group 3 received 100 mg/kg (PCA) only orally. Group 4 received 5.71 mg/kg (therapeutic dose) of nevirapine only orally. Group 5 received 5.71 mg/kg (therapeutic dose) of nevirapine and 50 mg/kg PCA orally. Group 6 received 5.71mg/kg (therapeutic dose) of nevirapine and 100 mg/kg PCA orally. The rats were sacrificed by cervical dislocation 24 hours after the last administration; blood was collected by cardiac puncture technique with the aid of clean needle and syringe into clean dry centrifuge tubes and allowed to coagulate by standing for 30 minutes. The blood samples were then centrifuged for 10 mins at

3000g using a bench centrifuge. The clear supernatant (serum) was collected and stored in the refrigerator.

Liver was quickly removed, rinsed in icecold 1.15% KCl, blotted and weighed. A portion of the liver was prepared for histological examination as described in section 3.2.3 and the remainder was then minced with scissors in 4 volumes of ice-cold 0.1M phosphate buffer pH 7.4 and homogenized using Potter-Elvehgen homogenizer. The homogenate was centrifuged at 10,000g for 15 minutes at 4°C and the supernatant termed post mitochondrial fraction (PMF) were aliquoted and used for the enzymes assays. The serum samples were used to determine ALT and AST activities according the method of Reitman and Frankel (1957), ALP by the method of Englehardt (1970) and GGT using the method of Szasz (1967). PMF of liver was used to determine reduced glutathione level according to the method of Beutler *et al.*, (1963), lipid peroxidation using the procedure of Vashney and Kale (1990), superoxide dismutase activity was determined as described by Misra and Fridovich (1972), catalase activity according to the method of Sinha (1971), protein concentration using biuret method as described by Gornal *et al.*, (1949), H_2O_2 concentration according to the method of Wolff (1994), GPx by the method of Rotruck *et al.*, (1973), Proteincarbonyl contents according to the methods of Uchida and Stadiman (1993) and total antioxidant activity in samples was determined by the method of Koracevic *et al.*, (2001).

The different assay procedures were previously described under materials and method in section 3.2.6 to 3.2.22.

RESULTS

Treatment with nevirapine caused elevation of serum levels of ALT, AST, ALP, and GGT ($p<0.05$; 155%, 26%, 161% and 128% increase respectively compared to control). Whereas, co-treatment with 50mg/kg and 100mg/kg PCA

respectively caused 8% and 25% reduction in ALT, 172% and 81% reduction in ALP, 13% and 8.4% reduction in AST, 54% and 60% ($p<0.05$) reduction in GGT levels (Table 3A).

Similarly, nevirapine decreased significantly the reduced glutathione (GSH) level ($p<0.05$, 133.6% decrease compared to control); whereas co-administration with 50mg /kg and 100mg/ kg PCA ameliorated this condition by increasing the GSH level significantly when compared to the nevirapine only group ($p<0.05$; 74.6% and 50% respectively) (Table 3B). Also, nevirapine induced significant lipid peroxidation demonstrated by the high concentration of MDA compared to control. Co-treatment with PCA was able to ameliorate this condition by causing a significant reduction in MDA concentration when compared with nevirapine alone group (Table 3B).

There was also a 16% increase in hydrogen peroxide concentration in nevirapine alone treated rats when compared with control. 50 mg/kg and 100 mg/kg PCA however, brought about a 9% and 5% decrease in the hydrogen peroxide concentration respectively when compared with the nevirapine only group (Table 3B). Vitamin C concentration was however lowered by 8.6% in nevirapine-treated rats when compared with control. Treatment with 50 mg/kg and 100 mg/kg PCA elevated the Vitamin C concentration by 14.9% and 23.6% respectively (Table 3C).

A 41% decrease in GPx activity was observed in the group of rats that took nevirapine alone. Treatment with 50 mg/kg and 100 mg/kg PCA improved the GPx activity by 35% and 33% respectively. However, a 15% increase was observed in GST activity of the nevirapine alone treated rats (Table 3D).

Nevirapine also caused 48% reduction in SOD activity compared to control. Upon treatment with 50mg/kg and 100mg/kg PCA SOD activity increased by 7% and 5% respectively. Nevirapine also lowered catalase activity by 31% while treatment

with 50mg/kg and 100mg/kg PCA reversed this condition by increasing the enzyme activity 29% and 30 % respectively (Table 3D).

Nevirapine also brought about a significant ($p<0.05$, 22.6%),) increase in protein carbonyl concentration and decrease ($p<0.05$; 36.9%) in total antioxidant capacity. Co-treatment with PCA at both doses was able to ameliorate these effects by causing a significant ($p<0.05$) reduction in protein carbonyl concentration and significant ($p<0.05$) increase in total antioxidant capacity (Table 3C).

We found that liver samples treated with nevirapine exhibited severe hepatic necrosis (Plate 3E) compared with control (Plate 3A). However, in PCA treated groups, integrity of the liver cells were well preserved (Plate 3B and 3D) while Plates 3C and F show that PCA attenuated the hepatic necrosis induced by nevirapine.

CONCLUSION

In conclusion, PCA exhibited the ability to alleviate liver injury associated with nevirapine drug administration by reducing oxidative stress and hepatic damage.

Table 3A. Effect of Protocatechuic Acid (PCA) on Biomarkers of Hepatic Damage in Rats Treated with Nevirapine

Treatment	ALT(U/L)	ALP (U/L)	GGT (U/L)	AST (U/L)
Control	3.2±0.7	15.2±2.0	2.70±0.7	8.1±0.8
PCA 50mg/kg	3.7±0.5(16.3%) ••	15.9±3.5(4.5%) ••	4.01±1.5(48.5%) ••	8.6±1.8(5.7%) ••
PCA 100mg/kg	4.3±1.1(34%) ••	17.4±4.2(14.6%) ••	4.2±1.8(55.6%) ••	8.5±1.0(4.4%) ••
NEVIRAPINE 5.71mg/kg	8.10±0.5*(155%) ••	40.0±9.8*(163.6%)••	6.2±0.7*(129%) ••	10.3±0.2(27.0%)***
PCA50mg/kg+ NEVIRAPINE 5.71mg/kg	7.4±0.7*(8.6%) •••	14.7±1.6^{a}(63.1%)•••	4.1±0.9^{a}(34.4%)•••	9.03 ±2.9*(13%)•••
PCA100mg/kg+ NEVIRAPINE 5.71mg/kg	6.4±1.3*(21.0%)•••	22.1±3.90^{a}(45%)•••	3.9±0.7^{a}(37%)•••	9.5±1.8(8.1%)•••

* p<0.05 when compared with control
^{a} p<0.05 when compared with nevirapine alone
•• Percentage change compared with control
••• Percentage change compared with nevirapine alone.

Table 3B. Effect of Protocatechuic Acid (PCA) on Liver Lipid Peroxidation (MDA), Reduced Glutathione (GSH) Level and Hydrogen Peroxide (H_2O_2) Concentration in Rats Treated with Nevirapine

Treatment	MDA (nmol/g liver)	GSH (mg/ml)	H_2O_2 (μmol H_2O_2/ min/mg protein)
Control	188.±8.7	1.6±0.4	0.228±0.04
PCA 50mg/kg	213+55.7 (13.3%) **	1.1±0.3 (35.2%) **	0.249±0.01(9.2%)**
PCA 100mg/kg	214.5±1.2 (13.8%)**	1.1±0.4 (30.9%) **	0.22±0.03(3.6%)**
NEVIRAPINE 5.71mg/kg	366±39*(94.6%) **	0.4±0.1* (78.4%) **	0.264±0.05(15.7%)**
PCA50mg/kg+ NEVIRAPINE 5.71mg/kg	245.5±20*(33.06%)**	1.37±0.7*# (29.1%) ***	0.24±0.03(9.09%)***
PCA100mg/kg+ NEVIRAPINE 5.71mg/kg	262.8±47*(28.4%)***	0.75±0.122*# (114%)***	0.25±0.07(5.3%)***

* p<0.05 when compared with control
# p<0.05 when compared with Nevirapine alone
** Percentage change compared with control
*** Percentage change compared with Nevirapine alone

Table 3C. Effect of Protocatechuic Acid (PCA) on Vitamin C (Vit C), Total Antioxidant Capacity and Protein Carbonyl Levels in Rats Treated with Nevirapine

Treatment	Vit C (µg/ml)	Total antioxidant capacity (µmol/mg protein)	Protein carbonyl (nmol/g tissue)
Control	0.139±0.019	0.650±0.006	0.100±0.015
PCA 50mg/kg	2.0±0.03*(42.3%)**	0. 72± 0.04	0.098±0.011
PCA 100mg/kg	0.3±0.03*(117%)**	0.60±0.09	0.1172±0.008
NEVIRAPINE 5.71mg/kg	0.13±0.02(8.6%)**	0.41±0. 16*	0.13±0.015*
PCA50mg/kg+ NEVIRAPINE 5.71mg/kg	0.11±0.005*(14.9%)***	0.69±0.06**	0.099±0.02**
PCA100mg/kg+ NEVIRAPINE 5.71mg/kg	0.157±0.023(23.6%)***	0.54± 0.12	0.102±0.016**

*p<0.05 when compared with control
*p<0.05 when compared with Nevirapine alone
** Percentage change compared with control
*** percentage change compared with Nevirapine alone
n=6.

Table 3D. Effect of Protocatechuic Acid (PCA) on Antioxidant Enzyme Activities in Rats Treated with Nevirapine

Treatment	GPx (unit/mg protein)	GST (U/L)	SOD (unit/mg protein)	CAT (μmol H_2O_2/min/mg protein)
Control	12.8±3.3	0.29±0.03	0.1±0.05	0.5±0.01
PCA 50mg/kg	11.6±1.5(10%)**	0.36±0.04(24.1%)**	0.14±0.02(4%)**	0.4±0.07(20%)**
PCA 100mg/kg	8.4±2.3(34.4)**	0.27±0.02(6.9%)**	0.14±0.04(4%)**	0.36±0.07(28%)**
NEVIRAPINE 5.71mg/kg	7.5±1.2*(41.47)**	0.34±0.03*(14.7%)**	0.06±0.03(40%)**	0.35±0.06*(30%)**
PCA50mg/kg+ NEVIRAPINE 5.71mg/kg	12.7±1.01^b(69.33%)****	0.22±0.007*(35.2%)***	0.064±0.008(6%)***	0.49±0.05^b (40%)***
PCA100mg/kg+ NEVIRAPINE 5.71mg/kg	12.1±2.8^a(61.33)***	0.32±0.006(5.9%)***	0.14±0.01^a(133%)***	0.50±0.1^a(42.9%)***

* p<0.05 when compared with control
^{a} p<0.05 when compared with Nevirapine
** Percentage change compared with control
*** Percentage change compared with Nevirapine alone
n=6.

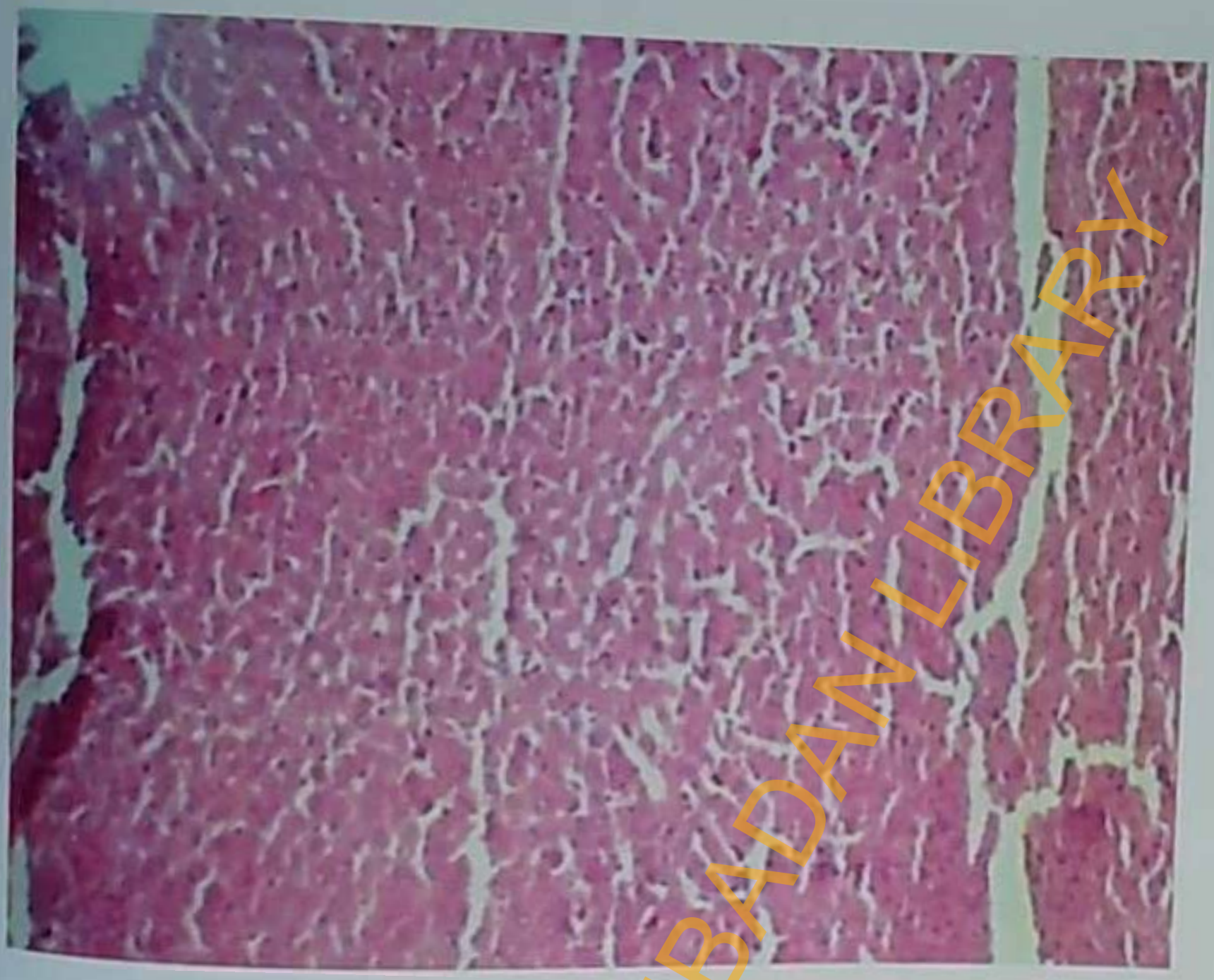

Plate 3A: Histological sections (x400) of normal liver from rat treated with water only (Control) showing no visible lesions.

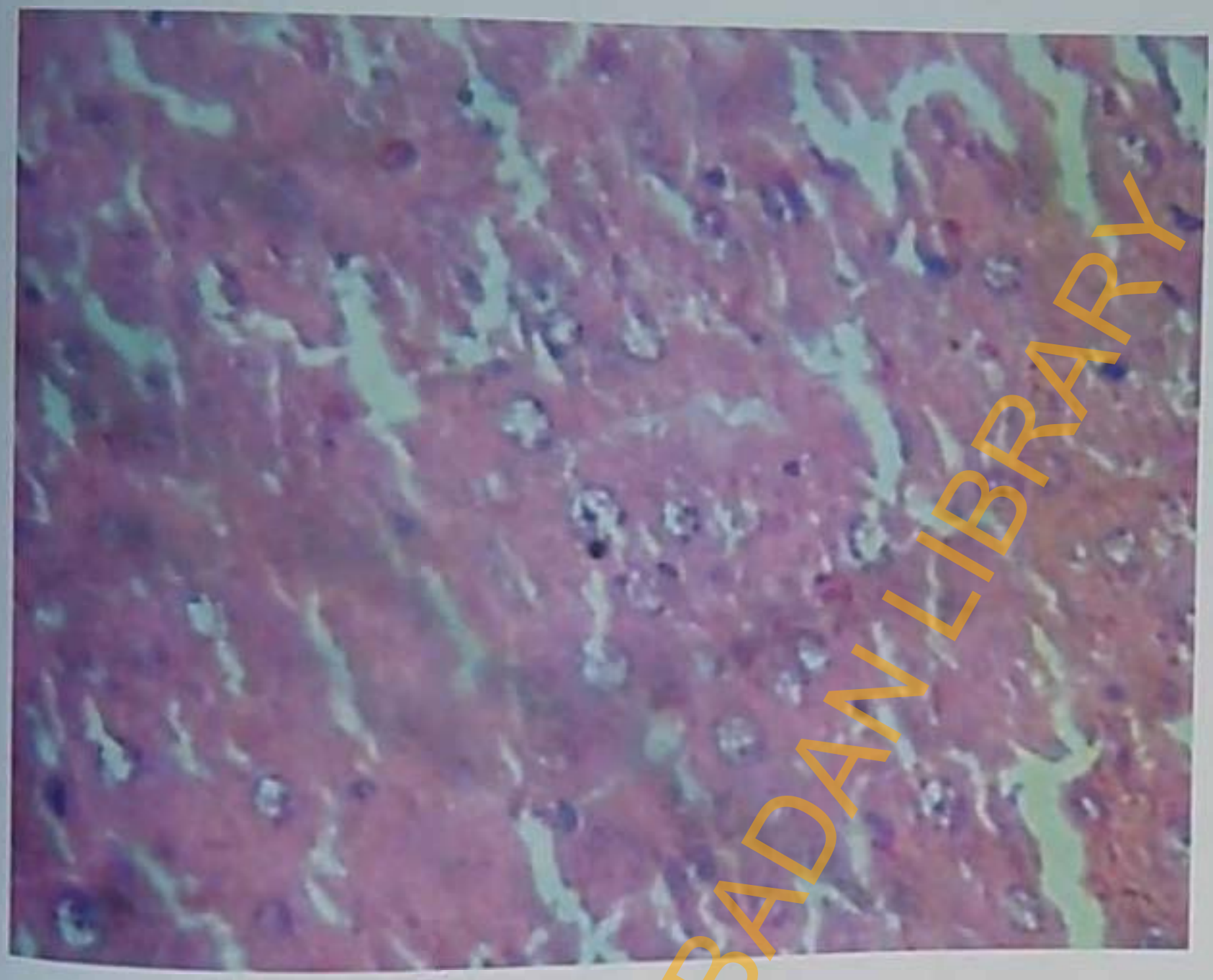

Plate 3B: Histological sections (x400) of liver from rat treated with 50mg/kg PCA alone orally for 3 weeks. Section shows no visible lesions.

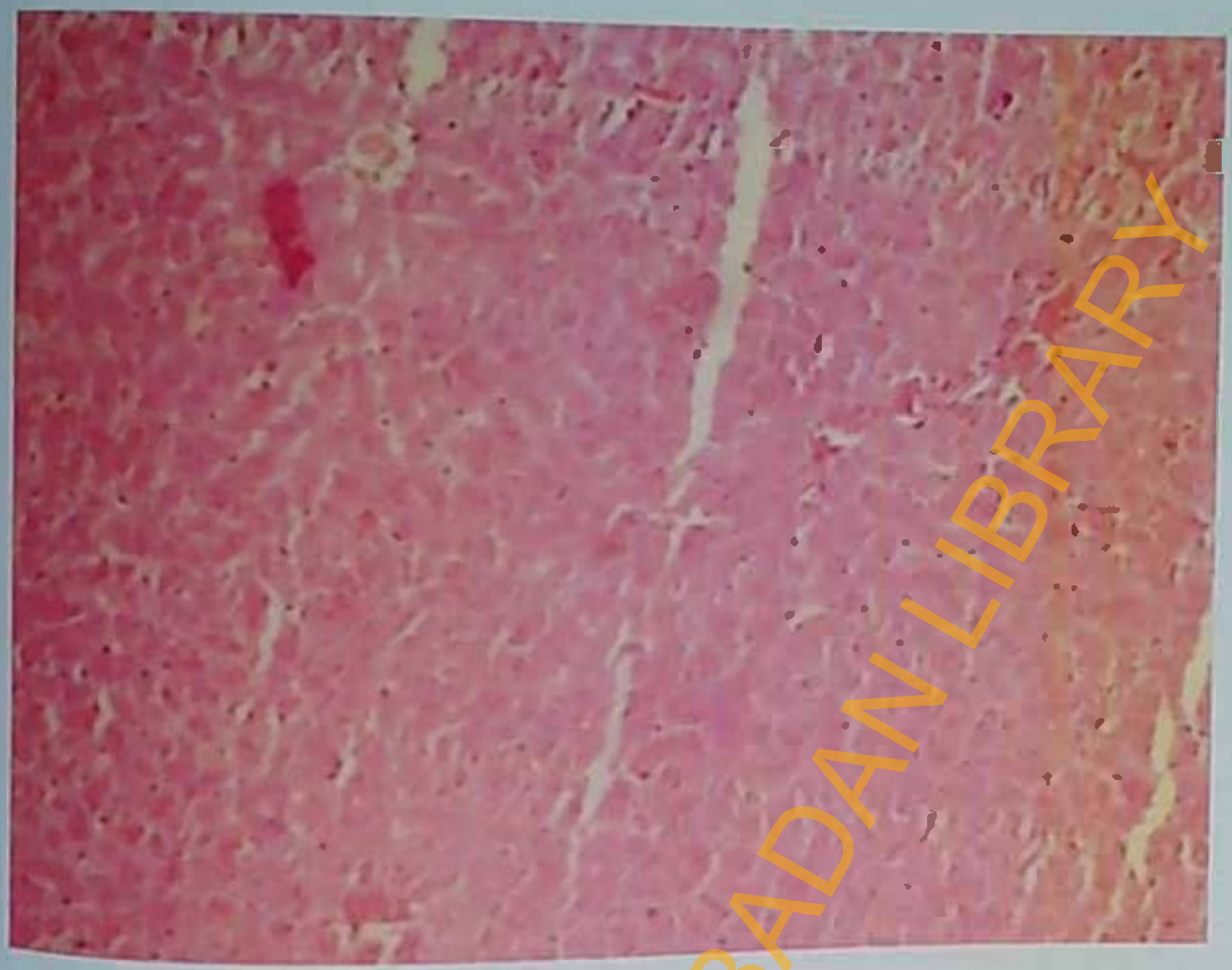

Plate 3C: Histological sections (x400) of liver from rat treated with 50mg/kg PCA and 5.71 mg/kg Nevirapine orally for 3 weeks. Section shows no visible lesions.

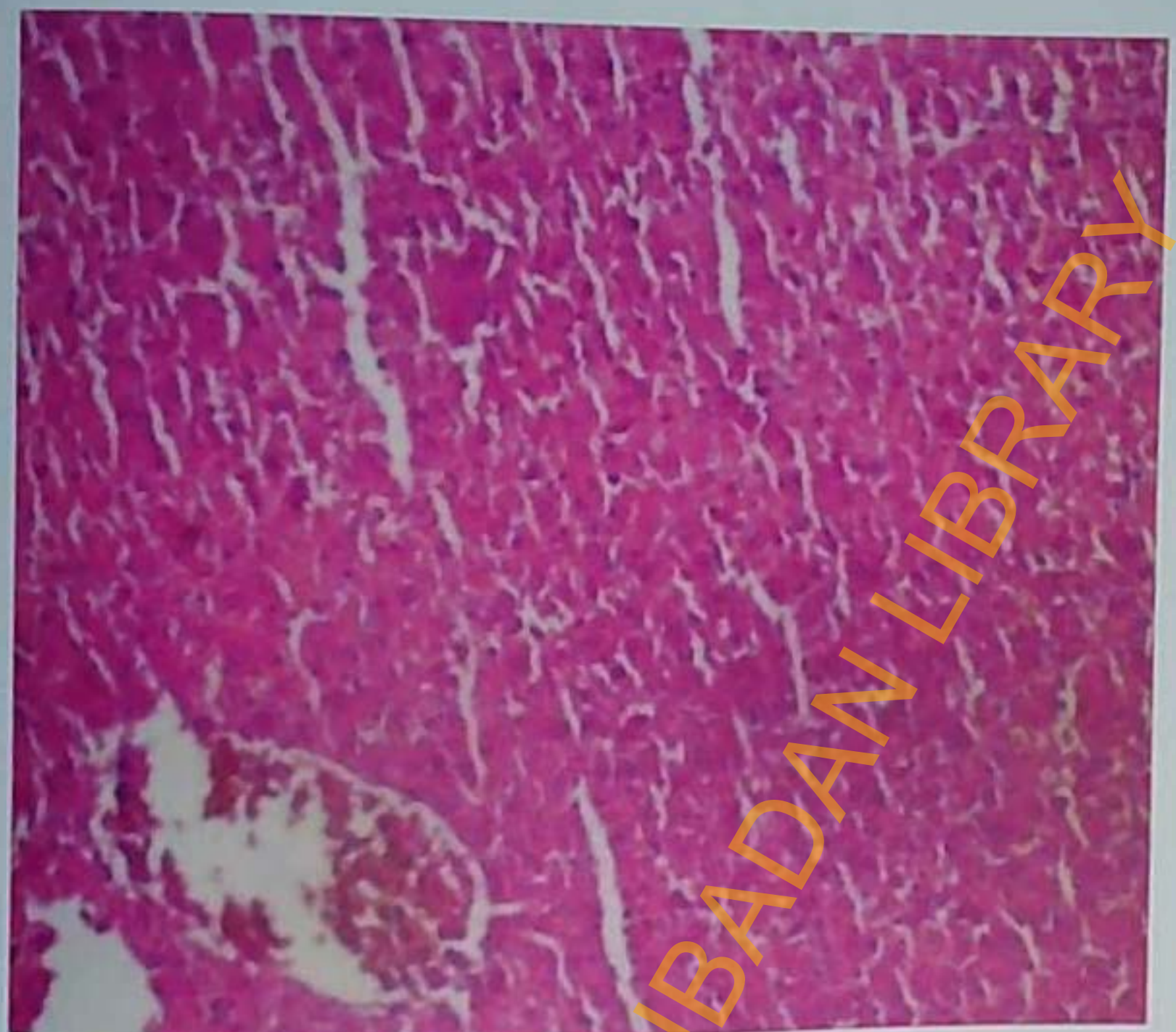

Plate 3D: Histological sections (x400) of liver from rat treated with 100mg/kg PCA alone orally for 3 weeks. Section shows no visible lesions.

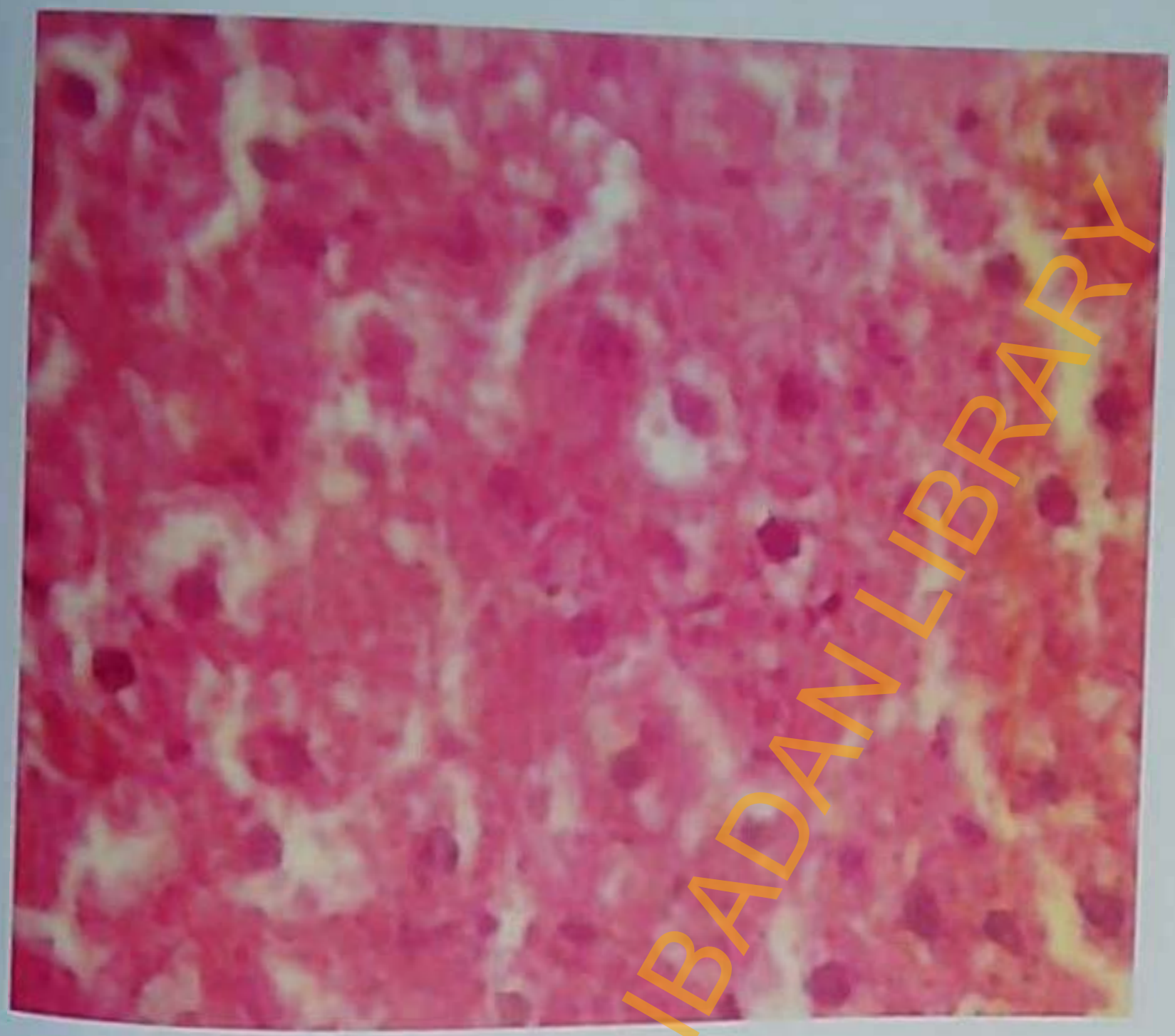

Plate 3E: Histological sections (x400) of liver from rat treated with 5.71mg/kg Nevirapine alone orally for 3 weeks. Section shows severe necrosis.

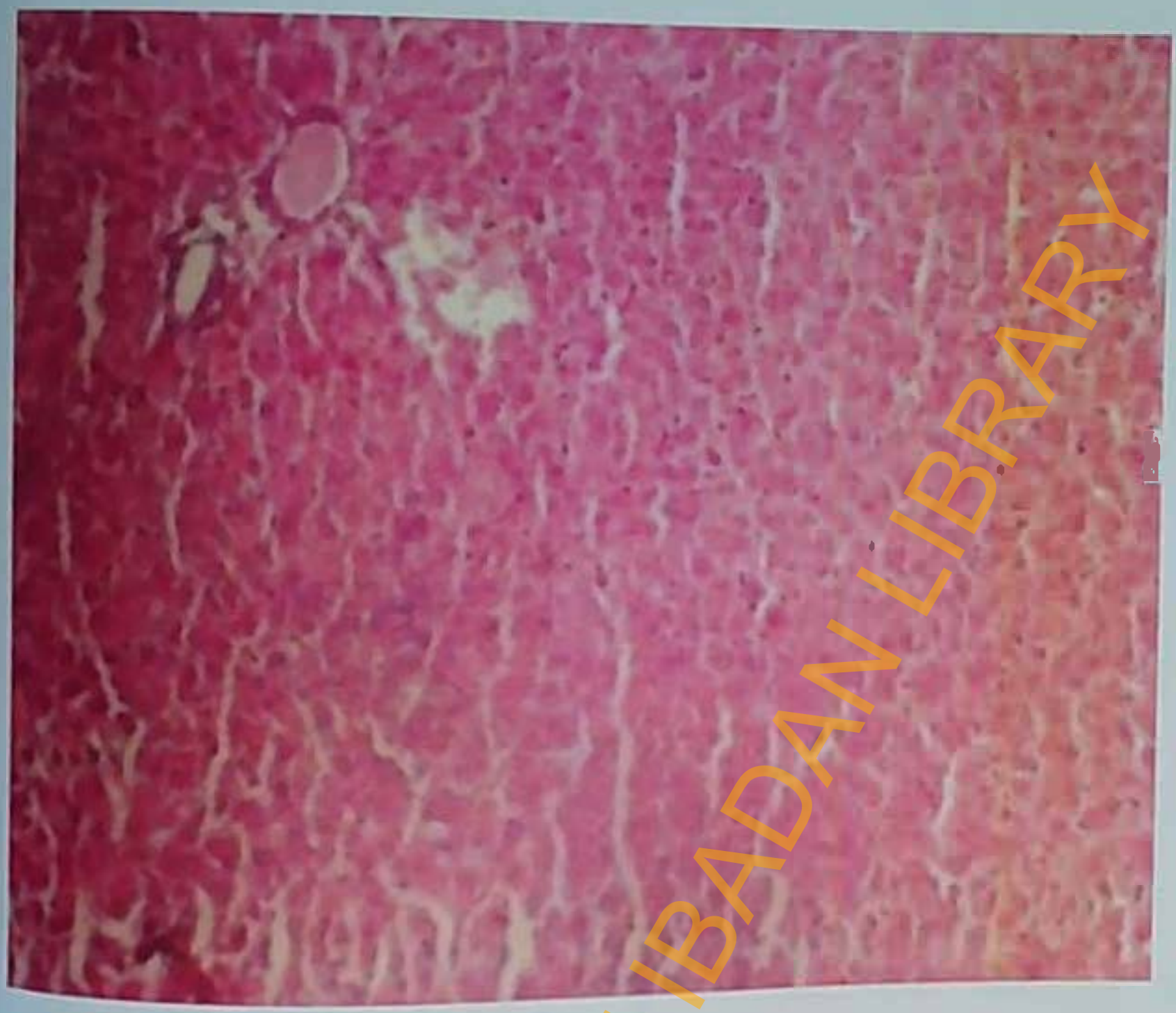

Plate 3F: Histological sections (x400) of liver from rat treated with 100mg/kg PCA and 5.71mg/kg Nevirapine orally for 3 weeks. Section shows severe no visible lesions.

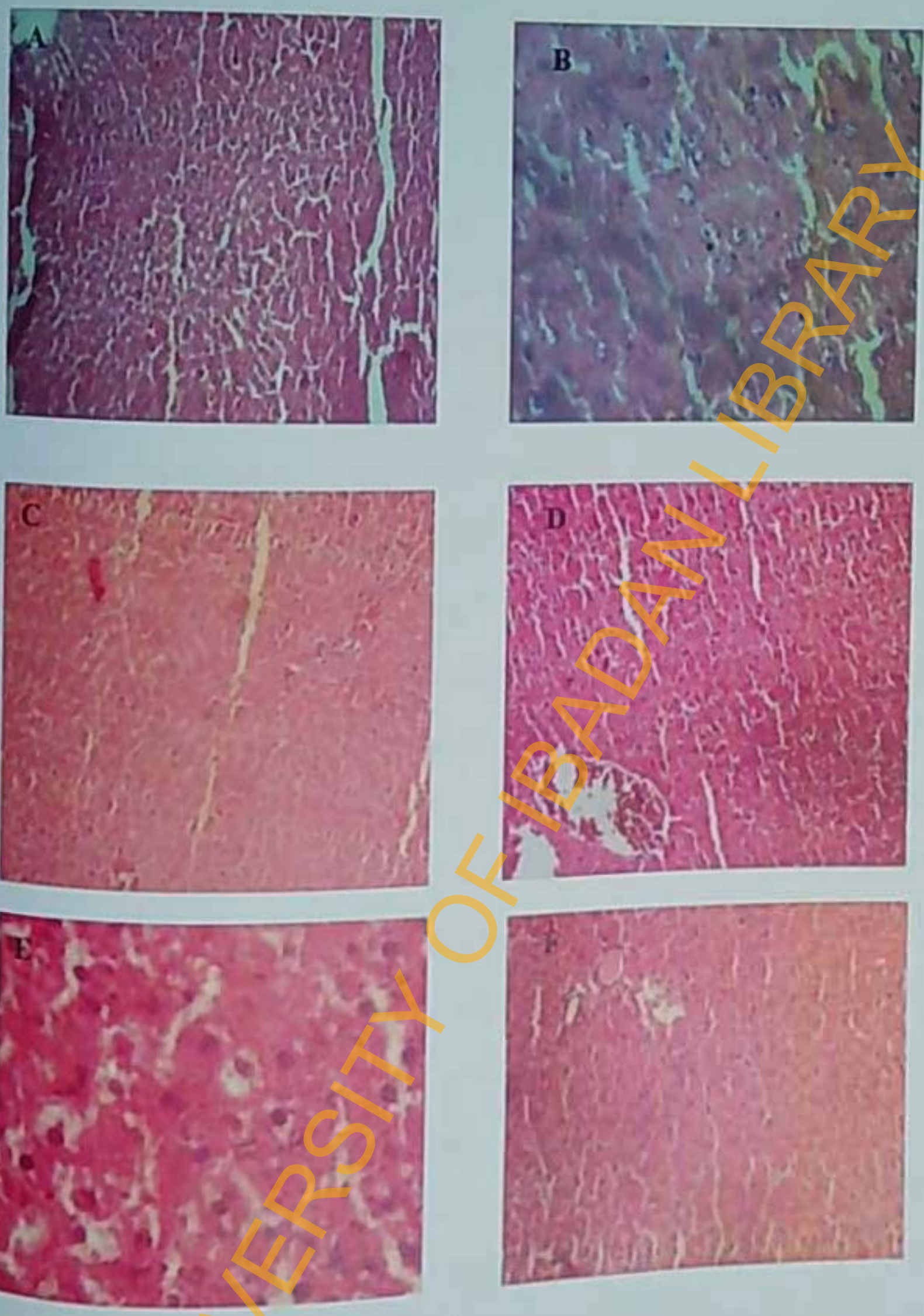

Plate 3G: Photomicrographs of rat liver sections (x400).
A- Control (water only) showing no visible lesions. B- 50mg/kg PCA alone shows no visible lesions. C- 50mg/kg PCA+5.71mg/kg Nevirapine shows no visible lesions. D- 100mg/kg PCA alone shows no visible lesions. E- 5.71mg/kg Nevirapine alone shows severe necrosis. F- 100mg/kgPCA+5.71mg/kg Nevirapine shows no visible lesions.

4.4 EXPERIMENT 4: THE PROTECTIVE EFFECT OF PROTOCATECHUIC ACID ON NEVIRAPINE INDUCED INFLAMMATION.

INTRODUCTION

Stimulation of inflammatory response is one of the molecular mechanisms involved in hepatic damage. Adducts that are large enough to serve as immune target may migrate to the surface of the hepatocyte, where they can induce the formation of antibodies (antibody-mediated cytotoxicity) or induce direct cytolytic T-cell responses (Robin *et al.*, 1997). The cytokine response is also evoked and this may cause inflammation and additional neutrophil-mediated hepatotoxicity (Jaeschke *et al.*, 2000).

It has been demonstrated that various inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-1β, (Blazka *et al.*, 1995; Blazka *et al.*, 1996; Ishida *et al.*, 2002) and proinflammatory enzymes like cyclooxygnase -2 and inducible nitric oxide synthase produced during drug-induced liver damage are involved in promoting tissue damage. These key mediators can activate signal transduction cascades as well as inducing changes in transcription factors, which mediate immediate cellular stress responses.

Based on the result of our previous study which suggests that nevirapine may exert its toxicity through the stimulation of inflammatory response; it is possible that a potent chemical agent that possesses antiinflammatory property is capable of reducing or modulating nevirapine- induced inflammation.

The frequent consumption of fresh fruit and vegetables is usually associated with a low incidence of hepatotoxicity and cancer. This may be attributable to the presence of some naturally occurring phenolic compounds that have antioxidative

properties. A natural phenolic compound, protocatechuic acid (3, 4-dihydroxybenzoic acid) is present in many edible and medicinal plants.

Protocatechuic acid has been shown to suppress the expression of inducible nitric oxide synthase (iNOS) and. cyclooxygenase-2 (COX2) involved in inflammation and carcinogenesis (Cichocki *et al*.. 2010).

Although several studies have shown PCA as a potent antinflammatory agent via its anticacinogenic activity, to the best of our knowledge there has been no study showing modulatory effect of PCA on nevirapine induced inflammation. Therefore. the specific aim of this study is to investigate modulatory capabilities of PCA on nevirapine induced inflammation.

PROCEDURE

Thirty six male albino rats weighing between 150-170 g were used for this study. They were purchased from the animal house of the Faculty of Basic Medical Sciences. University of Ibadan and housed in cages in the animal house of Biochemistry Department. University of Ibadan. The animals were given pellet feed and water *ad libitum*. They were randomly divided into 6 groups. The groups were treated for 3 weeks as follows. Group 1 control received only the water orally. Group 2 received 50 mg/kg (PCA) only orally. Group 3 received 100 mg/kg (PCA) only orally. Group 4 received 5.71 mg/kg (therapeutic dose) of nevirapine only orally. Group 5 received 5.71 mg/kg (therapeutic dose) of nevirapine and 50 mg/kg PCA orally. Group 6 received 5.71 mg/kg (therapeutic dose) of nevirapine and 100 mg/kg PCA orally. The rats were sacrificed by cervical dislocation 24 hours after the last administration; blood was collected by cardiac puncture technique with the aid of clean needle and syringe into clean dry centrifuge tubes and allowed to coagulate by standing for 30 minutes. The blood samples were then centrifuged for 10 mins at 3000

g using a bench centrifuge. The clear supernatant (serum) was collected and used to determine TNFα, IL-1β, PGE2 concentrations using ELISA technique as previously described in section 3.2.25. A portion of liver samples were processed and used for immunochemistry assay of COX-2 and iNOS enzymes as previously described in section 3.2.26 and from the remainder, post mitochondrial fraction (PMF) was obtained as previously described in section 3.2. The PMF was used to measure protein concentration using biuret method as described by Gomal *et al.*, (1949) myeloperoxidase (MPO) activity was according to the method of Eiserich *et al.*, (1998) and nitrite level as described by Navarro-Gonzalvez *et al.*, (1998). The different assay procedures are described under materials and method in section 3.2.6 to 3.2.25.

RESULTS

Treatment with Nevirapine alone resulted in elevation of serum levels of, IL-1β, TNF –α and PGE2 by 28, 27%, and 48%, compared to control respectively. However, co-treatment with PCA at 50mg /kg and 100mg/kg reversed the observed elevations by 12.5% and 19.1% ($p<0.05$) for IL-1β, 29.1% and 21.6 % ($p<0.05$) for TNF –α and 2.7% and 23% ($p<0.05$) for PGE2, respectively (Figures 3A, B and C respectively).

Similarly, tissue levels of MPO and NO were significantly elevated by 86% and 30 % respectively in the nevirapine alone group but co-administration with PCA at 50mg / kg and 100mg / kg ameliorated this condition by significantly decreasing the MPO levels by 39% and 28.5 %; and NO levels by 15.3% and 18.4% respectively when compared with control (Figures 3D and E respectively).

PCA also inhibited nevirapine-induced COX-2 and iNOS expressions. Immuno-histochemical staining verified inhibitory effect of PCA on Nevirapine

induced COX-2 and iNOS expressions. Intensity of the brown colour showed the extent to which the enzyme was expressed. The finding from this study shows that the nevirapine only group showed the most intense brown coloration; with the brownness toned down in the PCA co-administered groups (Plates 4A and 4B).

CONCLUSION

It can be therefore be inferred that nevirapine-induced hepatotoxicity associated with increased expression of COX-2 and iNOS, can be attenuated by protocatechuic acid as demonstrated by the result of our study. Result of this study therefore, demonstrates that PCA possess anti-inflammatory properties and can ameliorate inflammation mediated nevirapine induced hepatotoxicity in vivo in rats.

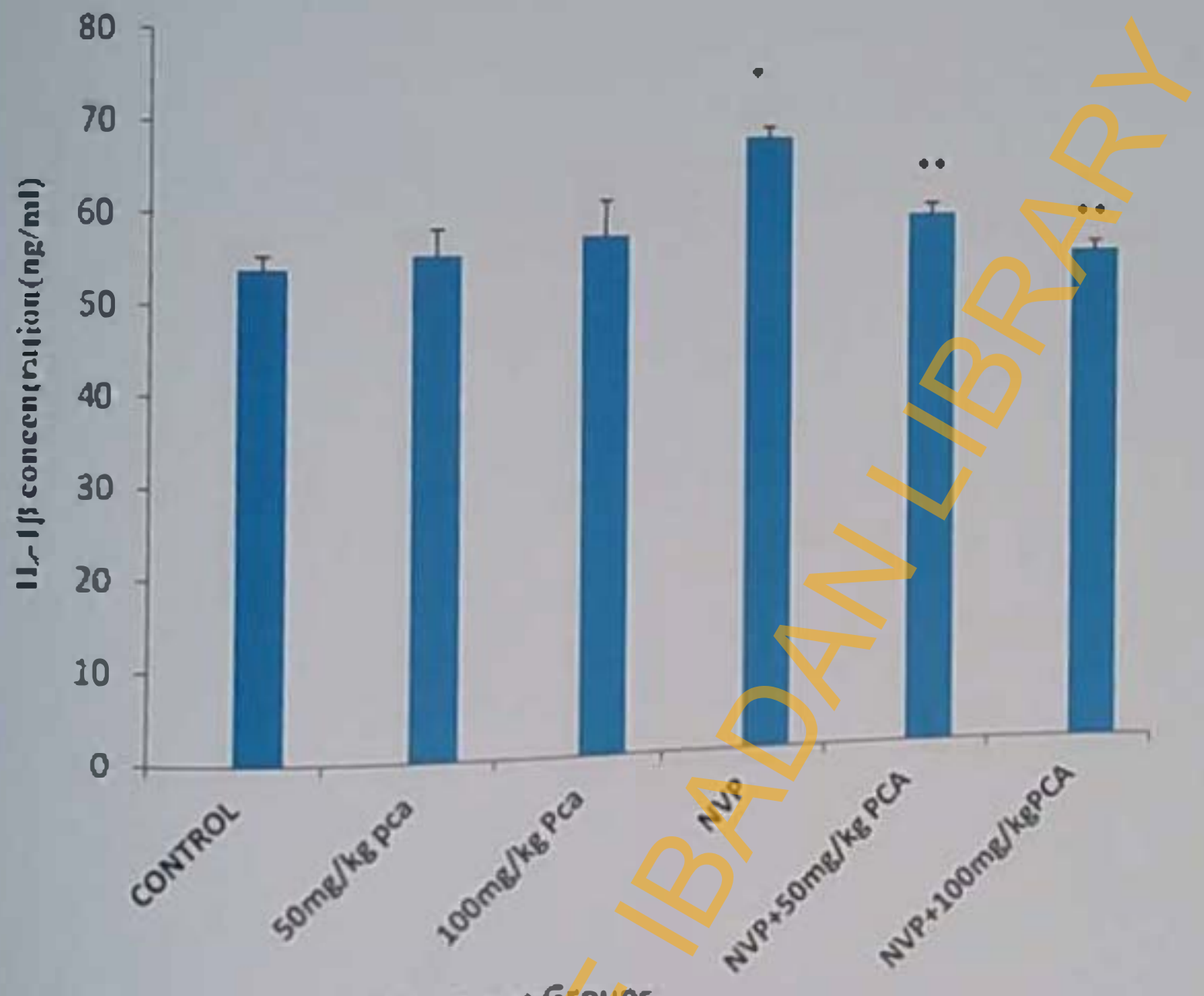

Figure 3A. Effect of Protocatechuic Acid on Nevirapine-Induced Elevation of Interleukin 1-beta Concentration in the Serum.

* p<0.05 when compared with Control

** p<0.05 when compared with Nevirapine alone

n=6

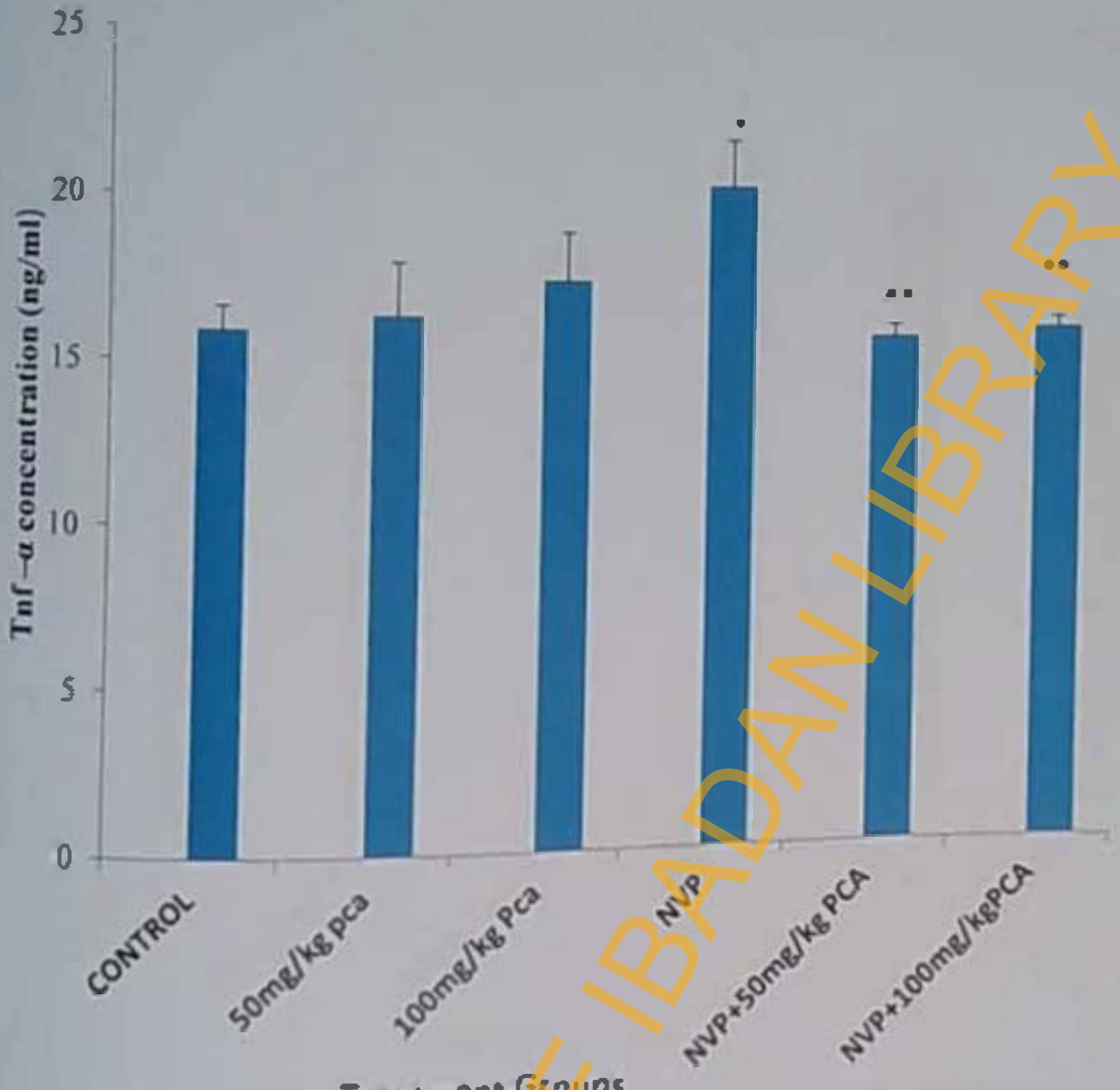

Figure 3B. Effect of Protocatechuic Acid on Nevirapine-Induced Elevation of Tumor Necrosis Factor Alpha Concentration in the Serum.

*p<0.05 when compared with control

**p<0.05 when compared with Nevirapine

n=6

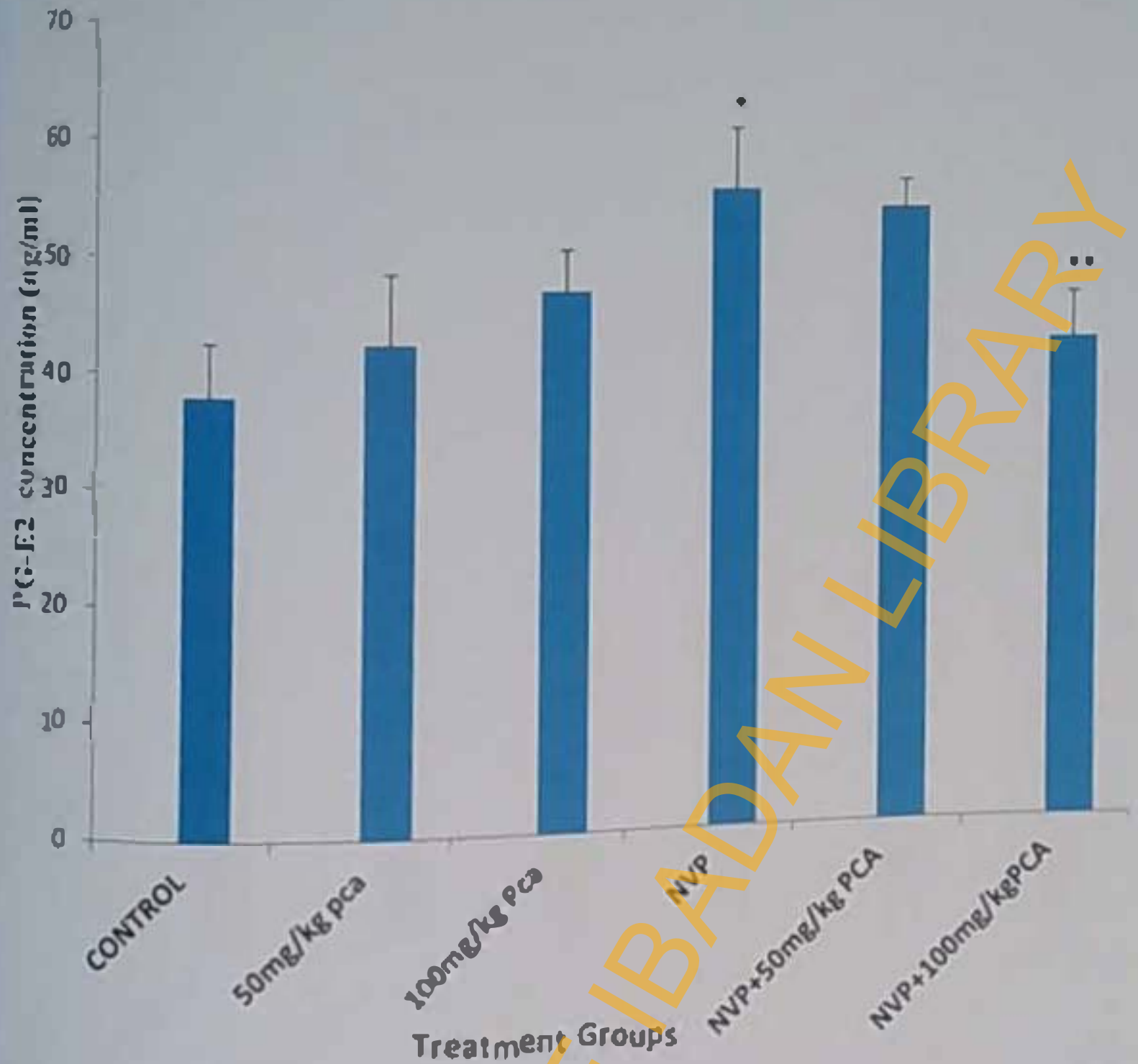

Figure 3C. Effect of Protocatechuic Acid on Nevirapine-Induced Elevation of PGE-2 Concentration in the serum

* P<0.05 when compared with control

** P<0.05 when compared with nevirapine

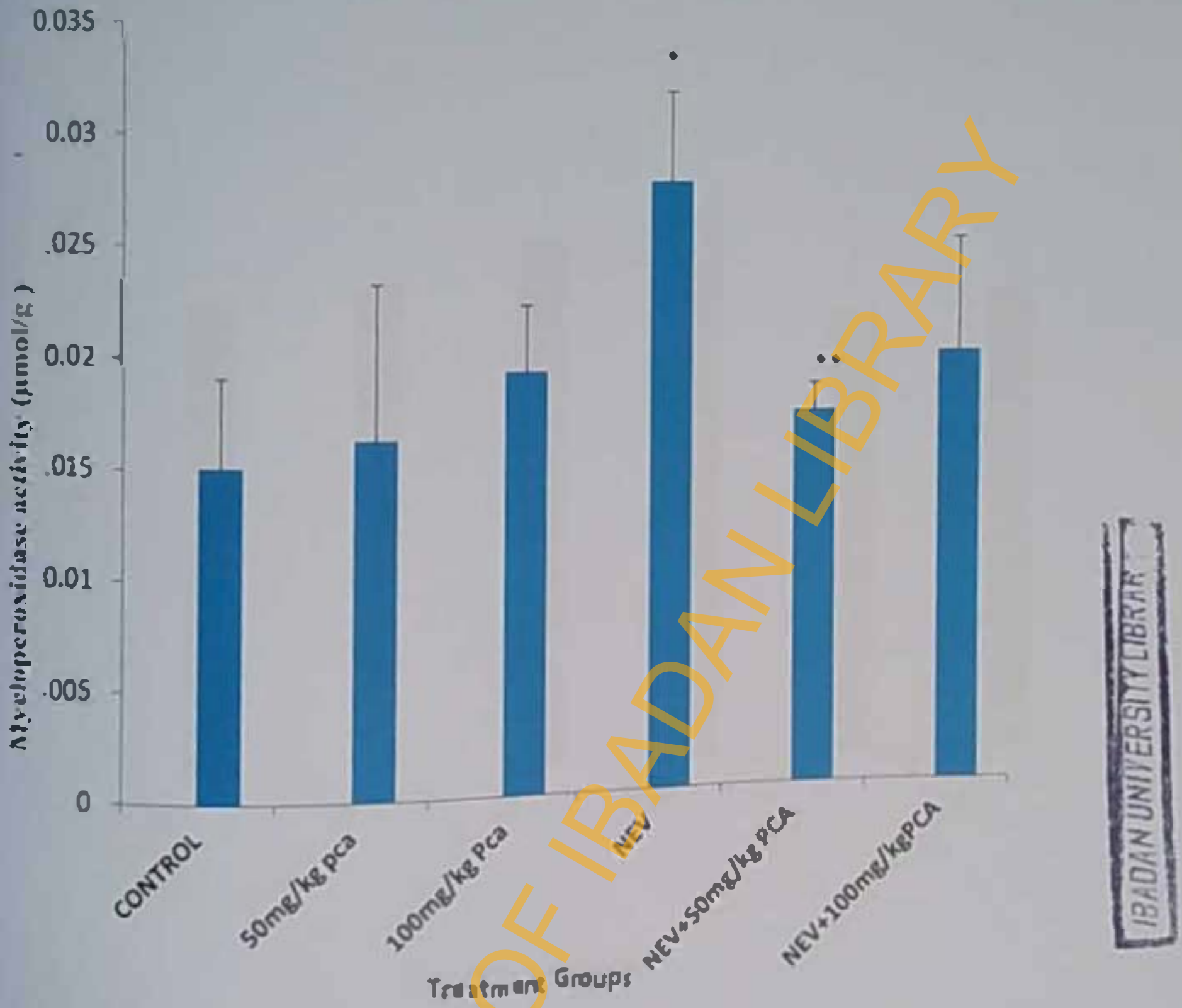

Figure 3D. Effect of Protocatechuic Acid on Nevirapine- Induced Elevation of Myeloperoxidase Activity in the Liver

* P<0.05 when compared with control

** P<0.05 when compared with nevirapine

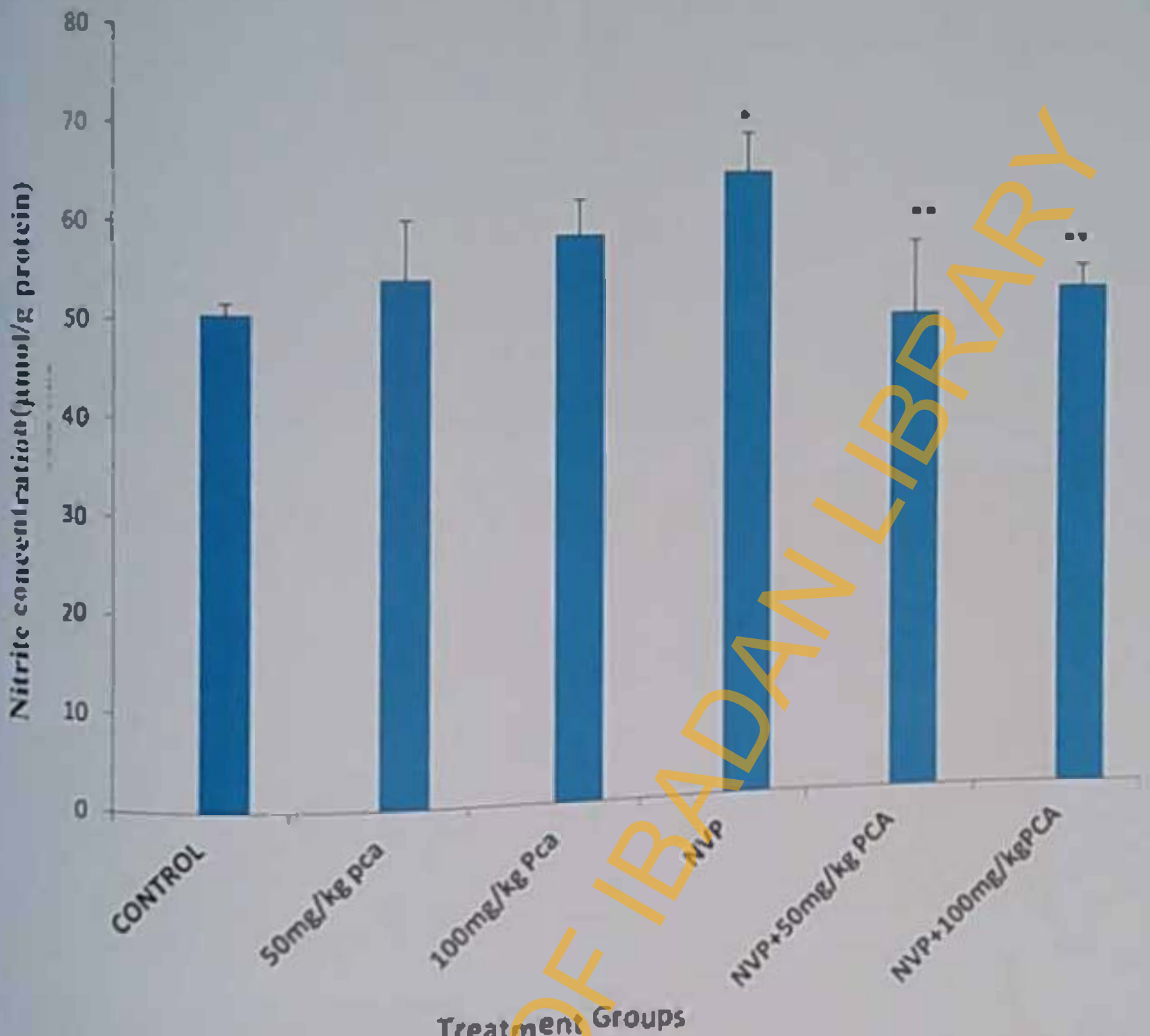

Figure 3E. Effect of Protocatechuic Acid on Nevirapine-Induced Elevation of Nitrite Concentration in the Liver

*p<0.05 when compared with control

**p<0.05 when compared with nevirapine

n=6

Plate 4A. Immunohistochemistry of COX-2 in the liver of Nevirapine and Nevirapine/protocatechuic acid treated rats

A: Control- Distilled water alone
B: 50mg/kg PCA alone.
C: 50mg/kg PCA+5.71mg/kg Nevirapine
D: 100mg/kg PCA alone.
E: 5.71mg/kg Nevirapine alone
F: 100mg/kgPCA+5.71mg/kg Nevirapine

Livers from treated rats were used for immunohistochemical analysis of COX-2, using goat polyclonal anti-rat COX-2 antibody as a primary antibody. Positive COX-2 staining yielded a brown-colored product. Intensity of the brown colour showed the extent to which the enzyme was expressed. The finding from this study shows that the nevirapine alone group showed the most intense brown colouration indicating increased expression of COX-2 compared to the other groups.

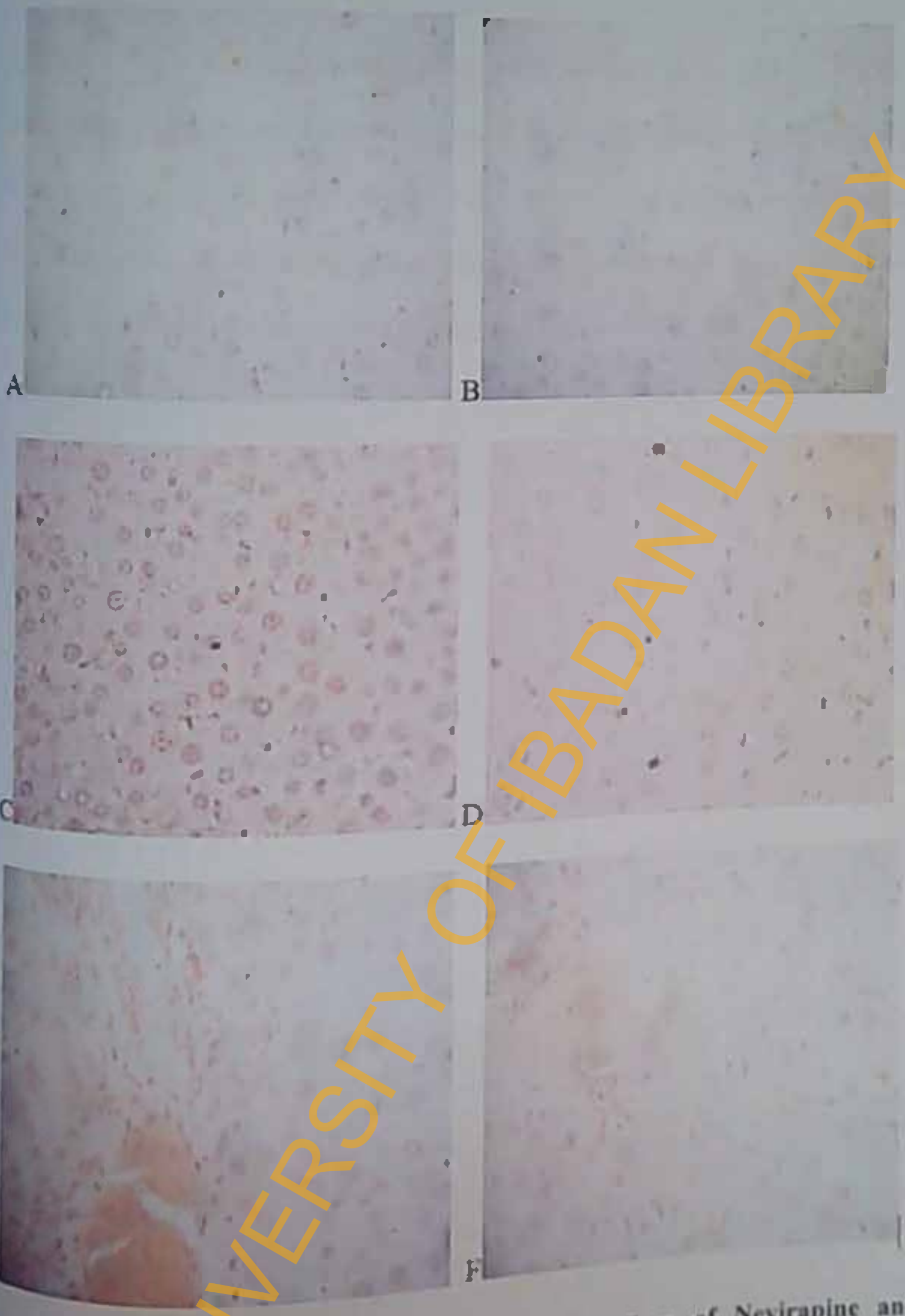

Plate 4B: Immunohistochemistry of iNOS in the liver of Nevirapine and Nevirapine/protocatechuic acid treated rats

A: Control- Distilled water alone
B: 50mg/kg PCA alone
C: 50mg/kg PCA+5.71mg/kg Nevirapine
D: 100mg/kg PCA alone
E: 5.71mg/kg Nevirapine alone
F: 100mg/kgPCA+5.71mg/kg Nevirapine

Livers from treated rats were used for immunohistochemical analysis of iNOS expression, using rabbit polyclonal anti-rat inos antibody as a primary antibody. Positive iNOS staining yielded a brown-coloured product. Intensity of the brown colour showed the extent to which the enzyme was expressed. The finding from this study shows that the nevirapine alone group showed the most intense brown colouration indicating increased expression of iNOS compared to the other groups.

4.5 EXPERIMENT 5: THE MODULATORY EFFECT OF PROTOCHATECUIC ACID (PCA) ON NEVIRAPINE-INDUCED APOPTOSIS.

INTRODUCTION

Programmed cell death (apoptosis) can occur in concert with immune-mediated injury, destroying hepatocytes by way of the tumor necrosis factor (TNF) and the FAS pathways, with cell shrinkage and fragmentation of nuclear chromatin (Reed, 2001). Certain chemicals may be able to trigger apoptosis by direct stimulation of the pro-apoptotic pathways in hepatocytes. On the other hand, other pathways including immune-mediated events can lead to release of TNF or activate the FAS pathways, and cholestasis is known to stimulate apoptosis through action of pro-apoptotic bile acids such as glycodeoxcholic acid (GCDC) (Lee, 2003).

Similarly, chemicals that damage mitochondria can also initiate apoptosis via release of cytochrome C (Bissel *et al.*, 2001; Jaeschke *et al.*, 2002). Thus induction of apoptosis is also be involved in the mechanisms of the toxicity of drug. Some antiretrovirals like zidovudine, stavudine and didanosine have been shown to induce apoptosis.

To the best of our knowledge no study has shown the involvement of apoptosis in nevirapine toxicity. However, it has been demonstrated that the mechanism by which protocatechuic acid offers it antioxidant protection is through regulating the genes controlling apoptosis (Tanaka *et al.*, 2011). Hence, the aim of this study is in two folds: firstly to investigate the involvement of apoptosis in nevirapine-induced hepatotoxicity and secondly the modulatory effect of PCA on nevirapine-induced apoptosis.

Procedure

Thirty six male albino rats of Wistar strain weighing between 150-170 g were

APOPTOSIS.

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Procedure

Thirty six male albino rats of Wistar strain weighing between 150-170 g were used for this study. They were purchased from the animal house of the Faculty of Basic

Medical Sciences. University of Ibadan and housed in cages in the animal house of Biochemistry Department, University of Ibadan. The animals were given pellet feed and water *ad libitum*. They were randomly divided into 6 groups. The groups were treated for 3 weeks as follows. Group 1(control) received only the water orally. Group 2 received 50 mg/kg (PCA) only orally. Group 3 received 100mg/kg (PCA) only orally. Group 4 received 5.71 mg/kg (therapeutic dose) of nevirapine only orally. Group 5 received 5.71 mg/kg (therapeutic dose) of nevirapine and 50mg/kg PCA orally. Group 6 received 5.71mg/kg (therapeutic dose) of nevirapine and 100mg/kg PCA orally. The rats were sacrificed by cervical dislocation 24 hours after the last administration; blood was collected by cardiac puncture technique with the aid of clean needle and syringe into clean dry centrifuge tubes and allowed to coagulate by standing for 30 minutes. The blood samples were then centrifuged for 10 mins at 3000 g using a bench centrifuge. The clear supernatant (serum) was collected and stored in the refrigerator. The serum samples were used to measure concentration of caspase 3, caspase 9, cytochrome C and p53 protein using ELISA assays technique as previously described section 3.2.25. The liver samples were processed and used for tunnel assay as previously described in section 3.2.4 and section 3.2.26.

RESULTS

Nevirapine caused a significant elevation of serum levels of caspase 9, cytochrome C, and tumor supressor p53 ($p<0.05$; 23%, 85% and5.2% control respectively). Co-treatment with 50mg /kg and 100mg/kg PCA caused a significant reduction (30.7% and 28.2 % ($p<0.05$) in caspase 9) and (50% and 51% in reduction cytochrome C $p<0.05$) and (2.6% ($p<0.05$) and 2.3% in tumor supressor p53) (Figures 4A, B and C respectively).

Nevirapine caused an elevation of serum levels of caspase 3 by, 33% and 12% when compared to contol). Co-treatment with 50mg /kg and 100mg/kg PCA caused a reduction (33.8% and 27.5.2% in caspasc 3) (Figure 4D).

The measure of nuclear DNA fragmentation is an important biochemical indicator of apoptosis in many cell types. Using a modified TUNEL assay showed that nevirapine induced apoptosis and this is depicited by the deep intensity of the brown coloration when compared with the control in (Plate 5A) as PCA at 50mg /kg and 100mg/kg was able to ameliorate this condition.

Conclusion

This result therefore shows the involvement of apoptosis in nevirapine induced hepatotoxicty and secondly ability of PCA to modulate nevirapine induced apoptosis.

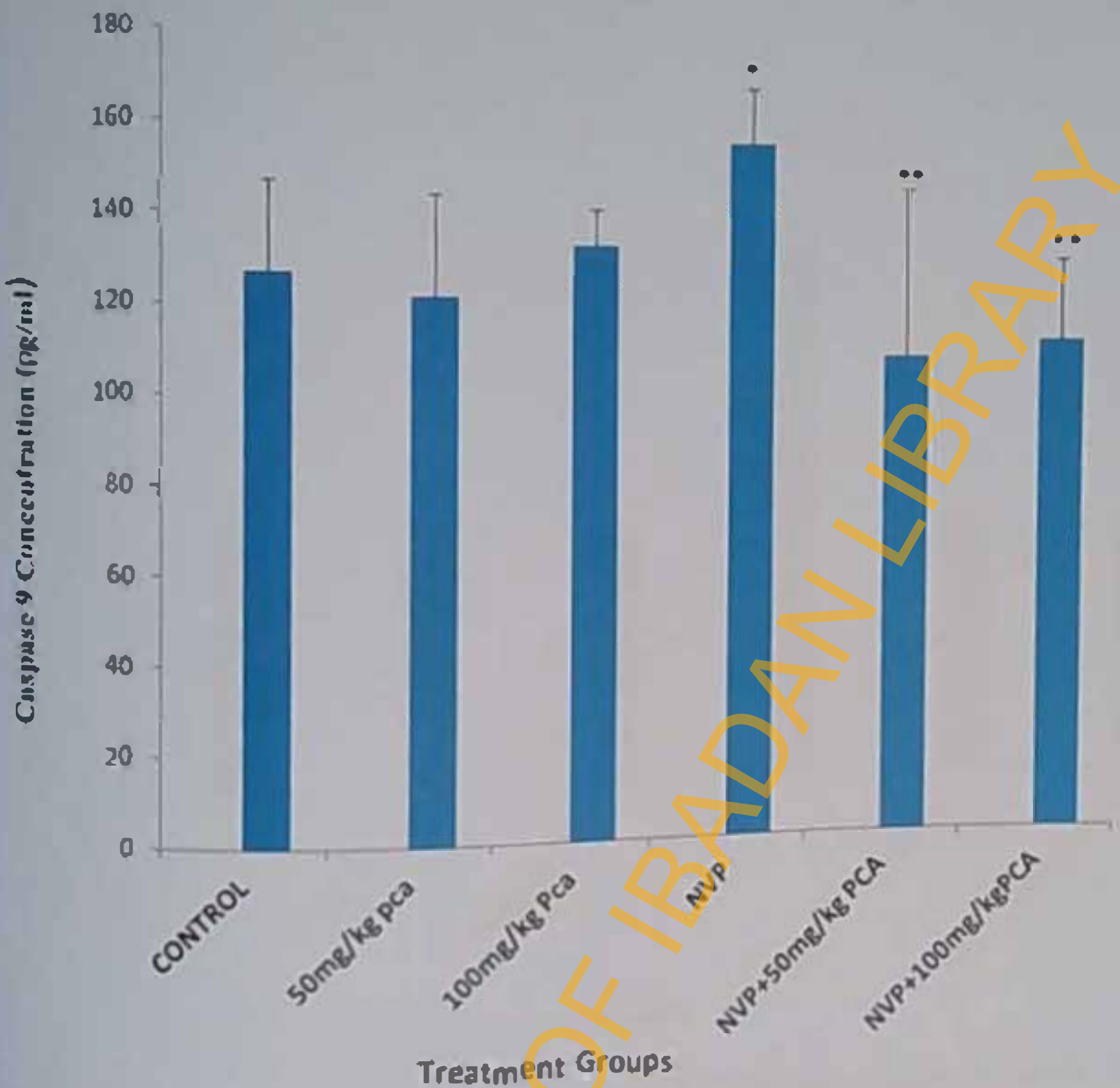


Figure 4A. Effect of Protocatechuic acid on Nevirapine-induced elevation of Caspase 9 Concentration in the Serum

* $p<0.05$ when compared with control

** $p<0.05$ when compared with Nevirapine

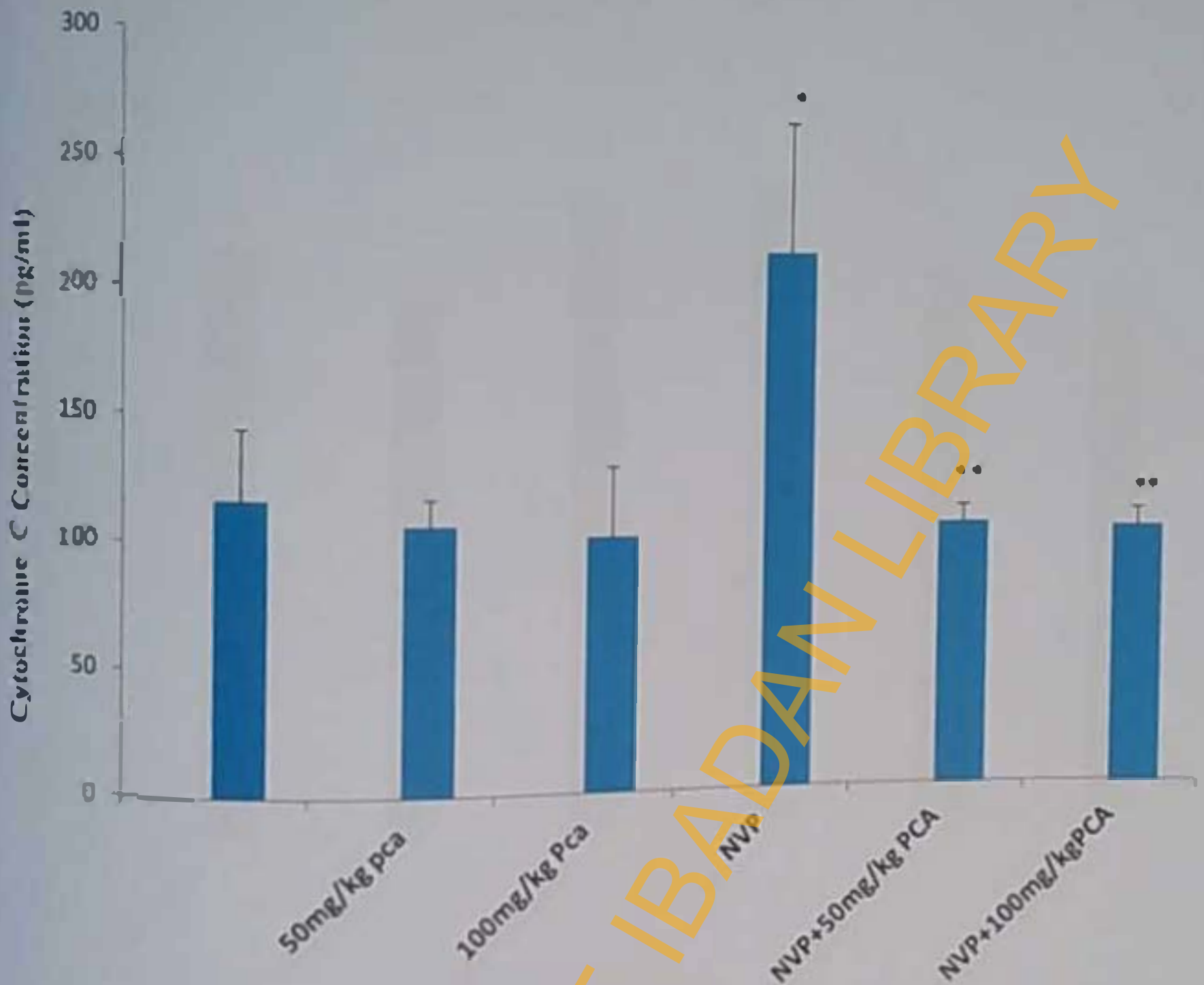


Figure 4B. Effect of Protocatechuic acid on Nevirapine-induced elevation of Cytochrome C Concentration in the Serum

*p<0.05 when compared with control

**p<0.05 when compared with Nevirapine

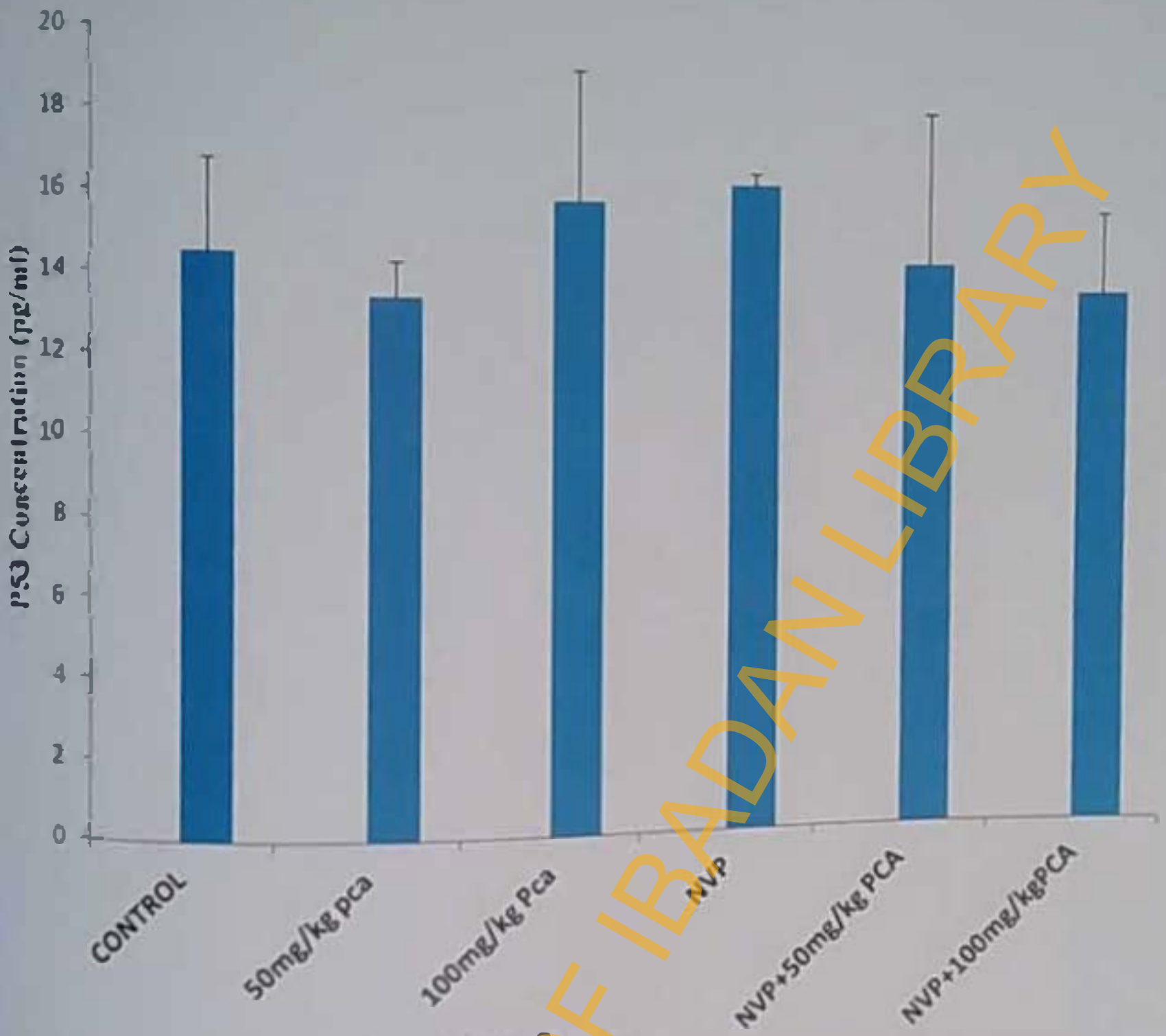


Figure 4C. Effect of Protocatechuic acid on Nevirapine-induced elevation of P53 Concentration in the Serum

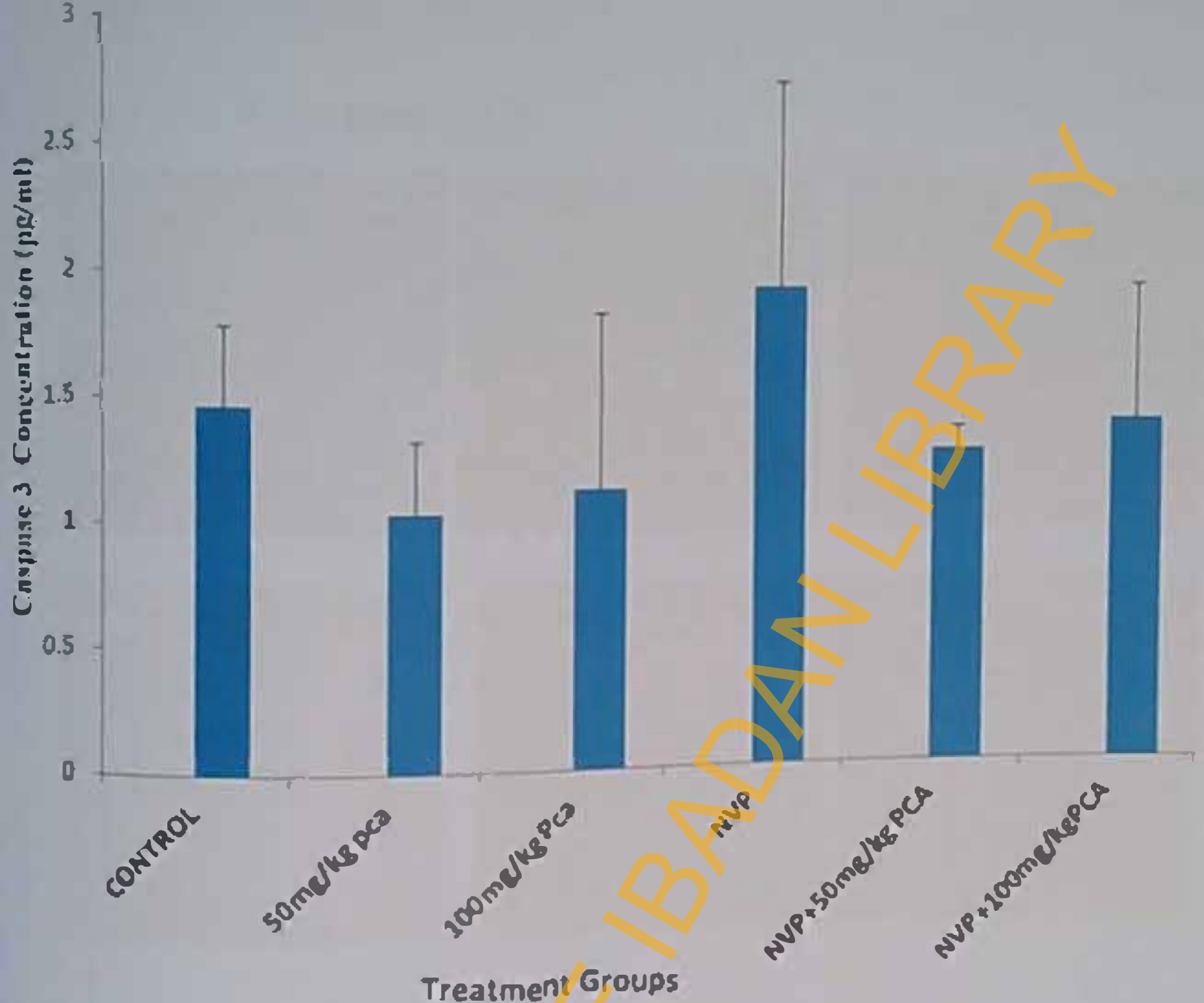
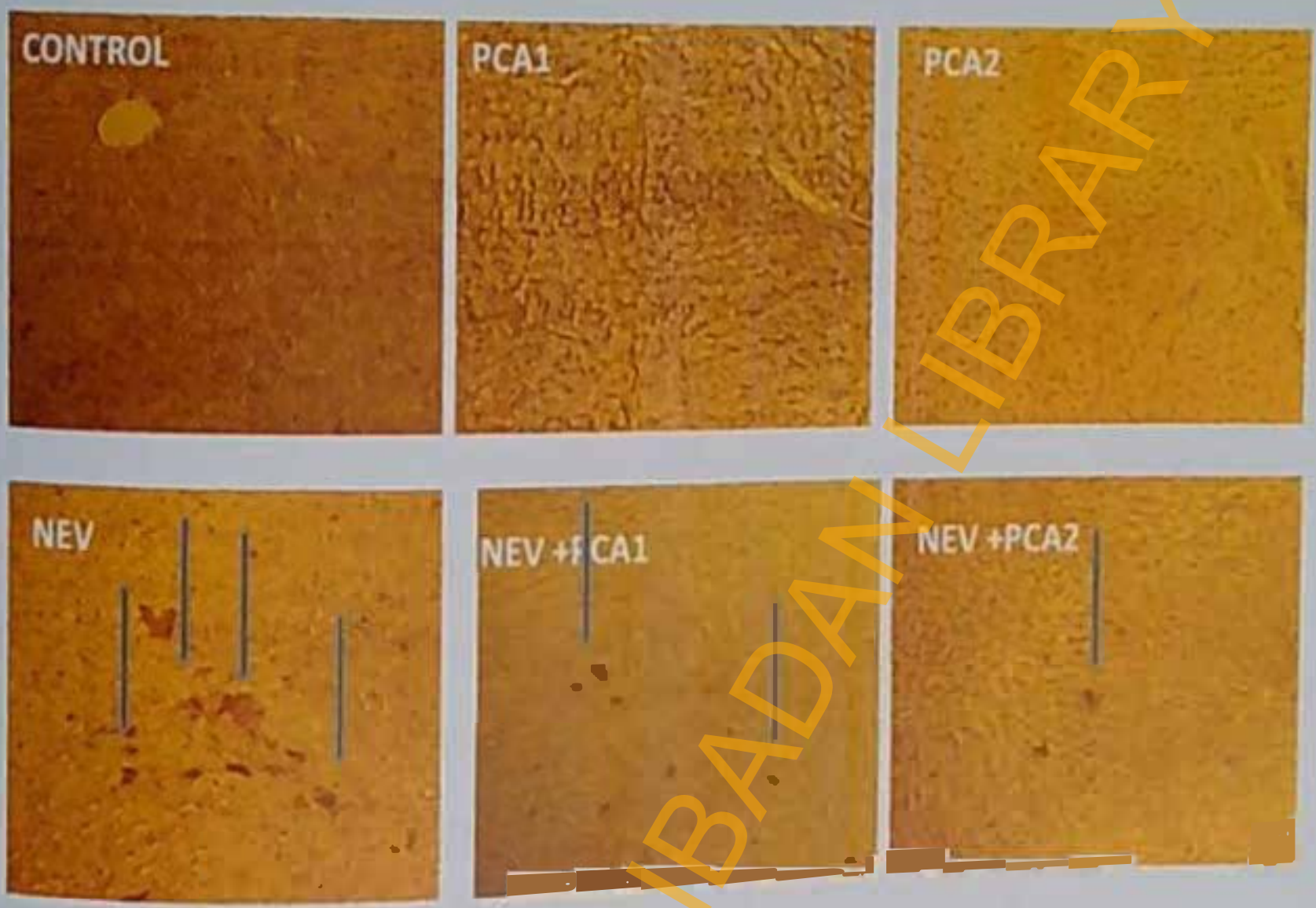


Figure 1D. Effect of Protocatechuic acid on Nevirapine- induced elevation of Caspase 3 Concentration in the Serum

TUNEL ASSAY RESULT



BROWN STAINING SHOWING APOPTOTIC CELL

Plate 5A. Effect of Protocatechuic acid on Nevirapine-induced Apoptosis using TUNEL assay

4.6 EXPERIMENT 6: PROTECTIVE EFFECT OF PROTOCATECHUIC ACID ON NEVIRAPINE-INDUCED GENOTOXICITY USING MICRONUCLEI ASSAY.

INTRODUCTION

The in vivo micronuclei assay is a mutagenic test system for the detection of chemicals which induce the formation of small membrane bond DNA fragments i.e. micronuclei in the cytoplasm of interphase cells.

Evidence gathered from our previous study showed nevirapine as having a genotoxic potential with a significant increase in number of miconucleated polychromatic erythrocyte when compared with control. Antioxidants are known to be important against DNA damage and mutations induced by reactive oxygen species (ROS). Evidence also abound that the antioxidant protocatechuic acid possess antimutagenic properties (Demetrios *et al*., 2004; Jaouad *et al*., 2011).

The objective of this work is to investigate the modulatory effect of protocatechuic acid on nevirapine-induced genotoxicity using micronuclei assay.

PROCEDURE

A total of 36 male albino rats aged between 6-8 weeks with body weight ranging from 40-70 g were used for this study. Animals were randomly divided into 6 groups. The groups were treated orally for 3 weeks as follows:

Group 1 control received only the water orally. Group 2 received 50 mg/kg (PCA) only orally. Group 3 received 100mg/kg (PCA) only orally. Group 4 received 5.71 mg/kg (therapeutic dose) of nevirapine only orally. Group 5 received 5.71 mg/kg (therapeutic dose) of nevirapine and 50mg/kg PCA orally. Group 6 received 5.71mg/kg (therapeutic dose) of nevirapine and 100mg/kg PCA orally. The rats were sacrificed by cervical dislocation. Bone marrow was flushed

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ON NEVIRAPINE-INDUCED GENOTOXICITY USING MICRONUCLEI ASSAY.

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stained with maygrunward stain. Bone marrow cells were then examined microscopically and scored per animal for micro-nucleated polychromatic erythrocytes (mnPCE). The the assay was carried out according to procedure previously described in materials and method section 3.2.27.

RESULTS

Similar to our previous experiment, nevirapine caused a significant increase ($p<0.05$; 68%) in the number of micro nucleated polychromatic erythrocytes when compared with control. However, co-treatment with PCA at both concentrations used in this study attenuated this effect. Our results show that PCA at 50mg/kg and 100mg/kg caused a significant reduction ($p<0.05$; 37.5% and 32.8%) respectively in number of micronucleated polychromatic erythrocytes.

CONCLUSION

The result of this experiment shows that nevirapine may be genotoxic and Protocatechuic acid may offer protection against nevirapine induced genotoxicity.

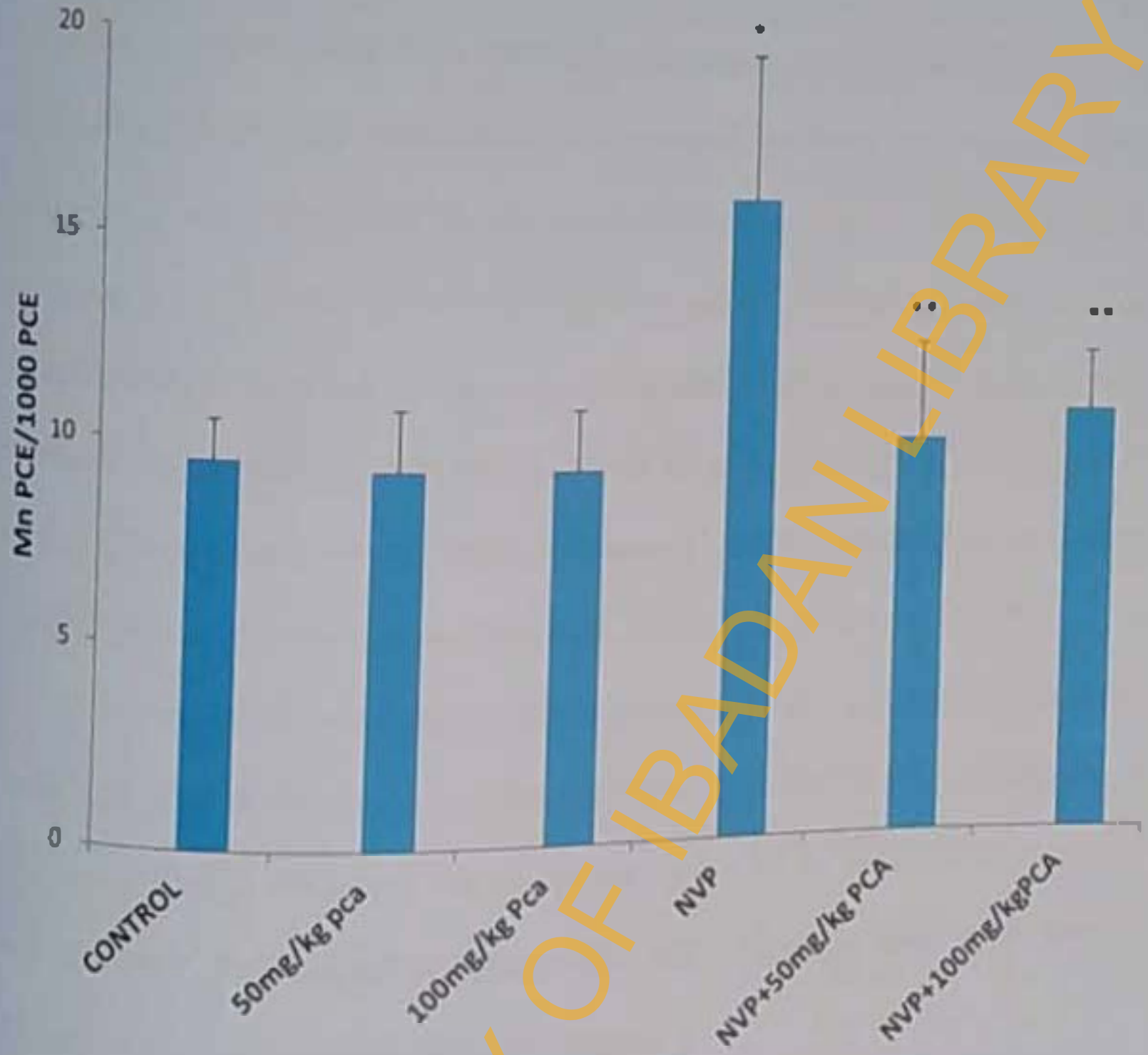

Figure 5A. Effect of Protocatechuic acid on Nevirapine-induced micronuclei formation

* P<0.05 when compared with control

** P<0.05 when compared with Nevirapine

mg

MNPCE - Micro-Nucleated Polychromatic Erythrocytes

PCE-Polychromatic Erythrocytes

CHAPTER FIVE

DISCUSSSION

Several antiretroviral drugs have been developed for the treatment of human immunodeficiency virus (HIV) infection. They are used to achieve the highest possible clinical benefit, and to diminish the risk of developing resistance. The use of these drugs however has been associated with toxicological effects which include hematologic disorders, myopathy hepatoxicity and cardiotoxicity just to mention a few. These effects are militating against the success of antiretroviral therapy and may result in decrease adherence to treatment which consequently leads to clinical failure, therapy discontinuation and even death (Domingo and Lozano, 2011; Bera *et al.*, 2012).

One of the major toxicological effects of antiretroviral agents is hepatotoxicity. The reason for this is due to the fact that the liver is central to the metabolism of virtually every foreign substance that enters the body. It is therefore necessary to identify therapies that adversely affect the liver, identify their toxicological mechanisms, and ways of ameliorating their effects.

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) used in the treatment of HIV-1 infection. Currently, it is the most widely used anti-HIV drugs in developing countries, both in combination therapy and to prevent mother-to-child transmission of HIV. Despite its efficacy against HIV, NVP produces a variety of toxic responses, including hepatotoxicity and skin rash. It is also associated with increased incidences of hepatocarcinogenesis in rodents (Antunes et al., 2013). It therefore becomes imperative to consider the inclusion of hepatoprotective agents in the treatment regimen.

5.1 EFFECT OF DURATION OF EXPOSURE ON NEVIRAPINE HEPATOTOXICITY

Nevirapine, the drug of interest in this study is associated with hepatotoxicity characterized by hepatocytes necrosis and elevation of transaminase serum levels (Elias and Brambaifa, 2013; Elias *et al.*, 2013). Although hepatotoxicity of nevirapine is established, effect of nevirapine on the liver with respect to duration of drug exposure has not been well-explored. It is therefore important to know if extent of the liver damage nevirapine induces is affected by the duration of exposure to the drug since it is used by the patients almost for a life time.

In the present study, therapeutic dose of nevirapine was administered for different durations (1-4 weeks) to assess the relationship between nevirapine-induced liver damage and duration of exposure to the drug. Various biomarkers of hepatic damage and oxidative stress were used to assess the extent of the damage. Results obtained showed that treatment with therapeutic dose of nevirapine caused marked acute liver damage as evident by the significant elevation of serum levels of alanine (ALT) and aspartate (AST) aminotransferases compared with control throughout the duration of the treatment.This finding agrees with the study of Umar *et al.*, (2008). Increased serum levels of ALT and AST directly reflect a major permeability problem or cell rupture (Benjamin, 1978; Umar *et al.*, 2008). AST exhibits high activity in cytoplasm, mitochondrion and microsomes of liver, heart, kidney and brain (Benjamin, 1978; Ringer *et al.*, 1979); while ALT is hepato-specific principally found in the cytosol of hepatocytes and elevated ALT levels are associated with acute liver and cardiac disease (Wolf, 2003).

In addition to transaminase elevation there were significant increases in serum level of direct bilirubin. Elevated serum bilirubin is indicative of impairment of the

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In addition to transaminase elevation there were significant increases in serum level of direct bilirubin. Elevated serum bilirubin is indicative of impairment of the

of the urinary tract. Histopathological analysis also showed increase in the severity of hepatic degeneration during the course of treatment.

Results from this study showed that the severity of liver damge increased with increased time of exposure. The 3rd and 4th weeks of exposure resulted in the most pronounced hepatic damage.

Reduced glutathione (GSH) was measured as a marker of oxidative stress in liver of rats. GSH is a tripeptide and the major endogenous antioxidant produced by cells. It helps to protect cells from reactive oxygen species (ROS), free radicals and peroxides (Pompella *et al.*, 2003). It is well established that ROS and electrophilic chemicals can damage DNA and that GSH can protect against this type of damage (Valko *et al.*, 2007). GSH can also directly detoxify carcinogens through phase II metabolism and subsequent export of these chemicals from the cell. We observed a decrease in GSH level when compared to control throughout the period of treatment but the most significant decrease was observed at weeks 2 and 3. This suggests the ability of the therapeutic dose of the drug to induce oxidative stress.

Superoxide dismutase (SOD) and catalase (CAT) play key roles in the detoxification of superoxide anion and hydrogen peroxide respectively, thereby protecting against ROS-induced damage (Eaton, 1991; Fridovich, 1995). Reductions in the activity of these enzymes result in an accumulation of superoxide anion and hydrogen peroxide in the liver. These reactive oxygen species have the potential of reacting with metal ions to form hydroxyl radical. This radical in turn reacts with cellular components like protein lipid and DNA and consequently damaging them.

Further, nevirapine caused a decrease in SOD activity throughout the course of the study with significant effect at week 1 and 4. Similarly, CAT activity decreased

liver, excessive hemolysis, or obstruction of the biliary tract. Histopathological analysis also showed increase in the severity of hepatic degeneration during the course of treatment.

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throughout the period of the study. The observed decrease in catalase activity was significant at week 1, 3 and 4. Taken together, the study indicates that Nevirapine induces oxidative stress and significantly with increased time of exposure.

Furthermore, nevirapine-induced lipid peroxidation as judged by the elevated concentration of malondialdehyde (MDA) compared to control throughout the course of the study. MDA, a product of membrane lipid peroxidation, has been shown to react with critical biomolecules such as nucleic acids, thus damaging the cells (Cuzzocrea *et al*., 2001).

From the aforementioned, nevirapine-induced hepatotoxicity and oxidative stress which is related to the duration of exposure to the drug. The third and fourth weeks of exposure to nevirapine showed the most severe damage when compared with control.

5.2 NEVIRAPINE-INDUCED HEPATOTOXICITY, OXIDATIVE STRESS AND INFLAMMATION

Having established from our previous study that nevirapine (at therapeutic dose) can induce hepatotoxicity by impairing the antioxidant system and that the extent of damage or toxicity becomes pronounced with prolonged exposure to the drug, further studies were carried out on the effect of nevirapine on some biomarkers of inflammation, phase II drug metabolizing enzymes, and other hepatic enzymes that are biomarkers of hepatic damage.

In this study, we investigated the effect of nevirapine on the expression of pro-inflammatory cytokines (tumor necrosis factor alpha (TNFα) and Interleukin 1 beta) in the serum, non enzymatic antioxidants (reduced glutathione and vitamin C), drug metabolizing enzymes and antioxidant enzymes (glutathione -S- tranferase, glutathione peroxidase), markers of liver damage (alkaline phosphatase, gamma glutamyl

transferase, alanine aminotransferase, aspartate amino transferase), lipid peroxidation and hydrogen peroxide concentration after three and weeks of drug administration.

Results obtained corroborate the finding of the previous study that nevirapine causes an elevation of biomarkers of acute liver injury such as ALT and AST. Likewise, the reduction in SOD and CAT activities by treatment with nevirapine was also reconfirmed in this study. Similarly, this study also reconfirmed the involvement of lipid peroxidation and GSH depletion in nevirapine-induced liver damage. Apart from all the reconfirmation, there was also a significant elevation ($p<0.05$) in serum ALP and GGT levels in this study. AST, ALT, ALP and GGT are considered as hepatic marker enzymes and their serum concentrations are used as diagnostic indicators of hepatic injury since they are related to the function of the hepatic cells. Alterations in the serum concentrations of these enzymes may be due to hepatocellular impairment and dysfunction (Mershiba *et al*., 2012).

The activity of the enzyme glutathione peroxidase (GPx) was also lowered in the nevirapine-treated group when compared with control.

Glutathione peroxidase (GPx) is a drug metabolizing enzyme and an enzymatic antioxidant which catalyses the reduction of hydrogen peroxide and hydroperoxide to non toxic products and scavenges the highly reactive lipid peroxides in the aqueous phase of cell membrane. GPx and the cellular NADPH generation mechanisms together form a system for removing hydroperoxide from the cell (Halliwell, 1977). A reduction in the activity of this enzyme therefore suggests that nevirapine can cause depletion in the endogenous defense system. The consequence of this reduction is accumulation of hydrogen peroxide and hydroperoxide resulting in increased in lipid peroxidation. The reduction in GPx activity in nevirapine- treated rats

can be correlated with GSH depletion (an important cofactor GPx needs to function), and also with increased lipid peroxidation observed in this study.

Nevirapine also caused marked elevation of hydrogen peroxide concentration. This can be associated with the reduction in catalase and glutathione peroxidase activity since they help to detoxify the cell of hydrogen peroxide.

Vitamin C concentration was also observed to be lowered by nevirapine. Vitamin C is a potent water soluble antioxidant in biological fluid (Frei *et al.*, 1990). Also, there are evidences that vitamin C regenerates other endogenous antioxidants like GSH, urate and β-carotene from their respective radical species (Halliwell, 1996). This result indicates that the administration of nevirapine seems to have overwhelmed the antioxidant defense mechanism thus causing n reduction of vitamin C concentration in the cells.

Treatment with nevirapine produced a significant elevation of tumor necrosis factor alpha (TNF α) and interleukin 1-beta (IL-1β) levels. It has been demonstrated that various inflammatory cytokines such as, TNF-α, interferon (IFN-γ) and IL-1β (Blakza *et al.*, 1995; Blakza *et al.*, 1996; Ishida *et al.*, 2002) are produced during drug-induced liver damage are involved in promoting tissue damage. This shows that this nevirapine provoked hepatic damage, may involve the induction of proinflammatory mediators. The results of this study indicate therefore that the mechanisms by which nevirapine exen its liver toxicity may be through the stimulation of inflammatory response and oxidative damage (due to free radical generation).

5.3 NEVIRAPINE-INDUCED OXIDATIVE STRESS AND PROTECTIVE EFFECT OF PROTOCATECHUIC ACID (PCA)

Antiretrovirals, the class of drugs to which nevirapine belongs, is associated with toxic effects and induction of oxidative stress via the generation of oxygen radicals, decrease in intracellular antioxidants and perturbation in the activities of antioxidant enzymes (Adikwu *et al.*, 2013). Phenolic compounds, which are widely distributed in plants, have been considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living systems (Herrog and Feskens, 1993).

In the present study, investigation of the effect of protocatechuic acid on nevirapine-induced hepatotoxicity and oxidative stress was carried out. Protocatechuic acid (3, 4-dihydroxybenzoic acid, PCA) - a natural phenolic compound found in many edible and medicinal plants (Justyna, 2005) and have been shown by several researchers to have hepatoprotective effects (Tanaka *et al.*, 2011; Chuen-Lan *et al.*, 2002).

The hepatic enzymes AST, ALT, ALP and GGT were used as biochemical markers for early acute hepatic damage and hepatic cholestasis. In this study, significant increases in the activities of AST, ALT, ALP and GGT were observed in nevirapine treated rats. This finding agrees with the study of Umar et al. (2008). Liver damage induced by nevirapine generally reflects disturbances of liver cell metabolism, which leads to characteristic changes in the serum levels of the marker enzymes. Increased serum levels of these enzymes directly reflect a major permeability problem or cell rupture (Benjumin, 1978; Umar, 2008). These enzymes are characteristic of liver damage; therefore their release into the serum indicted nevirapine-induced liver

Treatment with PCA attenuated these increased enzyme levels indicating that PCA *in vivo* has hepatoprotective properties against nevirapine-induced liver toxicity. This is consistent with the work of Thangaiyan et al., (2011) which showed that PCA could offer protection against elevation of these enzymes.

Similarly, nevirapine significantly decreased GSH levels and co-administration with 50 mg/kg and 100 mg/ kg PCA ameliorated the oxidative stress by significantly increasing GSH level when compared to the nevirapine only group. However, the 50 mg/kg was more effective than the 100 mg/kg dosage. This shows that PCA could affect GSH hepatic concentration in dose dependent manner. This is corroborated by Nakamura and co-workers. (2000) who demonstrated that high doses of PCA cause tyrosinase-dependent reduction of GSH level and antioxidative enzyme activities. The physiological role of GSH is as an essential intracellular reducing agent for the maintenance of thiol groups on intracellular proteins and for antioxidant molecules. It is well established that GSH, the most important biomolecule protecting against chemically induced cytotoxicity, can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction, or of free radicals by direct quenching.

Furthermore, the results for lipid peroxidation showed that nevirapine caused significant increase in lipid peroxidation as judged by the high concentration of MDA compared to control. Malondialdehyde (MDA) is one of the end-products of polyunsaturated fatty acid peroxidation and it is a good indicator of the degree of lipid peroxidation (Nakbi *et al.*, 2010). Co-treatment with PCA was able to ameliorate this increased lipid peroxidation significantly when compared with nevirapine alone treated group. This suggests PCA can protect the cells in liver tissue from injury by inhibiting

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membrane lipid peroxidation. These results are consistent with those of (Thangaiyan *et al*., 2011; Jong Moon Hur *et al*., 2003 and Chuen-Lan *et al*., 2002).

Increase in lipid peroxidation is an indication of the inability of the antioxidant defence mechanism to prevent excessive formation of free radicals. Treatment with PCA, which caused a significant decrease in lipid peroxidation level, suggests that PCA may exert stabilizing effect on liver cell membrane.

Glutathione peroxidase (GPx) is an enzymatic antioxidant which catalyses the reduction of hydrogen peroxide and hydroperoxides to nontoxic products and scavenges the highly reactive lipid peroxides in the aqueous phase of the cell membrane. GPx and the cellular NADPH generation mechanisms together form a system for removing hydroperoxides from the cell (Halliwell, 1977).

We observed a decrease in GPx activity of the rats that took nevirapine alone. This decrease in enzyme activity might be linked to the decreased availability of its substrate, GSH. A reduction in the activity of this enzyme therefore suggests that nevirapine can cause depletion in the endogenous defence system. Treatment with 50 mg/kg and 100 mg/kg PCA improved the GPx activity significantly to near-normal.

SOD, an enzyme that converts superoxide radicals into hydrogen peroxide, is widely distributed in cells and protecting cells against the toxic effect of superoxide anion (Fridovich, 1989). Superoxide anion is known to exert destructive effects on cellular components with lipid peroxidation being one of the consequences. The results obtained from our study showed that nevirapine caused reduction in SOD activity as compared to control with PCA being able to ameliorate this condition.

Catalase (CAT) is an enzyme found ubiquitously in nearly all living organisms and catalyses the decomposition of toxic hydrogen peroxide (H_2O_2) to water and oxygen. Nevirapine also significantly lowered catalase activity while both

concentrations of PCA restored the condition to normal by increasing the enzyme activity.

This study shows that PCA affects the activity of these enzymes in a dose dependent manner. Equilibrium between ROS and enzymatic antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are crucial and could be an important mechanism for preventing damage by oxidative stress.

The efficiency of enzymatic and non-enzymatic antioxidative systems could be detected by the determination of single components of this system which is known as total antioxidant capacity (TA) (Kankofer *et al.*, 2005). In the present study, a significant decrease in total antioxidant capacity in nevirapine treated rats was observed. This demonstrates the ability of nevirapine to suppress antioxidant status and induce oxidative stress. PCA, a polyphenolic compound with great antioxidant capacity was able to ameliorate this condition by significantly increasing total antioxidant capacity in the 50 mg/kg co-treatment group.

The formation of oxidized proteins is one of the highlights of oxidative stress. and the carbonyl groups (aldehydes and ketones) are produced on protein side chains when they are oxidized (Dalle-Donne *et al.*, 2003). The presence of carbonyl groups in proteins makes them susceptible to degradation by proteolytic enzymes leading to deficiency of proteins. The proteins may aggregate together. some proteins may become susceptible to degradation and this modification can cause many pathological conditions. Oxidatively modified proteins are not repaired and must be removed by proteolytic degradation. and a decrease in the efficiency of proteolysis will cause an increase in the cellular content of oxidatively modified proteins (Reckman and Crow, 1993).

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Nevirapine brought about a significant increase in protein carbonyl concentration. Co-treatment with PCA at both doses was able to ameliorate this condition by causing a significant reduction in protein carbonyl concentration.

In conclusion, PCA exhibited the ability to assuage liver injury associated with nevirapine drug administration by reducing oxidative stress and hepatic damage. In view of the present findings, fruits rich in protocatechuic acid could be consumed by patients under nevirapine antiretroviral therapy. PCA could prevent nevirapine-induced oxidative damage because it is considered an antioxidant that exerts direct effects by scavenging free radicals (Chuen-Lan et al., 2002) and indirect effects by inducing the expression of antioxidant enzymes.

5.4 MODULATORY EFFECT OF PROTOCATECHUIC ACID (PCA) ON NEVIRAPINE-INDUCED INFLAMMATORY RESPONSE

In our previous study we demonstrated the involvement of inflammation in Nevirapine induced hepatotoxicity. The present study seeks to reaffirm the involvement of inflammation in nevirapine- induced hepatotoxicity and investigate the modulatory effect of protocatechuic acid on the drug- induced hepatotoxicity.

Hepatocyte damage usually result in the release of signals that stimulate activation of other cells, particularly those of the innate immune system, including Kupffer cells (KC), natural killer (NK) cells, and NKT cells. These cells contribute to the progression of liver injury by producing proinflammatory mediators and secreting chemokines to further recruit inflammatory cells to the liver.

It has been demonstrated that various inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-1β (Blazka *et al.*, 1995; Blazka *et al.*, 1996; Ishida *et al.*, 2002) produced during drug- induced liver

injury and involved in promoting tissue damage. Cytokines are low-molecular-weight proteins that regulate diverse physiological processes, such as growth, development, differentiation, wound healing, and immune response (Miki *et al*., 2007; Lu *et al*., 2006). In response to inflammation, cytokines are secreted from cells of the immune system.

Some cytokines stimulate or even aggravate inflammation, whereas others attenuate inflammatory responses. Several proinflammatory cytokines, especially IL-6 and TNF-α, have been implicated in inflammation-associated carcinogenesis and hepatoxicity (Lin *et al*., 2007; Noguchi *et al*., 1998; Rigby *et al*, 2007; Tsetepis *et al*., 2002).

The results from this study further reveal that nevirapine-induced hepatotoxicity is associated with a significant increase in proinflammatory cytokines expression (TNF-α and IL-1β). Treatment with PCA reduced the expression of these cytokines. This ability of PCA to lower the expression of proinflammatory cytokine may be linked to its antioxidant capacity since induction of inflammatory cytokines (TNF-α, IL-1, IL-6) have also been reported to play a role in oxidative stress-induced inflammation (Hussain *et al*., 2007).

This sustained inflammatory / oxidative environment leads to a vicious circle, which can damage healthy neighbouring cells and over a long period of time may lead to carcinogenesis (Federico et al., 2007).

In addition to these two proinflammatory cytokines, the effect of Nevirapine on other proinflammatory mediators like cyclooxygenase 2 (COX-2), inducible nitinc oxide sythase (iNOS), prostaglandin E-2 (PGE2), myeloperoxidase and nitrite level was also investigated.

Cyclooxygense-2 is a proinflammatory mediator and the rate limiting enzyme in prostaglandin biosynthesis from arachidonic acid. It has been implicated in liver inflammation and fibrosis (Horrillo et al., 2008). Inducible nitric oxide synthase is an inducible member of the three nitric oxide synthase isoforms (endothelial nitric oxide synthase (eNOS), neural nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS)). They catalyze the oxidation-reduction reaction of L-arginine in the presence of oxygen to form nitric oxide (NO) and L-citrulline. Under pathological conditions, however, iNOS is strongly up regulated and large amounts of NO are generated in the liver.

In this study, it was observed that treatment with Nevirapine brought about a significant induction of COX-2 and iNOS expression as verified by immunohistochemical staining. The increase in the expressions of these enzymes explains why nevirapine also caused a significant induction of PGE-2 and NO levels. PGE-2 and NO and are direct products of enzymatic activities of COX-2 and iNOS respectively. The findings from this study, further lend support to the role of COX-2 and iNOS in nevirapine- induced hepatic damage.

Nitric oxide (NO) formed from L-arginine by nitric oxide synthase plays an important role as a secondary messenger. Pathological conditions associated with release of cytokine e.g. inflammation and sepsis can increase NO production (Nathan, 1992). Nitric oxide ($NO^{\cdot}$) is a well known regulator of physiological processes (Pacher et al., 2007). However, the overproduction of $NO^{\cdot}$, accompanied by the enhanced superoxide generation, may result in the formation of peroxynitrite and a subsequent nitration of protein tyrosine residues leading to cell damage and organ dysfunction (Pacher et al., 2007).

Thus, the suppression of NO overproduction represents the therapeutic approach to nevirapine-mediated liver injury. PGE-2, a product of arachidonic acid breakdown via COX- 2 pathway has also been implicated in inflammation. It is one of the effector molecules through which COX-2 links inflammation to cancer.

Protocatechuic acid has been shown to suppress the expression of iNOS and COX-2 involved in inflammation and carcinogenesis (Cichocki *et al.*, 2011). Co-treatment with PCA in our study achieved a reduction in the levels of these proinflammatory mediators. This further demonstrates that PCA via its anti-inflamatory property is capable of ameliorating nevirapine- induced inflammation.

Results from the present study also show that nevirapine-induced hepatic injury can bring about polymorphonuccar leukocyte infiltration and accumulation as indicated by significant increase in myeloperoxidase (MPO) activity in nevirapine-treated rats. Myeloperoxidase (MPO) is a lysosomal enzyme present in the azurophilic granules of polymorphonuclear leukocytes (PMNs) and is unique to neutrophils and monocytes. MPO utilizes H_2O_2 produced by the neutrophils to oxidize a variety of aromatic compounds to give substrate radicals for bacterial activity (Hampton et al., 1998). This enzyme is unique however in that it can oxidize chloride ions to produce a strong non-radical oxidant, hypochlorous acid (HOCl). HOCl is the most powerful bactericidal produced by neutrophils. Excessive production of these radicals can cause oxidative stress leading to oxidative tissue injury.

The ability of PCA to lower the hepatic of MPO activity suggests its protective role is linked to its ability to reduce leukocytes infiltration and subsequently lower the expression of proinflammatory mediators that may exacerbate hepatic injury. The work of Tanaka and coworkers. (2011) corroborates this finding. They showed that PCA

could reduce activity of myeloperoxidase in the skin induced by the administration of 12-O-tetradecanoylphorbol 13-acetate (TPA).

5.5 NEVIRAPINE-INDUCED APOPTOSIS AND PROTECTIVE ROLE OF PROTOCATECHUIC ACID

Apoptosis is a genetically encoded form of cell suicide central to the development and homeostasis of multicellular organisms (Zhang et al. 2003; Ravagnan et al, 2002). Findings suggest that increased hepatocyte apoptosis is an important mechanism contributing to inflammation and fibrogenesis of the liver (Wu 2006).

In this study, we measured the expression of pro-apoptotic proteins caspase 3, caspase 9, cytrochrome C and p53 as biomarkers for detection of hepatic apoptosis. Also, the immunohistochemical analysis for apoptosis was performed by TUNEL method.

The caspases (cysteinyl aspartate specific proteases) - a group of proteases, play a central role as executors of the cell death programme (Nicholson and Thornberry, 1997). Caspases are constitutively expressed as inactive proenzymes and generally require proteolytic processing for their activation. Apoptotic caspases belong to two classes; the first is the initiators of apoptosis while the other is the executioners of apoptosis caspases (Guicciardi and Gores, 2005).

Activated downstream or execution caspases are responsible for degradation of several cellular substrates associated with the morphological changes of apoptosis, including nuclear degradation, cytoskeleton alterations, and membrane blebbing.

It was observed that the serum levels of the apoptotic marker caspase 9 in the nevirapine treated rats were markedly increased compared to the normal healthy control rats. This data shows that apoptosis was initiated in the nevirapine- treated rats. To

further investigate the involvement of apoptosis in nevirapine-induced liver injury, caspase 3 was assayed for as a representative of effector / executional caspases in this study. The serum level caspase 3 was also elevated in the nevirapine-treated rats when compared with the control. Results from this study is comparable to the work of Tarek *et al.*,(2014) which reported the elevation of serum caspase 3 in rats treated with carbon tetrachloride a standard hepatotoxin. Thus, it can be inferred from these results that there is induction of apoptosis in nevirapine- induced liver damage.

The level cytochrome C, an important apoptogenic factor (Kluck *et al.*, 1997) which interacts with the scaffolding protein Apaf-1 and subsequently activates caspase-9 (Li *et al.*, 1997) was also measured. There was a marked significant increase in the level of cytochrome C in nevirapine- treated rats when compared with control.

The tumor suppressor p53 has been implicated in the induction of the expression of apoptotic genes or direct activation of the apoptotic cascade (Vousden and Lu, 2002). The activation of effector caspase 3 (an executor of apoptosis) is at least in part, activated by ROS-induced activation of p53 (Bragado et al., 2007; Boatright and Salvesen, 2003). Our result shows that there was an activation of tumor suppressor p53 in nevirapine induced liver damage.

The immunohistochemical analysis for apoptosis was performed by TUNEL method. The chromogen-generated brown stain is an indication of apoptotic cells. More intense brown staining was observed in the nevirapine- treated rat when compared with the control. While less intense brown staining was observed in PCA treated rats. The result obtained from the liver tunnel assay serves as confirmatory test for the expression of pro-apoptotic proteins in nevirapine- treated rats.

Conversely, treatment with PCA resulted in a decrease in serum levels of these enzymes. This is an indication that PCA possesses protective properties against

nevirapine induced apoptosis. Although some studies have shown the involvement PCA in promoting apoptosis, ours showed the anti-apoptotic ability of PCA which is in consonance with the studies of Morikawa *et al.*, (1999); Lin, (2003) and Yen, (2000). To the best of our knowledge, this is the first time a study will show the involvement of apoptosis in nevirapine induced toxicity and the protective role of protocatechuic acid.

5.6 NEVIRAPINE-INDUCED GENOTOXICITY AND PROTECTIVE ROLE OF PROTOCATECHUIC ACID.

PCA is known for its free radical scavenging and antioxidant activities. These attributes make it a good candidate for preserving the integrity of biological molecules like protein and DNA by preventing their oxidation. The micronuclei test is one of the most widely applied short term test used in genetic toxicology and has become one of the most important tests implemented by the regulatory entities of different countries to evaluate mutagenicity of, and sensitivity to, xenobiotics (OECD, 1997; EPA, 1998).

The finding from our previous study shows that nevirapine is likely to be mutagenic due to the presence of increased number of micronucleus polychromatic erythrocytes. There was a marked elevation in the frequency of micro nucleated polychromatic erythrocytes observed in the nevirapine alone group when compared with control. This may be attributed to the presence of a Phase II metabolite which is a reactive electrophile, and therefore is expected to react directly with bionucleophiles (e.g., DNA) yielding covalent adducts. Antunes and coworker (2013) showed that nevirapine metabolite has a potential of forming DNA adduct and could be genotoxic.

Treating the animals with PCA resulted in a marked reduction in the frequency of micro nucleated polychromatic erythrocytes observed in the nevirapine alone group. This is corroborated by the works of Demetrios *et al.*, (2004) and Jaouad *et al.*, (2009)

that showed the antimutagenic and antigenotoxic activity of PCA. In conclusion, it can be inferred from this study that PCA can protect against nevirapine-induced genotoxicity.

CONCLUSION

The results of our study demonstrate that nevirapine can induce hepatotoxicity and with pronounced severity upon extended duration of exposure to the drug. Also, the results showed that the mechanisms by which nevirapine exert its toxicity may be through the stimulation of inflammatory response and oxidative damage due to free radical generation. Also, the observed increase in formation of micronuclei following treatment with nevirapine suggests that it may possess genotoxic potentials. Nevirapine also increased the concentration of apoptotic biomarkers. This shows that the mechanism of the toxicity of this drug may involve induction of apoptosis.

Furthermore, the study demonstrated that PCA can ameliorate nevirapine-induced hepatotoxicity *in vivo* by its antioxidant activity and also by inactivation of oxidative stress signaling pathway. This protection of nevirapine-induced hepatotoxicity by protocatechuic acid is associated with its ability to lower the levels of pro-inflammatory cytokines and prostaglandin E2 as shown by the results of the study. Similarly, protocatechuic acid offered protection against nevirapine-induced apoptosis and treatment with PCA reversed the formation of micronuclei.

Therefore, from the aforediscussed, this study showed that nevirapine possess hepatotoxic properties even at therapeutic dose, and protocatechuic acid can offer protection by ameliorating its effects.

CONTRIBUTION TO KNOWLEDGE

In this study, efforts were made to elucidate the possible molecular mechanisms that are involved in nevirapine- induced hepatotoxicity. The findings of the present study showed that prolonged oral treatment with therapeutic dose of nevirapine damaged the liver. Results showed that this nevirapine-mediated toxicity occurs via generation of free radicals, stimulation of inflammatory responses and initiation of apoptosis. The hepatoprotective role of protocatechuic acid was also explored; and results demonstrated that it can offer protection via its antioxidant property when co-administered with nevirapine.

There has been no study from literature, showing modulatory effect of PCA on nevirapine-induced inflamination. Data from this study indicate that PCA has modulatory capabilities on nevirapine-induced inflammation by attenuating the nevirapine-induced increased expression of COX-2 and iNOS.

Furthermore, there is no study from literature that has shown the involvement of apoptosis in nevirapine-induced toxicity. Data from this study suggest the involvement of apoptosis in nevirapine-induced hepatotoxicty and secondly that PCA has modulatory effect on nevirapine-induced apoptosis. This observed protective effect of PCA is via significant reduction in serum levels of apoptotic proteins - caspase 9 and 3, cytochrome c, and tumor supressor p53 in the PCA treatment groups. Similarly, the TUNEL assay result of this study showed that co-administration of PCA ameliorated nevirapine-induced nuclear DNA fragmentation (an important biochemical indicator of apoptosis in many cell types).

Also, this study used micronuclei assay to demonstrate that nevirapine could be genotoxic and that PCA can protect against nevirapine-induced genotoxicity.

REFERENCES

Adaramoye O. A., Adesanoye O. A., Adewumi O. M., Akanni O. (2011). Studies on the toxicological effect of nevirapine, an antiretroviral drug, on the liver, kidney and testis of male Wistar rats. *Human & Experimental Toxicology*. 31(7):676-85.

Adikwu Elias., Brambaifa Nelson., Deo Oputiri., Oru-Bo Precious Geoffrey (*2013*). Antiretroviral toxicity and oxidative stress. *American Journal of Pharmacology and Toxicology*. 8 (4): 187-196.

Adolph L., Lorenz R. (1982). Enzyme diagnosis in diseases of the heart, liver, and pancreas. *New York: S Karger*. 9–27.

Akande A.A., Olaosebikan O.F., Jimoh A.K., Abdulazeez Olawumi H.O. (2007). Lamivudine therapy and hepatotoxicity as seen in a Nigerian tertiary antiretroviral treatment centre. *African Journal of Biochemistry Research*. 1 (6): 090-094.

Antunes Alexandra M.M., Wolf Benjamin., Conceição Oliveira M., A. Belan Frederick., Matilde Marque M. (2013). 2'-Deoxythymidine Adducts from the Anti-HIV Drug Nevirapine. *Molecules* 18: 4955-4971.

Anthony B. (2001). Hepatitis, HIV and Your Liver. *AIDS Community Research Initiative of America community forum summary*.

Baer-Dubowska W., Szaefer H., Krajka-Kuzniak V. (1998). Inhibition of murine hepatic cytochrome P-450 activities by natural and synthetic phenolic compounds. *Xenobiotica*. 28:735–43.

Baeuerle P.A., Baltimore D. (1996). NF-kB: ten years after. *Cell*. 87:13–20.

Baldwin A.S. Jr. (1996). The NF-kappa B and I Kappa B protein: new discoveries and insights. *Ann. Rev. Immunol*. 14: 649-83

Beckman J.S. (1996). Oxidative and tyrosine nitration from peroxy nitrite. *Chem Res Toxicol*. 9: 836 – 844.

Beckman J.S., Crow JP. (1993). Pathological implications of nitric oxide superoxide and peroxynitite fonnation. *Biochem Soc Trans* 21, 330-334.

Bellini C., Keiser O., Chave J.P., Furrer H.J., Bucher H., Kaiser L., Telenti A., Cavassini M. (2003). Frequent liver dysfunction after lamivudioc withdrawal in hiv-hepatitis b co-infection. *Antivir Ther*. 8 : abstract no. 992.

Benjamin M.N. (1978). Outline of Veterinary Clinical Pathology. *University Press, IOWA, USA*. pp 229-232.

Bera E., Naidoo D., Williams M. (2012). Maternal deaths following nevirapine-based antiretroviral therapy. *South Afr. J. HIV Med.* 13 196-197.

Bersoff-Matcha S.J., Miller W.C., Aberg J.A. (2001). Sex differences in nevirapine rash. *Clin Infect Dis* 32:124-129.

Beutler E., Duron O. Kelly B.M. (1963). Improved method for the determination of blood glutathione. *J Lab Clin Med*. 61: 882-8.

Bissel D.M., Gores G.J., Laskin D.L., Hoofnagle J.H. (2001). Drug-induced liver injury: mechanisms and test systems. *Hepatol*. 39:1009–13.

Blazka M.E., Elwell M.R., Holladay S.D., Wilson R.E., Luster M.I. (1996). Histopathology of acetaminophen-induced liver changes: role of interleukin 1 alpha and tumor necrosis factor alpha. *Toxicol Pathol*. 24:181–189.

Blazka M.E., Wilmer J.L., Holladay S.D., Wilson R.E., Luster M.I. (1995) Role of proinflammatory cytokines in acetaminophen hepatotoxicity. *Toxicol Appl Pharmacol*. 133:43-52.

Blomhoff Rune. (2005). Dietary antioxidants. *Current Opinon in Lipidology*. 16:47-54.

Boatright K.M., Salvesen G.S. (2003). Mechanisms of caspase activation. *Curr Opin. Cell Biol.* 15: 725–731.

Bohrer H., Qin F., Zimmermann T. (1997). Role of NF-kB in the mortality of sepsis. *J Clin. Invest.* 100: 972–85.

Bondy S.C. (1992). Ethanol toxicity and oxidative stress toxicity. *Toxicolol Letter* 63:231-241.

Bragado P., Armesilla A., Silva A. (2007). Apoptosis by cisplatin requires p53 mediated p38alpha MAPK activation through ROS generation. *Apoptosis* 12:1733–1742.

Bruccoleri A., Gallucci R., Germolec D.R., Blackshear P., Simeonova P., Thurman R. G., Luster M. I. (1997). Induction of early-immediate genes by tumor necrosis factor alphacontribute to liver repair following chemical-induced hepatotoxicity. *Hepatology* 25: 133–141.

Burman W.J., Gallicano K., Peloquin C. (1999). Therapeutic implications of drug interactions in the treatment of human immunodeficiency virus-related tuberculosis. *Clinical Infectious Diseases*. 28: 419–430.

Carr A., Cooper D.A. (1996). Current clinical experience with nevirapine for HIV infection. *Adv. Exp. Med. Biol.* 394: 299-304.

Carr A., Vella S., de Jong M.D., Sorice F., Imrie A., Boucher C.A., Cooper D.A. (1996). A controlled trial of nevirapine plus zidovudine versus zidovudine alone in p24 antigenaemic HIV infected patients. *AIDS* 10: 635–641.

Carton J.A., Maradona J.A., Asensi V. (1999). Lamivudine for chronic hepatitis B and HIV coinfection. *AIDS*.13: 1002-1003.

Cheeseman S., Havlir D., McLaughlin M.M. (1995). Phase I/II evaluation of NVP alone and in combination with zidovudine for infection with human immunodeficiency virus. *Journal of Acquired Immune Deficiency Syndrome* 8:141–151.

Chen J., Mannarguli B.M., Xu L., Uetrecht J. (2008). Demonstration of the metabolic pathway responsible for nevirapine-induced skin rash. *Chem. Res. Toxicol.* 21:1862–1870.

Ching-Lung L., Rong-Nan C., Nancy W.Y., Leung Ting-Tsung C., Richard Guan, Dnr I.T., Keng-Yeen N., Pui-Chee W., Julie C.D., Judy Barber, Sally L.S., Fraser D.G. (1998). A One-Year Trial of Lamivudine for Chronic Hepatitis B. *The New Engl. J. Med.* 339:61-62.

Chu S.C., Marks-Konczalik J., Wu H.P. *(1998)*. Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: characterization of differences between human and mouse iNOS promoters. *Biochem. Biophys. Res. Commun.* 248:871–8.

Chuen-Lan Liua., Jin-Ming Wangb., Chiu-Yih Chub., Ming-Tzong Chenge., Tsui-Hwa Tsengb. (2002). In vivo protective effect of protocatechuic acid on tert-butyl hydroperoxide-induced rat hepatotoxicity. *Food and Chemical Toxicology*. 40:635–641.

Cichocki M., Blumczynska J., Baer-Dubowska W. (2010). Naturally occurring phenolic acids inhibit 12-O-tetradecanoylphorbol-13-acetate-induced

NF-kappa B, iNOS and COX-2 activation in mouse epidermis. *Toxicology*. 268:118-24.

Colette J.S., Sabin C.A., Lampe F.C. (2005). The relationship between CD4 cell count nadirs and the toxicity profiles of antiretroviral regimens. *Antivir Ther* 10:459-67.

Cominacini L., Pasini A.F., Garbin U., Davoli A., Tosetti M.L., Campagnola M. (2004). Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. *J Biol Chem*. 275:12633-8.

Cuzzocrea S., Riley D.P., Caputi A.P., Salvemini D. (2001). Antioxidant therapy: a new pharmacological approach in shock, inflammation and ischemia / reperfusion injury. *Pharmacol. Rev.* 53:135-9.

Czaja M.J., Xu J., Alt E. (1995). Prevention of carbon tetrachloride-induced rat liver injury by soluble tumor necrosis factor receptor. *Gastroenterology*. 108:1849-1854.

Dalle-Donne I., Giustarini D., Colombo R., Rossi R., Milzani A. (2003) Protein carbonylation in human diseases. *Trends Mol.Med* 9:169-176.

D'Aquila R.T., Hughes M.D., Johnson V.A., Fischl M.A., Sommadossi J.P., Liou S-H., Timpone J., Myers M., Basgoz N., Niu M. Hirsch M.S. (1996). Nevirapine, zidovudine, and didanosine compared with zidovudine and didanosine in patients with HIV-1 infection. *Annals of Internal Medicine*. 124: 1019-1030.

Demetrios Stagos., Spyridon Kouris., Demetrios Kouret. (2004). Plant Phenolics Protect from Bleomycin-induced Oxidative Stress and Mutagenicity in Salmonella typhimurium TA102. *Anticancer Research*. 24: 743-746.

Dianzani M.U. (1985). Lipid peroxidation in ethanol poisoning : A critical reconsideration. *Alcohol* 20, 2161-173.

Domingo P., Lozano F. (2011). Management of antiretroviral drug toxicity. *Enfermedades Infecciosas Mic. Clin.* 29: 535-544.

Eaton J.W. (1991). Catalases and peroxidases and glutathione and hydrogen peroxide: mysteries of the bestiary. *J. Lab. Clin Med.* 118:3-4.

Edwards C. K., III, Borcherding S.M., Zhang J., Borcherding D.R. (1991). Role of tumor necrosis factor α in acute and chronic inflammatory responses: novel therapeutic approaches. In *Xenobiotics and Inflammation* (L. B. Schook, and D. L. Laskin, Eds.). pp. 97–147. Academic Press. Boca Raton.

Eiserich J.P., Hristova M., Cross C.E., Jones A.D., Freeman B.A., Halliwell B., vander Vliet A. (1998). Formation of nitric oxide derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391:393–397.

Elias A., Brimbaifa N. (2013). Concentration-effect. incidence and mechanism of nevirapine hepatotoxicity. *Am. J. Pharmacol. Toxicol.* 8: 2030.

Elias A., Deo O., Geoffrey O., Enimeya D.A. (2013). Nevirapine hepatotoxicity: Implications of risk factors. *Am. J. Pharm. Toxicol* 8: 51-63.

Environmental Protection Agency (EPA) (1998). Health Effects Test Guidelines OPPTS 870.5395. Mammalian Erythrocyte Micronucleus Test. USA. 1-12.

European medicines agency. (2006) Non-clinical guideline on drug-induced hepatotoxicity. London, Doc. Ref. EMEA/CHMP/SWP/150115/2006.

Faromhi E.O., Shrotriya S., Surh Y. (2009). Kolaviron inhibits dimethyl nitrosamine–induced liver injury by suppressing COX-2 and iNOS expression via NF-KB and AP-1. *Life sciences* 84:149-155.

Federico A., Morgillo F., Tuccillo C., Ciardiello F., Loguercio C. (2007). Chronic inflammation and oxidative stress in human carcinogenesis. *Int. J. Cancer.* 121: 2381--2386.

Frei B., Stocker R., England L. Ames B.N. (1990): Ascorbate: The Most Effective Antioxidant in Human Blood Plasma. *Adv. Exp Med. Bio.* 264:155-163.

French M., Amin J., Roth N. (2002). Randomized, open-label, comparative trial to evaluate the efficacy and safety of three antiretroviral drug combinations including two nucleoside analogues and nevirapine for previously untreated HIV-1 infection: the O2Combo 2 study. *HIV Clin Trials* 3:177–85.

Fridovich I. (1989). Superoxide Dismustases. An Adaptation to paramagnetic. *Gas. J. Biol Chem.* 264: 7761 – 7764.

Fridovich I. (1995). Superoxide radical and superoxide dismutases. *Annual Rev. Biochem.* 64: 97–112.

Fromenty B., Pessayre D. (1995). Inhibition of mitochondrial betaoxidation as a mechanism of hepatotoxicity. *Pharmac. Ther.* 67: 101–54.

Gansler R.W., Taylor B.S., Shuo L. (2001). Complex regulation of manInducible nitric oxide synthase gene transcription by Stat 1 and NF-kappa B. *Proc. Natl. Acad. Sci. USA* 2001. 98: 8638–43.

Geller D.A., Lowenstcrin C.J., Shapiro R.A. (1993). Molecular cloning and expression of nitric oxide synthase from human hepatocytes. *Pro. Natl. Acad. Sci. USA* 1993 . 90 (8): 3491-5.

González de Requena D., Núñez M., Jiménez-Nacher I., Soriano V. (2002). Liver toxicity caused by nevirapine. *AIDS.* 16: 290-291.

Gornal A.G., Barawill J.C., David M.M. (1949). Determination of serum protein by means of biuret reaction. *J. Biol. Chem* 177: 751-761.

Guengerich F.P. (2001). Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol* 14:611-50.

Guengerich F.P., Kim D.H., Iwasaki M. (1990). Role of human cytochrome P450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem. Res. Toxicol.* 14:168-179.

Guicciardi M.E., Gores G.J. (2005). Recent advances in basic Science. Apoptosis: a mechanism of acute and chronic liver injury. *Gut* 54:10241033.

Gunawan B., Kaplowitz N. (2004). Clinical perspectives on xenobiotic-induced hepatotoxicity. *Drug Metab Rev.* 36:301-312.

Habig W.H., Pabst M.J., Jacoby W.B. (1974). Glutathione -S- transferase. The first enzymatic steps in mercapturic acid formation. *J.Biol.Chem.* 249: 7130-7139.

Haliwel B. (1977). Antioxidants and human disease: a general introduction. *Nutr. Rev.* 55: 544-9.

Halliwell B., Gutteridge J.M. (1989) Lipid peroxidation: a radical chain reaction. *Free Radicals in Biology and Medicine*. 188-276.

Halliwell B. (1996). Oxidative stress, nutrition and health experimental strategies for optimization of Nutritional antioxidant intake in humans. *Free Radical Res.* 25:57 – 74.

Hammer S.M. (2005). Single-dose nevirapine and drug resistance: the more you look, the more you find. *J. Infect. Dis.* 192: 1-3.

Hampton M.B., Kettle A.J., Winterbour I.C. (1998). Inside the neutrophil phagosome: oxidants myeloperoxidase and bacterial killing. *Blood* 92(9): 3007-17.

Haroon Saloojee (2002). Nevirapine - godsend or a drug from hell? *Science in Africa-.*

Harris M., Durakovic C., Rae S., Raboud J., Fransen A., Shillington A., Conway B., Montaner J.S.B. (1998). A pilot study of nevirapine, indinavir, and lamivudine among patients with advanced human immunodeficiency virus disease who have had failure of combination nucleoside therapy. *Journal of Infectious Diseases.* 177: 1514–1520.

Havlir D., Cheeseman S.H., McLaughlin M.M., Murphy R., Erice A., Spector S.A., Greenough T.C., Sullivan J.L., Hall D., Myers M. (1995). High-dose nevirapine: safety, pharmacokinetics, and antiviral effect in patients with human immunodeficiency virus infection. *Journal of Infectious Diseases.* 171: 537–545.

Hertog M.G.L., Feskens E.J.M., Hollman P.C.H., Katan M.B., Kromhout D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet* 342: 1007-1010

Hirose Y., Tanaka T., Kawamori T., Ohnishi M., Makita H., Mori H., Satoh K. (1995). Chemoprevention of urinary bladder carcinogenesis by the

Halliwell B. (1996). Oxidative stress, nutrition and health experimental strategies for optimization of Nutritional antioxidant intake in humans. *Free Radical Res.* 25:57 – 74.

Hammer S.M. (2005). Single-dose nevirapine and drug resistance: the more you look, the more you find. *J. Infect. Dis.* 192: 1-3.

Hampton M.B., Kettle A.J., Winterbour I.C. (1998). Inside the neutrophil phagosome: oxidants myeloperoxidase and bacterial killing. *Blood* 92(9): 3007-17.

Haroon Saloojee (2002). Nevirapine - godsend or a drug from hell? *Science in Africa-*.

Harris M., Durakovic C., Rae S., Raboud J., Fransen A., Shillington A., Conway B., Montaner J.S.B. (1998). A pilot study of nevirapine, indinavir, and lamivudine among patients with advanced human immunodeficiency virus disease who have had failure of combination nucleoside therapy. *Journal of Infectious Diseases.* 177: 1514–1520.

Havlir D., Cheeseman S.H., McLaughlin M.M., Murphy R., Erice A., Spector S.A., Greenough T.C., Sullivan J.L., Hall D., Myers M. (1995). High-dose nevirapine: safety, pharmacokinetics, and antiviral effect in patients with human immunodeficiency virus infection. *Journal of Infectious Diseases.* 171: 537–545.

Hertog M.G.L., Feskens E.J.M., Hollman P.C.H., Katan M.B., Kromhout D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet* 342: 1007-1010

Hirose Y., Tanaka T., Kawamori T., Ohnishi M., Makita H., Mori H., Satoh K. (1995). Chemoprevention of urinary bladder carcinogenesis by the

natural phenolic compound protocatechuic acid in rats. *Carcinogenesis*. 16:2337-42.

Holt M.P., Ju C. (2006). Mechanisms of drug-induced liver injury. *APS Journal*. 8(1): E48-E54.

Hussain S.P., Harris C.C. (2007). Inflammation and cancer: an ancient link with novel potentials. *Int. J. Cancer*. 121:2373–2380.

Ignatowicz E., Balana B., Vulimiri S.V., Szaefer H., Baer-Dubowska W. (2003). The effect of plant phenolics on the formation of 7. 12-dimethylben2[a]anthracene-DNA adducts and TPA-stimulated polymorphonuclear neutrophils chemiluminescence *in vitro*. *Toxicology*. 189:199-209.

Ikeda Y., Taniguchi N. (2005). Gene expression of gamma-glutamyl transpeptidase. *Meth. Enzymol*. 401:408–425.

Ishida Y., Kondo T., Ohshima T., Fujiwara H., Iwakura Y., Mukaida N. (2002). A pivotal involvement of IFN-gamma in the pathogenesis of acetaminophen-induced acute liver injury. *FASEB J*. 16:1227-1236.

Jaeschke H., Cores G.J., Cederbaum A.I., Hinson J.A., Pessayre D., Lemasters J.J. (2002). Mechanisms of hepatotoxicity. *Toxicol. Sci*. 66:166-76.

Jaeschke H. (2000). Reactive oxygen and mechanisms of inflammatory liver injury. *J. Gastroenterol. Hepatol*. 15:718–24.

Jakota S.K., Dani H.M. (1982). A new colorimetric technique for the estimation of vitamin C using folin phenol reagent. *Anal biochem*. 127: 178-182.

Jauad Anter., Magdalena Romera-Jiménez., Zahler Fernández-Bedmar., Myriam Villafero-Pulido., Mohamed Analla., Angeles Alonso-

Moraga., Andrés Muñoz-Serrano. (2011) Antigenotoxicity, cytotoxicity, and apoptosis Induction by Apigenin, Bisabolol, and Protocatechuic Acid. *Journal of Medicinal Food.* 14 (3): 276-283.

Jendrassik L., Grof P. (1938). Colorimetric Method of Determination of bilirubin. Biochem. 297:81.

John M. Cullen (2005). Mechanistic Classification of Liver Injury. *Toxicologic Pathology.* 33:6–8.

Jong Moon Hur., Ju Gwon Park., Ki Ho Yang., Jong Cheol Park., Jeong Ro Park, Soon Sil Chun., Jae Sue Choi., Jong Won Choi (2003). Effect of methanolic extract of Zanthoxylum piperitum leaves and of its compound, protocatechuic acid, on hepatic drug metabolizing enzymes and lipid peroxidation in rats. *Biosci. Biotechnol. Biochem.* 67 (5): 945-950.

Ju C., Reilly T.P., Bourdi M., Radonovich M.F., Brady J.N., George J.W., and Pohl L.R. (2002). Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem Res Toxicol* 15: 1504-1513.

Jung K., Wischke U.W. (1984). Electrophoretic variants of alanine aminopeptidase, alkaline phosphatase, an-glutamyl transferase in urine. *Clin. Chem.* 30:856-9.

Jung K., Mildner D., Jacob D, Scholz D., Precht K. (1981). On the pyridoxal-5-phosphate stimulation of AST and ALT in serum and erythrocytes of patients undergoing chronic hemodialysis and with kidney transplants. *Clin. Chim. Acta* 115:105–10.

Justyna Szumilo. (2005). Protocatechuic acid in cancer prevention. *Postepy Hig Med Dosw.* 59: 608-615.

Kampa M., Alexaki V.I., Notas G., Nifli A.P., Nistikaki A., Hatzoglou A., Bakogeorgou E. Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast Cancer cells: potential mechanisms of action. *Breast Cancer Res.* 6: R63-74.

Kayama F., Yoshida T., Elwell M.R., Luster M.I. (1995). Role of tumor necrosis factor in cadmium-induced hepatotoxicity. *Toxicol. Appl. Pharmacol.* 13: 224–234.

Kaukofer M., Lipko J., Zduneczyk S. (2005).Total antioxidant capacity of bovine spontaneously released and retained placenta. *Pathophysiology* 11, 215- 219.

Kim S.O., Kundu J.K., Shin Y.K., Park J.H., Cho M.H., Kim T.Y., Surh Y.J., (2005). 6-Gingerol inhibits COX-2 expression by blocking the activation of p38 MAP kinase and NF-kappaB in phorbol ester-stimulated mouse skin. *Oncogene* 24:2558-2567.

Klaunig J.E., Kumenalulis L.M. (2004). The role of oxidative stress in carcinogenesis. *Annu Rev. Pharmacol. Toxicol.* 44:239.67.

Kluck R.M., Bossy-Wetzel E., Green D.R., Newmeyer D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science.* 275 5303:1132-1136.

Koop D.R. (1992). Oxidative and reductive metabolism by cytochrome P4502E1. *FASEB J.* 6: 724-730.

Koracevic D., Koracevic G., Djordjevic V., Andrejevic S., Cosic V. (2001). Methods for the measurement of antioxidant activity in human fluids. *J. Clin. Pathol.* 54: 356-61.

Krasowska A., Stasiuk M., Oswiecimska M., Kozubek A., Bien M., Witek S., Sigler K. (2001). Suppression of radical –induced lipid preoxidation in a model system by alkyl ester of Cinnamatre quaternary ammonium salts. *Z. Natur Forsch.* 56c:878-885.

Lanping M., Zaiqun L., Bo Z., Li Y., and Zhongli L. 2000. Inhibition of free radical induced oxidative hemolysis of red blood cells by green tea polyphenols. *Chinese Science Bull.* 45: 2052-2056.

Laskin D.L., Heck D.E., Gardner C.R. *(1994).* Distinct patterns of nitric oxide production in hepatic macrophages and endothelial cells following acute exposure of rats to endotoxin. *J. Leukoc. Biol.* 56 (6):751-8.

Lee W. (2003). Drug-induced hepatotoxicity. *N. Engl. J. Med.* 349: 474–85.

Lewis J.H. (2000). Drug-induced liver disease. *Med. Clin. North Am.* 84:1275–1311.

Li J., Billiar T.R. (1999). Nitric oxide. IV. Determinants of nitric oxide protection and liver injury. *Am. J. Physo.* 276(5): G1069-73.

Li P., Nijhawan D., Budihardjo I. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell.* 91 (4):479-489.

Lin W.L., Hsieh Y.J., Chou F.P., Wang C.J., Cheng M.T., Tseng T.H. (2003). Hibicus protocatechuic acid Inhibit lipopolysaccharide-induced rat hepatic damage. *Arch. Toxicol.* 77:42-7.

Lin W.W., Karin M. (2007). A cytokine-mediated link between innate immunity, inflammation, and cancer. *J. Clin Invest.* 117:1175–1183.

Lott J.A., Wolf P.L. (1986). Alanine and aspartate aminotransferase (ALT and AST). Clinical enzymology: a case-oriented approach. *Chicago: Year Book Medical Publishers*. 1986:111–38.

Lu H., Ouyang W., Huang C. (2006). Inflammation, a key event in cancer development. *Mol. Cancer Res.* 4:221–233.

Mae-Wan Ho. (2007). Beyond the HIV-Causes-AIDS Model. The Institute of Science in Society. ISIS Press Release .

Marina Núñez., Daniel González-Reiruena., Juan González-Lahoz., Vincent Soriano. (2003). Interactions between Nevirapine Plasma Levels, Chronic Hepatitis C, and the Development of Liver Toxicity in HIV-Infected Patients. *AIDS Research and Human Retroviruses* 19(3): 187-188.

Marseille E., Kahn J.G., Mmiro F., Guay L., Musoke P., Fowler M.G., Jackson, J.B. (1999). Cost effectiveness of single-dose nevirapine regimen for mothers and babies to decrease vertical HIV-1 transmission in sub-Saharan Africa. *Lancet* 354:803–809.

Masella R., Vari R., D' Archivio M, Di Benedetto R., Matarrese P., Malorni. (2004). Extra Virgin Oil Biophenls Inhibit Cell-Mediated Oxidation Of LDL By Increasing The mRNA Transcription Of Glutathione-Related Enzymes. *J. Nutr.* 134:785-91.

Merluzzi K.D., Hargrave K., Labadia M., Grozinger K., Skoog M., Wu J.C., Shih C.K., Eckner K., Hattox S., Adams J., Rosenthal A.S., Faanes R., Eckner R.J, Koup R.A., Sullivan J.L. (1990). Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. *Science (Washington DC)* 250:1411–1413.

Mershiba S., Dassprakash M., Saraswathy S. (2012). Protective effect of naringenin on hepatic and renal dysfunction and oxidative stress in arsenic intoxicated rats. *Mol. Biol. Rep.* 40(5): 3681-3691.

Michael P. Holt., Cynthia Ju (2006). Mechanisms of drug-induced liver injury. *AAPS Journal.* 8(1): E48-E54.

Miki C., Inoue Y., Araki T., Uchida K., Kusunoki M. (2007). Cytokines and cancer development. *J. Surg. Oncol.* 95:10–11.

Miller V., Staszewski S., Boucher C.A.B., Phair J.P. (1997). Clinical experience with non-nucleoside reverse transcriptase inhibitors. *AIDS* 11: 157–164.

Misra H.P., Fridovich I.J. (1972). The univalent reduction of oxygen by reduced flavins and quinines. *J. Biol. Chem.* 247: 188-192.

Miura T., Matsuda Y., Tsuji A., Katunuma N. (1981). Immunological cross reactivity of gamma-glutamyl transpeptidase from human and rat kidney. *J Biochem (Tokyo)* 89: 217–22.

Mocroft A., Ledergerber B., Katlama C. (2003). Decline in the AIDS and death rates in the EuroSIDA study: an observational study. *Lancet* 362:22–9.

Mohan S., Epstein J.B. (2003). Carcinogenesis and cyclooxygenase: the potential role of COX-2 inhibition in upper aerodigestive tract cancer. *Oral Oncology* 39:537–546.

Montaner J.S.G., Reiss P., Cooper D., Vella S., Harris M., Conway B., Wainberg M.A., Lange J.M.A. (1998). A randomized, double-blind trial comparing combinations of nevirapine, didanosine, and zidovudine for HIV-infected patients: the INCAS trial. *Journal of the American Medical Association.* 279: 930–937.

Moos A. B., Baccher-Steppan L., Kerkvliet N.I. (1994). Acute inflammatory response to sheep red blood cells in mice treated with 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin: The role of proinflammatory cytokines. IL-1 and TNF. *Toxicol. Appl. Pharmacol.* 127:331–335.

Morikawa A., Kato Y., Sugiyama T., Koide N., Chakravortty D., Yoshida T., Yokochi T. (1999). Role of nitricoxide in lipopolysaccharide-induced hepatic injury in D-galactosamine-sensitized mice as anexperimenta endotoxic shock model. *Infect Immun.* 67:1018-24.

Murphy R., Montaner J. (1996). Nevirapine: a review of its development, pharmacological profile and potential for clinical use. *Expert Opinion on Investigational Drugs* 5: 1183-1199.

Murphy R., Sommadossi J.P., Lamson M.J., Hall D., Myers M., Dusek A. (1999). Antiviral effect and pharmacokinetic interaction between nevirapine and indinavir in HIV-1 infected individuals. *Journal of Infectious Disease.* 179: 1116-1123.

Nakamura Y., Torikai K., Ohto Y., Murakami A., Tanaka T., Ohigashi H (2000). A simple phenolic antioxidant protocatechuicacid enhances tumor promotion and oxidative stress in female ICR mouse skin: dose and timing-dependent enhancement and involvement of bioactivation by tyrosinase. *Carcinogenesis* 21:1899-907.

Nakbi A., Tayeb W., Grissa A., Issaoui M., Dabbai S., Chargui I., Elhouez M., Mileda., Hammami M. (2010). Effects of oliveoil and its fraction on oxidative stress and liverfatty acid composition on 2,4-dichlorophenoxyacetic acid treated rats. *Nutrition and Metabolism* 7:80.

Nathan C. (1992). Nitric Oxide as a Secretory Product of Mammalian Cells. *FASEB.* 6:3051–64.

Navarro-Gonzalvez J.A., Garcia-Benayas C., Arenas J. (1998): Semi automated measurement of nitata in biological fluids. *Clinc Chem.* 44(3): 679-681.

Nicholson D.W., Thornberry N.A. (1997). Caspases: killer protcases. *Trends Biochem Sci* 22:299–306.

Nicole J. Fernandez, Beverly A. Kidocy (2007). Alkaline phosphatase: beyond the liver. *Vet Clin Pathol.* 36:223–233.

Noguchi M., Hiwatashi N., Liu Z., Toyota T. (1998). Secretion imbalance between tumour necrosis factor and its inhibitor in inflammatory bowel disease. *Gut* 43: 203–209.

Nora Heisterkamp., John GroVen., David Warburton., Tam P. Sneddon (2008). The human gamma-glutamyltransferase gene family. *Hum Genet* DOI 10.1007/s00439-008-0487-7.

Organization for Economic Operation and Development (1997). Guidelines for Testing of Chemicals n. 474. Mammalian Erythrocyte Micronucleus Test. Paris. pp 1-10.

Pacher P., Beckman J.S., Liaudet L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev.* 87: 315–424.

Palella F.J., Delaney K.M., Moorman A.C. (1998). Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med* 338:853–60.

Panoutsopoulos G.I., Beedham C. (2005). Enzymatic oxidation of vanillin, isovanillin and protocatechuic aldehyde with freshly prepared Guinea pig liver slices. *Cell Physiol Biochem.* 15:89-98.

PDR staff. VIRAMUNE® (nevirapine). In Physicians' Desk Reference, 63rd ed., Physicians' Desk Reference Inc.: Montvale, NJ, USA. 2009: . 873–881.

Pessayre D., Mansouri A., Haouzi D., and Fromenty B. (1999). Hepatotoxicity due to mitochondrial dysfunction. *Cell Biol Toxicol* 15: 367–73

Pompella A., Visvikis A., Paolicchi A., DeTata V., Casini A.F. (2003). The changing faces of glutathione, a cellular protagonist. *Biochem Pharmacol.* 66:1499–1503.

Popovic M., Caswell J.L., Mannargudi B., Shenton J.M., Uetrecht J.P. (2006). Study of the sequence of events involved in nevirapine-induced skin rash in Brown Norway rats. *Chem. Res. Toxicol.* 19:1205–1214.

Pozniak A.L., Miller R. Ormerod L.P. (1999). The treatment of tuberculosis in HIV-infected persons. *AIDS* 13: 435–445.

Raquel Horrillo, Anna Planagumà, Ana González-Périz, Natàlia Ferré, Esther Titos, Rosa Miquel, Marta López-Parra, Jaime L. Masferrer, Vicente Arroyo, Joan Clària. (2008). Comparative protection against liver inflammation and fibrosis by a selective cyclooxygenase-2 inhibitor and a nonredox-type 5-lipoxygenase inhibitor. *The Journal of pharmacology and experimental therapeutics.* 323(3):778-86.

Ravagnan L., Roumier T., Kroemer G. (2002). Mitochondria, the killer organelles and their weapons. *Journal of Cellular Physiology.* 192: (2):131–137.

Reed J.C. (2001). Apoptosis-regulating proteins as targets for drug discovery. *Trends Mol Med* 7:3149.

Reitman S., Frankel S. (1957). A colorimetric method for the determination of serum GOT and GPT. *Amer. J. Clin. Path.* 28: 56.

Rej R. (1984). Measurement of aminotransferases. Part I. Aspartate aminotransferase. *CRC Crit Rev Clin Lab Sci* 21:99–106.

Richman D., Rosenthal A.S., Skoog M., Eckner R.J., Sabo J.P, Merluzzi V.J. (1991). BI-RG-587 is active against zidovudine-resistant human immunodeficiency virus type I and synergizes with zidovudine. *Journal of Antimicrobial Agents and Chemotherapy* 35: 305–308.

Rigby R.J., Simmons J.G., Greenhalgh C.J., Alexander W.S., Lund P.K. (2007). Suppressor of cytokine signaling 3 (SOCS3) limits damage-induced crypt hyperproliferation and inflammation-associated tumorigenesis in the colon. *Oncogene* 26:4833–4841.

Ringer D.H., Dabich L. (1979). Haematology and Clinical Biochemisity In: the laboratory rats vol. I. Baker HJ, Lindsey JR, Weisbroth SH. *Academic Press London* 105-116.

Riska P., Lamson M., Macgregor T., Sabo J., Hattox S., Pav J., Keirns J. (1999). Disposition and Biotransformation of the Antiretrovital drug Nevirapine in humans. *The American Society for Pharmacology and Experimental Therapeutics* 27: 895-901.

Riska P.S., Joseph D.P., Dinallo R.P, Keirns J.J. (1996). Metabolism of nevirapine, a nonnucleoside reverse transcriptase inhibitor, in mouse, rat, dog, rabbit and monkey. *ISSX Proceedings* 10: 290

Robin M.A., Le Roy M., Descatoire V., Pessayre D. (1997). Plasma membrane cytochromes P450 as neoantigens and autoimmune targetsin drug-induced hepatitis. *J Hepatol* 26: Suppl 1:23-30.

Rotruck, J.T, Pope, A.L, Ganther, H.E, Swanson, A.B, Hafeman, D.G and Hockstra W.G. (1973). Selenium: biochemical role as a component of glutathione peroxidise. *Science* 179: 588-90.

Ruiz L., Negredo E., Domingo P., Pareles R., Francia E., Balagué M., Gel S., Bonjoch A., Fumaz C.R., Johnston S. (2001). Antiretroviral treatment simplification with nevirapine in protease inhibitor-experienced patients with HIV-associated lipodystrophy: 1-year prospective follow-up of a multicenter, randomized, controlled study. *J. Acquir. Immune Defic Syndr.* 27:229–236.

Rust C., Cores G.J. (2000). Apoptosis and liver disease. *Am J Med* 108:567–74.

Sahai J., Cameron W., Saigo M., Stewart F., Myers Lamson M.J. and Gagnier P. (1997). Drug interaction study between saquinavir and nevirapine (Abstract). *International AIDS Conference (abstract no.614)* 4: 178.

Sasz G. A kinetic photometric method for serum γ- glutamy transferase. *J Clin Chem* 15:124

Schafer J.J., Ravi S., Rowland E.V., Leon N., (2011). The expanding class of non-nucleosidereverse transcriptase inhibitors for the treatment of HIV-1 infection. *P T.* 36: 346-364.

Schmidt W. (1975). The Micronucleus test. *Mutat. Res.* 31:9-15.

Schmeda-Hirschmann G., Tapia A., Theoduloz C., Rodriguez J., Lopez S., Feresin G.E. (2004). Free radical scavengers and antioxidants from Tagetes mendocina. *Z. Naturforsch C.* 59:345-53.

Seddon J.N., Vare J.R., (1988). Endotheluim–Derived Relaxing Factor Reduces Platelet Ahesion to Bovine Endothelium Cells. *Ibid* 85: 1341 –1344.

Sichenlist U., Franzoso G., Brown K. (1994). Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol* 10: 405-55.

Simon P. Wolff, Anthony Garner., Roger T. Dean (1986). Free radicals, lipids and protein degradation. *Trends in biochemical sciences* 11(1):27-31

Sinha K.A. (1972). Colorimetric assay of catalase. *Anal Biochem* 47: 389-394.

Srivastava A., Lian L.Y., Maggs J.L., Chaponda M., Pirmohamed M., Williams D.P., Park B.K. (2010). Quantifying the metabolic activation of nevirapine in patients by integrated applications of NMR and mass spectrometries. *Drug Metab Dispos.* 38:122–132.

Sroka Z., Cisowski W. (2003). Hydrogen peroxide scavenging, antioxidant and antiradical activity of some phenolic acids. *Food Chem Toxicol.* 41:753-8.

Syakalima M., Takiguchi M., Yasuda J., Hashimoto A. (1998). The canine alkaline phosphatases: a review of the isoenzymes in serum, analytical methods and their diagnostic application. *Jpn J Vet Res.* 46:3–11.

Szefer H., Jodynis-Liebert J., Clebucki M., Matuszewska A., Baer-Dubowska W. (2003). Effect of naturally occurring planiphenolics on the induction of drug metabolizing enzymes by o-toluidine. *Toxicology.* 186:67-77.

Tanaka Takuji., Tanaka Takahiro., Tanaka Mayu (2011). Potential cancer chemoprevention of protocatechuic acid. *J Exp Clin Med* 3(1):27

Tanaka T., Kawamori T., Ohnishi M., Okamoto K., Mori H., Hara A., (1994).Chemoprevention of 4-nitroquinoline-1-oxide-induced oral

carcinogenesis by dietary protocatechuic acid during initiation and post-initiation phase. *Cancer Research* 54:2359-2365.

Tanaka T., Kojima T., Kawamori T., Mori H. (1995). Chemoprevention of digestive organs carcinogenesis by natural product protocatechuic acid. *Cancer*. 75 (Suppl.) 1433–1439.

Tanaka T., Kojima T., Kawamori T., Yoshimi N., Mori H. (1993). Chemoprevention of diethylnitrosamine-induced hepatocarcinogenesis by a simple phenolic acid. protocatechuic acid in rats. *Cancer Research* 53:2775–2779.

Tarek K Motwi., Samia A Ahmed., Nadia S Metwally., Reem M Samy., Noha N Nabir (2014). Edible plants induced recovery from hepatic disorder through Matrix metalloproteinase 9 elevation. *Int. J. Pharm. Sci. Rev. Res.*, 24(2): 65-78.

Tate S.S., Meister A. (1981). Gamma-Glutamyl transpeptidase: catalytic, structural and functional aspects. *Mol Cell Biochem* 39: 357-68.

Thagaiyan Radhig., Kodukkur Viswanathan Pugalendi (2011). Potential beneficial effect of protocatechuic acid on hepatic marker, lipid peroxidation and antioxidant status against D-galactosamine induced toxicity in rats. *Journal of Pharmacy Research* 4 (1):222-225.

Trauner M., Meier P.J., Boyer J.L. (1998). In: Mechanisms of Disease (F. H. Epstein, ed.). Molecular pathogenesis of cholestasis. *N Eng J Med* 339: 1217–27.

Trautwein C., Rakemann T., Brenner D.A. (1998). Concanavalin A-induced liver cell damage: activation of intracellular pathways triggered by tumour necrosis factor in mice. *Gastroenterology* 114: 1035-45.

Tselepis C., Perry I., Dawson C., Hardy R., Darnton S.J., McConkey C., Stuart R.C., Wright N., Harrison R., Jankowski J.A. (2002). Tumour necrosis factor-α in Barrett's oesophagus: a potential novel mechanism of action. *Oncogene*. 21:6071–6081.

Tseng T.H., Hsu J.D., Lo M.H., Chou F.P., Huang C.L., Chu C.Y., Wang C.J., (1998). Inhibitory effect of Hibiscus protocatechuic acid on tumor promotion in mouse skin. *Cancer Letters* 126: 199–207.

Tseng T.H., Kao T.W., Chu C.Y., Chou F.P., Lin W.L., Wang C.J. (2000). Induction of apoptosis by hibiscus protocatechuic acid in human leukemia cells via reduction of retinoblastoma (RB) Phosphorylaion and Bcl-2 expression. *Biochem Pharmacol* 60:307-15.

Tseng T.H., Wang C.J., Kno E.S., Chu H.Y., (1996). Hibiscus protocatechuic acid protects against oxidative damage induced by tertbutylhydroperoxide in rat primary hepatocytes. *Chemico-Biological Interactions* 101:137–148.

Tsuda T., Horio F., Osawa T. (1999). Absorption and metabolism of cyanidin 3-O-beta D-glucoside in rats. *FEBS Lett.* 449:179-82.

Uchida K., Stadtman E.R. (1993). Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase: A possible involvement of intra- und inter molecular cross-linking reaction. *J. biol. Chem.* 268:6388-6393.

Uma Maheswari M., Rao P. G. M. (2005). Antihepatotoxic effect of grape seed oil in rat. *Indian J Pharmacol* 37: 179.182.

Umar R.A., Hassan S.W., Ludu M.J., Mutazu I.K., Shehu H., Shehu R.A., Muhammed L.G., Mojeho E.I. (2008). Adverse hepatic effects

associated with administration of antiretroviral drugs (nevirapine, lamivudine and stavudine) to albino rats: implication for management of patients with HIV/AIDS. *Asian Journal of Biochemistry* 1: 19-25.

Valentova K. Cvak L., Muck A., Ulrichova J., Simanek V. (2003). Antioxidant activity of extracts from the leaves of Smallanthus sonchifolius. *Eur J Nutr.* 42:61-6.

Valko M., Leibfritz D., Moncol J., Cronin M.T., Mazur M., Telser J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* 39:44-84.

Varshney R., Kale R.K. (1990). Effect of calmodulin antagonists on radiation induced lipid peroxidation in microsomes. *Int. J. Rad. Biol.* 58: 733-743.

Von Gadow A., Joubert E., Hansmann C.F. (1997). Comparison of antioxidant activity of aspalathin with that of other plant phenols of Rooibosed tea (Aspalathon linearis). α-tocopherol, BHT and BHA. *J. Agric. Food Chem.* 45: 632-638.

Vousden K.H., Lu X. (2002). Live or let die, the cell's response to p53. Nat. Rev. *Cancer* 2:594-604.

Watanabe S., Phillips M.J. (1986). Acute phalloidin toxicity in living hepatocytes. Evidence for a possible disturbance in membrane flow and for multiple functions for actin in the liver cell. *Am J Pathol* 122: 101-11.

Weinshilboum R. (2003). Inheritance and drug response. *N Engl J Med* 348: 529-37.

Wen B., Chen Y., Fitch W.L. (2009). Metabolic activation of nevirapine in human liver microsomes: Dehydrogenation and inactivation of cytochrome P450 3A4. *Drug Metab. Dispos.* 37:1557-1562.

associated with administration of antiretroviral drugs (nevirapine, lamivudine and stavudine) to albino rats: implication for management of patients with HIV/AIDS. *Asian Journal of Biochemistry* 1: 19-25.

Valentova K. CvakL., Muck A., Ulrichova J., Simanck V. (2003). Antioxidant activity of extracts from the leaves of Smallanthus sonchifolius. *EurJ Nutr.* 42:61-6.

Valko M., Leibfritz D., Moncol J., Cronin M.T., Mazur M., Telser J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* 39:44–84.

Varshney R., Kale R.K. (1990). Effect of calmodulin antagonists on radiation induced lipid peroxidation in microsomes. *Int. J. Rad. Biol.* 58: 733-743.

Von Gadow A., Joubert E., Hansmann C.F. (1997). Comparison of antioxidant activity of aspalathin with that of other plant phenols of Rooibosed tea (Aspalathon linearis), a-tocopherol, BHT and BHA. *J. Agric. Food Chem.* 45: 632-638.

Vousden K.H., Lu X. (2002). Live or let die, the cell's response to p53. Nat. Rev. *Cancer* 2:594–604.

Watanabe S., Phillips M.J. (1986). Acute phalloidin toxicity in living hepatocytes. Evidence for a possible disturbance in membrane flow and for multiple functions for actin in the liver cell. *Am J Pathol* 122: 101–11.

Weinshilboum R. (2003). Inheritance and drug response. *N Engl J Med* 348:529-37.

Wen B., Chen Y., Fitch W.L. (2009). Metabolic activation of nevirapine in human liver microsomes: Dehydrogenation and inactivation of cytochrome [illegible] 1562.

Wester C.W., Kim S., Bussmann H. (2005). Initial response to highly active antiretroviral therapy in HIV-1C-infected adults in a public sector treatment program in Botswana. *J Acquir Immune Defic Syndr* 40:336–43.

Wolf L.D. (2003). Update on the clinical liver function testing. San Diego Health System, University of California, USA.

Wolff S.P (1994). Ferrous ion oxidation in the presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol* 233: 182-89.

Wroblewski F. (1958). The clinical significance of alterations in transaminase activities of serum and other body fluids. *Adv Clin Chem* 1:313–51.

Wu D., Zhai Q., Shi X. (2006). Alcohol-induced oxidative stress and cell responses." *Journal of Gastroenterology and Hepatology*. 21, 3:S26–S29.

Xue-Lian Ma., Yue-Hua Li., Jian-Xin Guo., Jing Li, Lin Guo., Cui-Zhen Wu. (2008). Expression of inducible nitric oxide synthase in the liver is under the control of nuclear factor kappa B in concanavalin A-induced hepatitis. *Journal of Gastroenterology and Hepatology* 23:e231–e235.

Yamada Y., Fausto N. (1998). Deficient liver regeneration after carbon tetrachloride injury in mice lacking type 1 but not type 2 tumor necrosis factor receptor. *Am. J. Pathol.* 152:1577–1589.

Yun J.J., Jung J.S., Hong Y.J., Moon Y.S., Suh H.W., Kim Y.H., Yun-Choi H.S. (2004). Protective effect of protocatechuic acid isopropyl ester against murine models of sepsis: inhibition of TNF-alpha and nitric oxide [illegible] oIL-10. *Biol Pharm Bull* 27:2024-7.

antiretroviral therapy in HIV-1C-infected adults in a public sector treatment program in Botswana. *J Acquir Immune Defic Syndr* 40:336–43.

Wolf L.D. (2003). Update on the clinical liver function testing. San Diego Health System. University of California, USA.

Wolff S.P (1994). Ferrous ion oxidation in the presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol* 233: 182-89.

Wroblewski F. (1958). The clinical significance of alterations in transaminase activities of serum and other body fluids. *Adv Clin Chem* 1:313–51.

Wu D., Zhai Q., Shi X. (2006). Alcohol-induced oxidative stress and cell responses." *Journal of Gastroenterology and Hepatology*. 21, 3:S26–S29.

Xue-Lian Ma., Yue-Hua Li., Jian-Xin Gao., Jing Li, Lin Guo., Cui-Zhen Wu. (2008). Expression of inducible nitric oxide synthase in the liver is under the control of nuclear factor kappa B in concanavalin A-induced hepatitis. *Journal of Gastroenterology and Hepatology*. 23:e231–e235.

Yamada Y., Fausto N. (1998). Deficient liver regeneration after carbon tetrachloride injury in mice lacking type 1 but not type 2 tumor necrosis factor receptor. *Am. J. Pathol.* 152:1577–1589.

Yan J.J., Jung H.S., Hong Y.J., Moon Y.S., Suh H.W., Kim Y.H., Yun-Choi H.S. (2004). Protective effect of protocatechuic acid isopropyl ester against murine models of sepsis: inhibition of TNF-alpha and nitric oxide production and augmentation of IL-10. *Bio Pharm Bull* 27:2024-7.

Yazdanian M., Ratigan S., Joseph D. (1999). Blood-brain barrier properties of human immunodeficiency virus antiretrovirals. *Journal of Pharmaceutical Sciences* 88: 950–954.

Yen G.C., Chen H.Y. (1995). Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J. Agr. Food Chem.*, 43: 27–32.

Yen G.C., Hsieh C.L. (2000). Reactive oxygen species scavenging activity of Du-zhong (Eucommia ulmoides oliv.) and its active compounds. *J Agric Food Chem* 48:3431-6.

Yin M.C., Lin C.C., Wu H.C., Tsao S.M., Hsu C.K. (2009). Apoptotic effects of protocatechuic acid in human breast, lung, liver, cervix, and prostate cancer cells: potential mechanisms of action. *J Agric Food Chem.* 57:6468-73.

Yoshino M., Murakami K. (1998). Interaction of iron with polyphenolic compounds application to antioxidant characterization. *Anal Biochem.* 257:40-4.

Zhang J., Dong M., Li L. (2003). Endonuclease G is required for early embryogenesis and normal apoptosis in mice. Proceedings of the National Academy of Sciences of the United States of America. vol. 100. no. 26. 15782–15787.

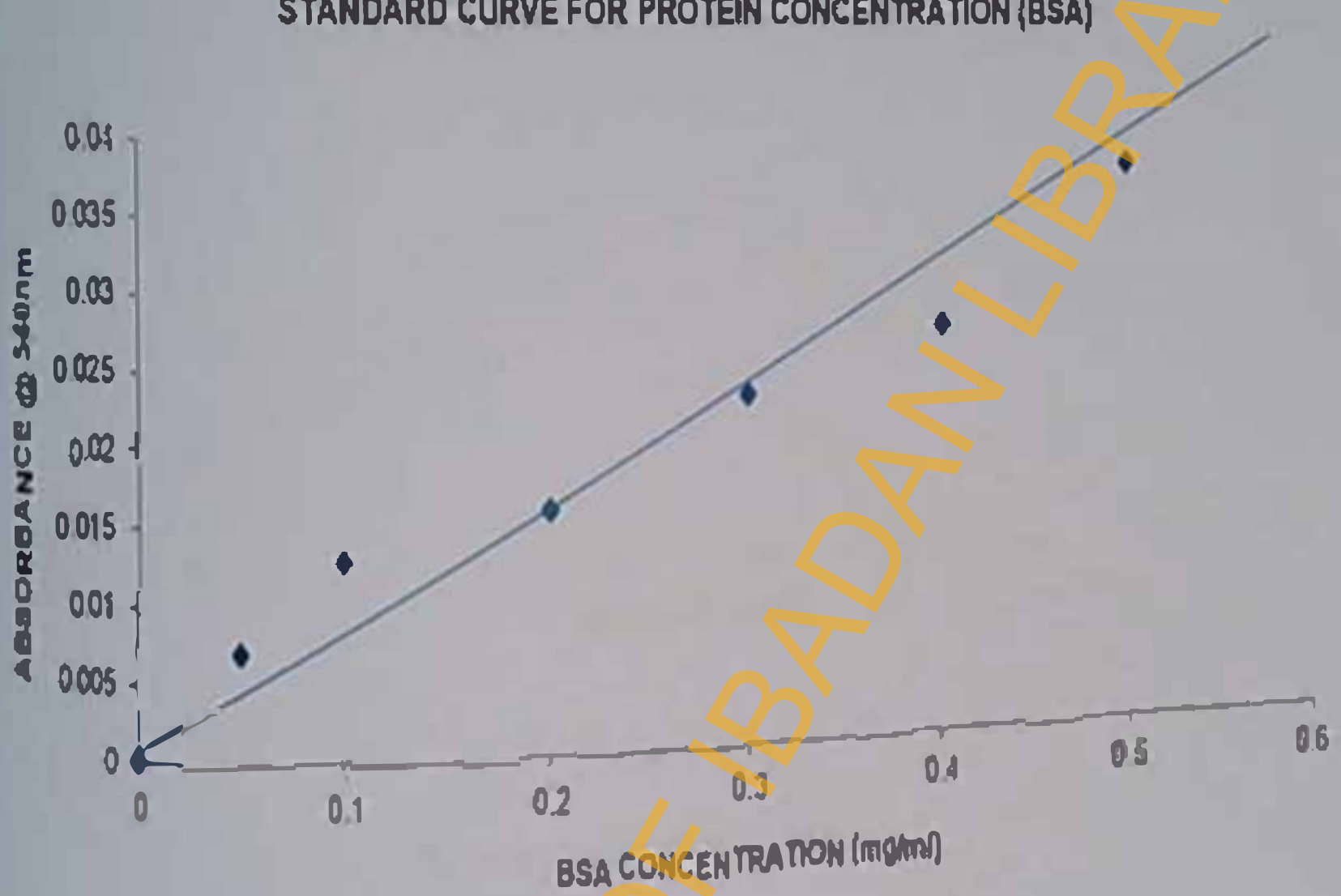
Zhang J., Huang W., Chua S.S., Wei P., Moore D.D. (2002). Modulation of acetaminophen-induced hepatotoxicity by the xenobiotic receptor CAR. *Science* 298:422–24.

Zhou J., Kumarasamy N., Ditangco R. (2005). The tread Asia HIV observational database: baseline and retrospective data. *J Acquir Immune Defic Syndr* 38:174–9.

Zhou-Stache J., Buettner R., Artmann G., Mittermayer C., Bosserhoff A.K. (2002). Inhibition of TNF-alpha induced cell death in human umbilical vein endothelial cells and Jurkat cells by Protocatechuic acid. *Med Biol Eng Comput*. 40:698-703.

APPENDIX

FIGURES



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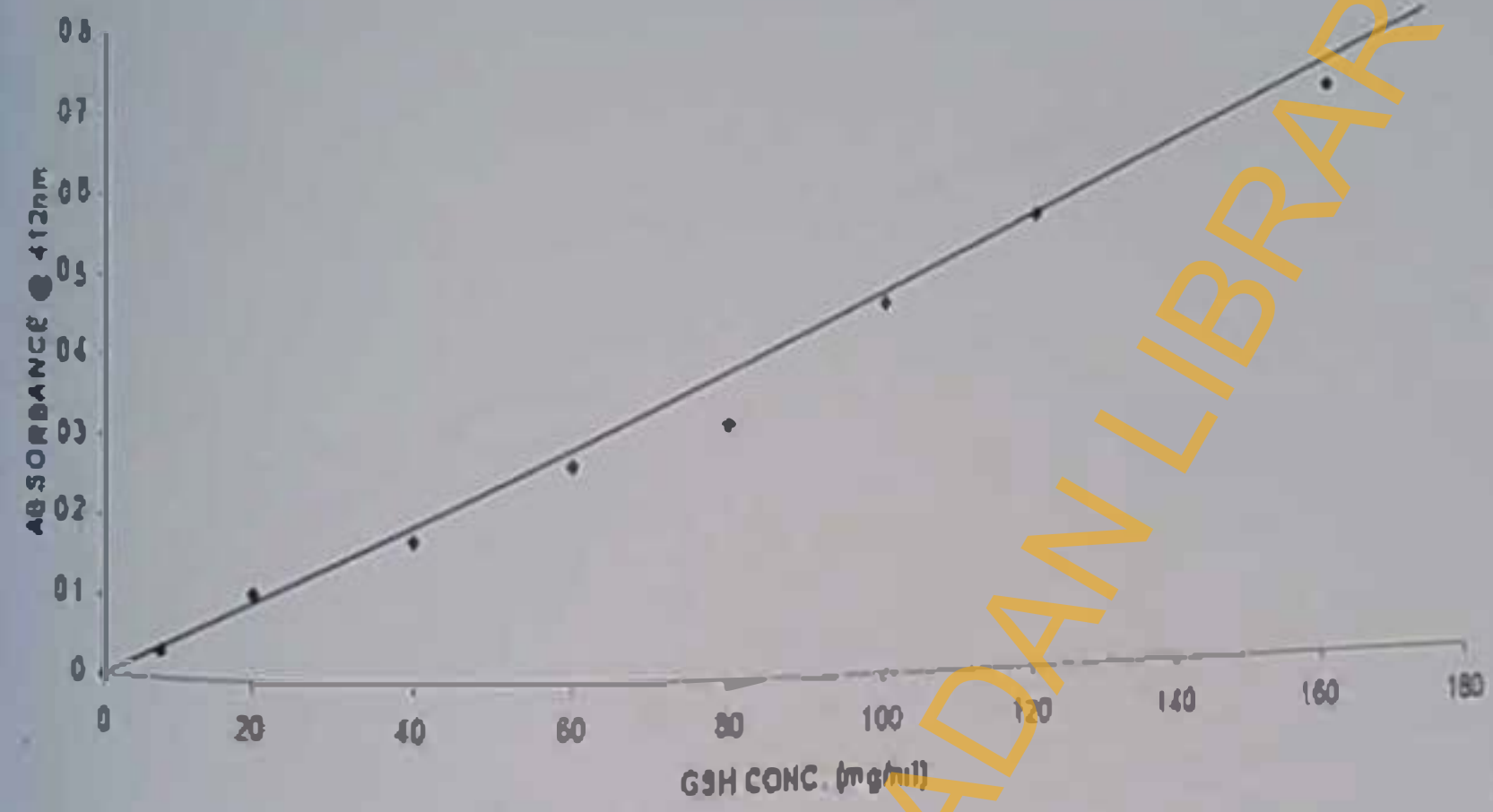

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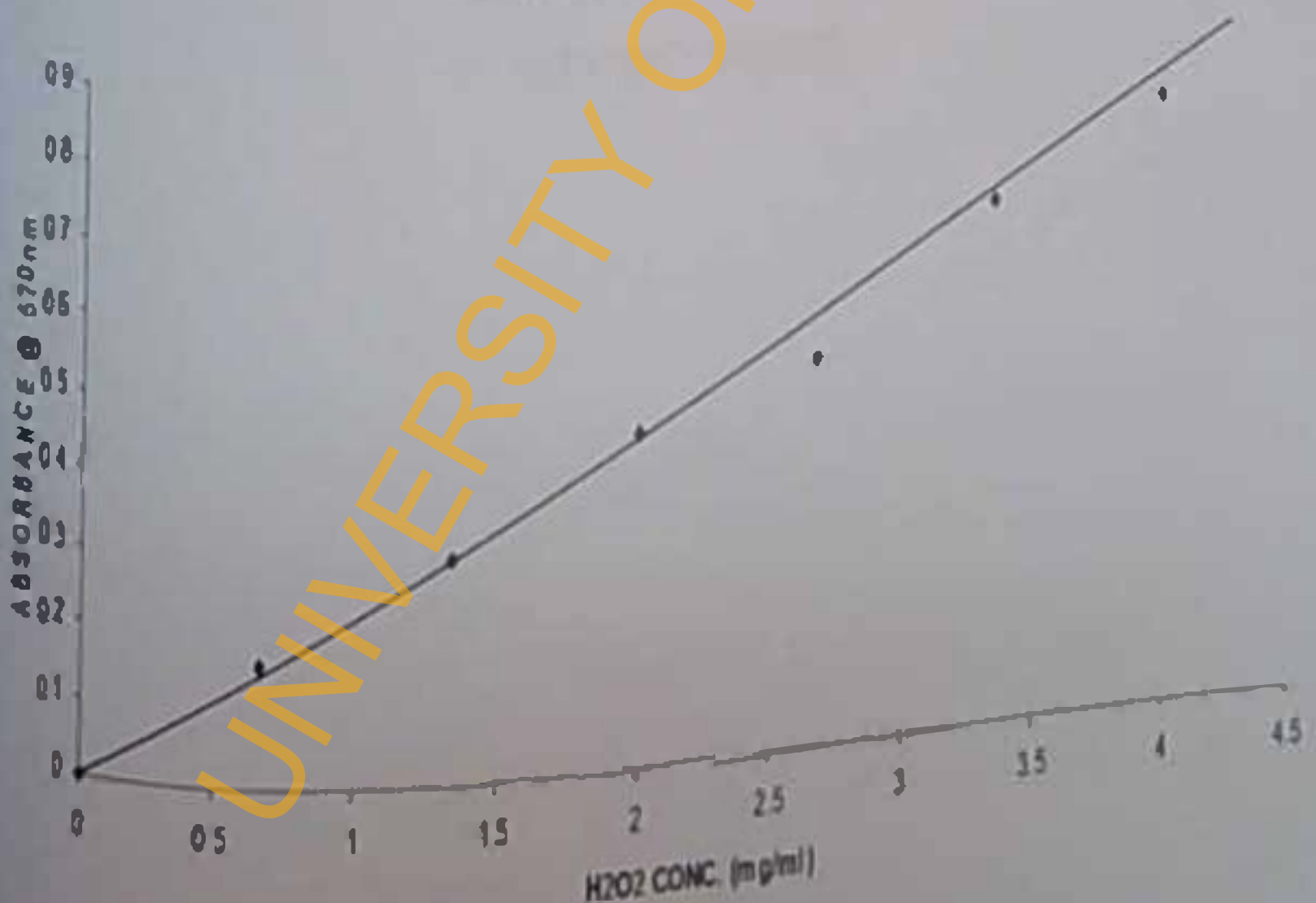

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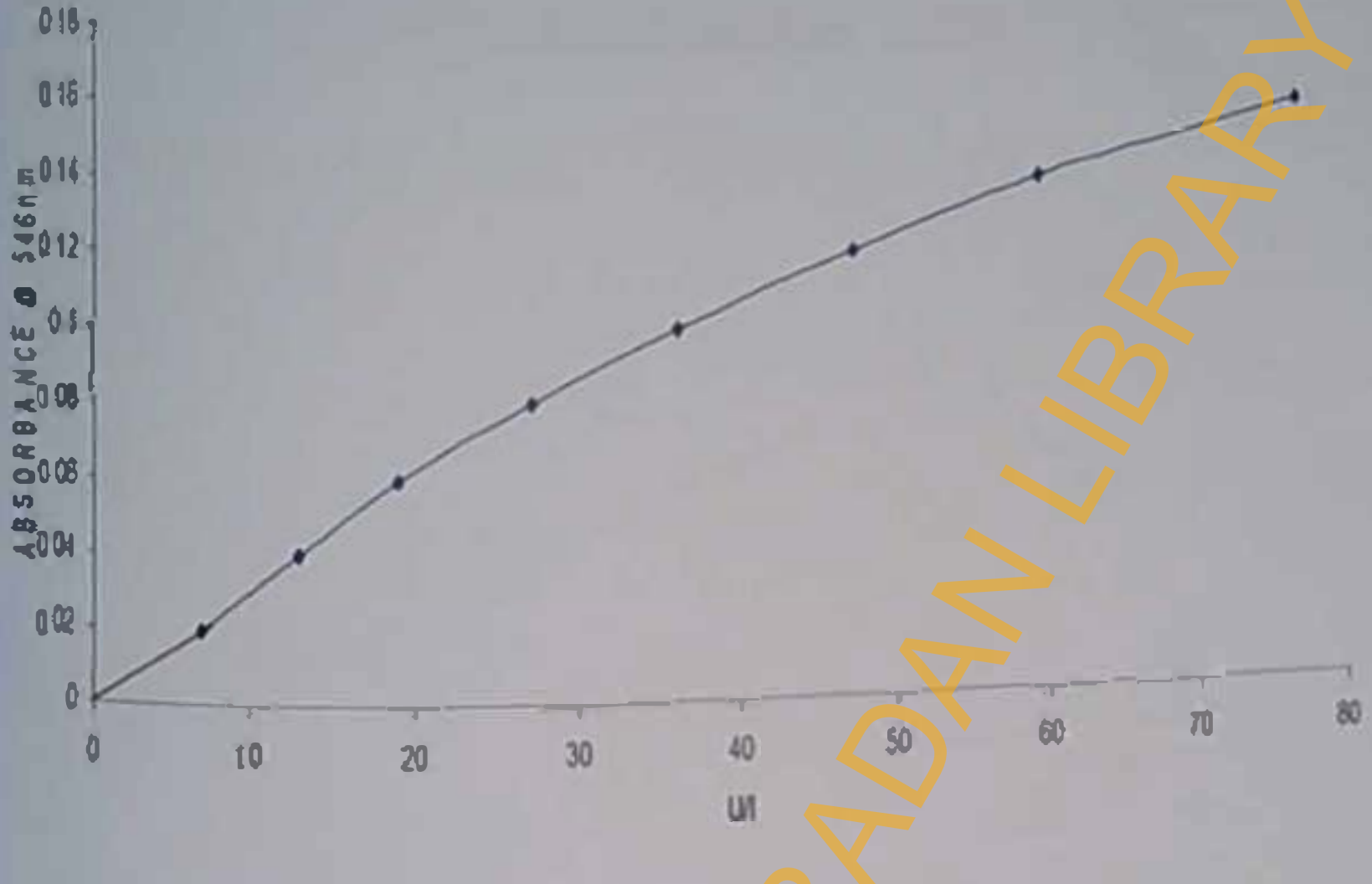

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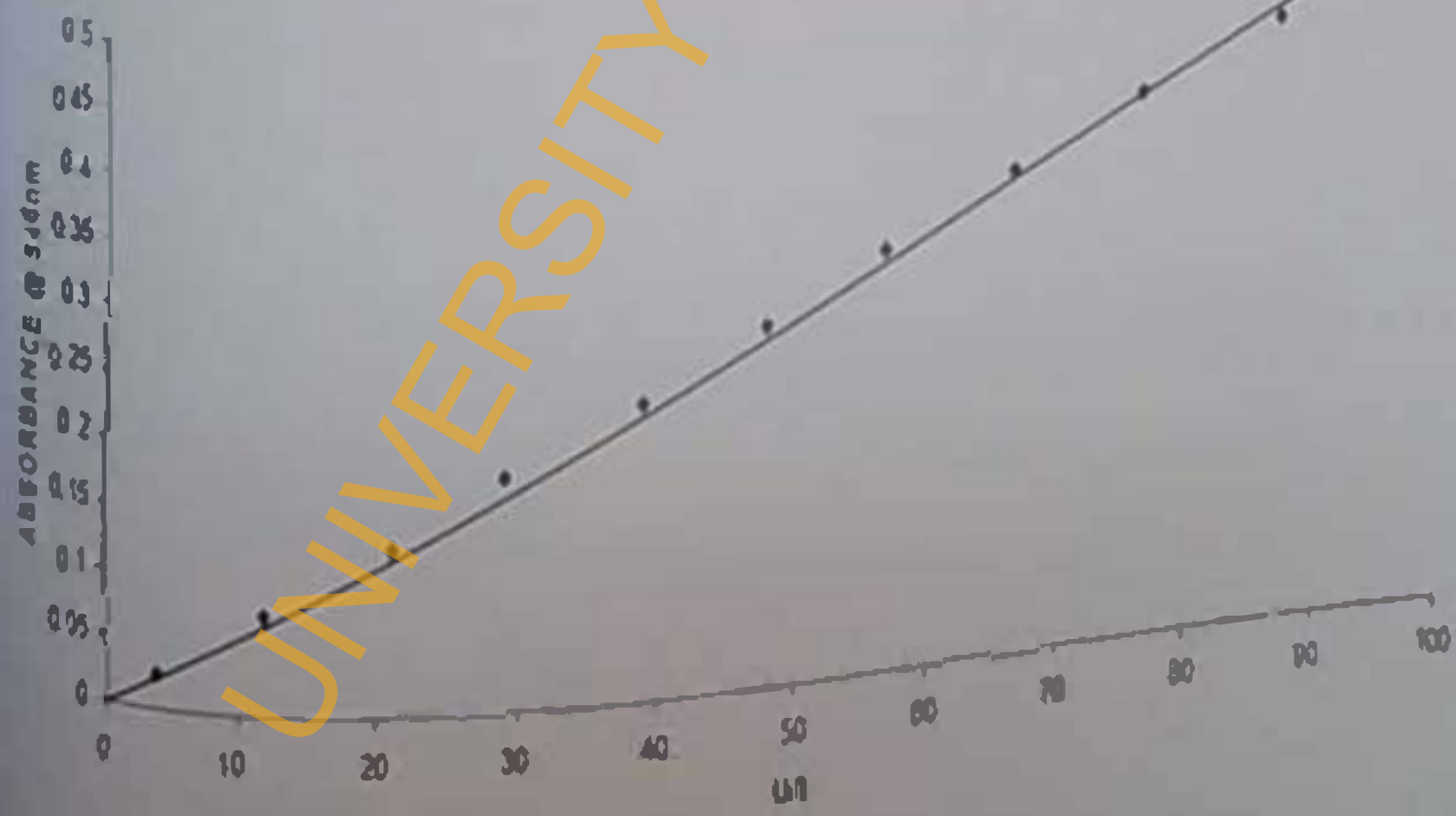
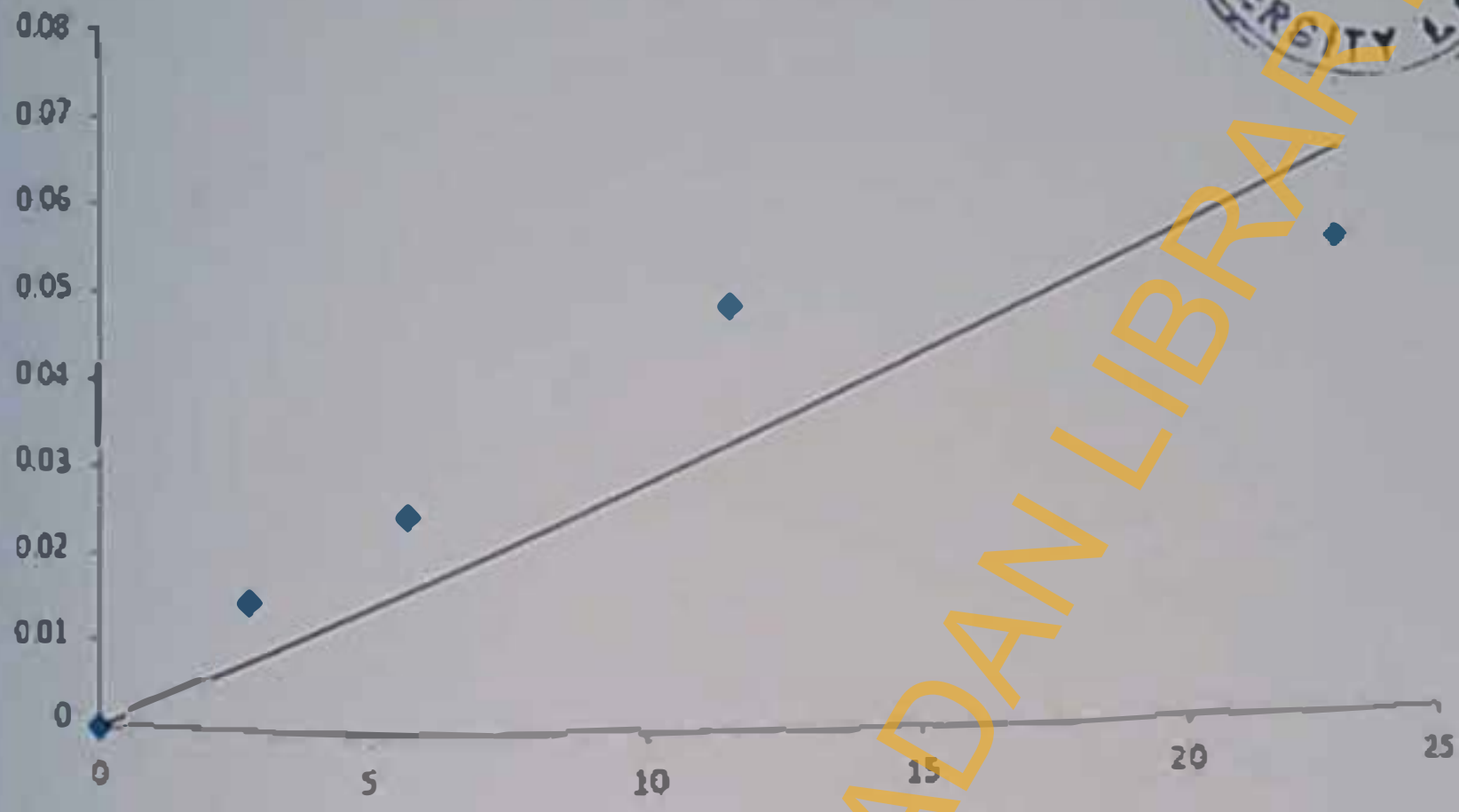


Nitrite calibration curve



Nitrite calibration curve