HYPOLIPIDAEMIC, ANTIOXIDATIVE AND HEPATOPROTECTIVE EFFECTS OF *Persea americana* (Lauraceae) LEAF EXTRACTS IN RATS.

BY

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ABSTRACT

Hyperlipidaemia and oxidative stress are important factors in the pathogenesis of chronic degenerative and inflammatory diseases. *Persea americana* (Lauraceae) has been widely used in ethnomedicine in the treatment of various ailments including hypertension. This study evaluated the hypolipidaemic, antioxidative and hepatoprotective potentials of Aqueous Extract of *P. americana* (AEPA) and Methanolic Extract of *P. americana* (MEPA) respectively in rats.

Hyperlipidaemia and hepatotoxicity were induced by feeding 4-week old male rats with high lipid diet containing cholesterol and cholic acid, and carbon tetrachloride (CCl_4) respectively. Hyperlipidaemic rats were administered AEPA or MEPA orally at 10 mg kg^{-1} body weight for 8 weeks while three groups of rats pre-treated with Reducdyn® (consisting of acetyl-homocysteine-thiolactone and cysteine) 100 mg and AEPA 100 or 200 mg kg/body weight were intoxicated with CCl_4. Control rats received standard chow and water only. Hypolipidaemic and antioxidant effects of the extracts were assessed by determining the levels of plasma lipids, antioxidant enzymes and glutathione (GSH) respectively. The hepatoprotective effect of *P. americana* was evaluated by assay of liver enzymes, bilirubin and histopathology of the liver. Phytochemical constituents of the extracts were determined by qualitative analysis. Data were analyzed using ANOVA.

Administration of AEPA reduced total plasma cholesterol (T-CHOL), low density lipoprotein cholesterol (LDL) and triglycerides (TG) by 8%, 19% and 35% respectively, while MEPA lowered T-CHOL (4%) and LDL (20%). Plasma high density lipoprotein cholesterol (HDL) level was increased while the index of atherogenicity (LDL/HDL) was markedly reduced in the treated rats compared to the hyperlipidaemic control. The extracts

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lowered oxidative stress as shown by significant decline in plasma malondialdehyde (MDA) and increase in GSH. The extracts elicited an increase in the activities of catalase and superoxide dismutase (SOD) compared to the hyperlipidaemic control rats. Hepatoprotective effect of AEPA was indicated by significant decrease in total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in the treated rats compared to the control. AEPA was substantially hepatoprotective against CCl_4-induced liver damage at 100 mg kg^{-1} body weight. The highest percentage protection was against AST (94%). Pre-treatment with 200 mg kg^{-1} body weight AEPA protected the rats against liver damage (AST, 127%; ALT, 74%; bilirubin, 106%). Pre-treatment with AEPA also lowered T-CHOL and TG while total protein concentration was restored. Administration of AEPA reversed the increases in the levels of MDA, GSH, catalase and SOD caused by CCl_4-intoxication. Leukocytes counts also increased significantly after pre-treatment with 100mg kg^{-1} body weight AEPA. Histopathological analysis of CCl_4-intoxicated rats showed that AEPA reduced the severity of necrosis, cellular infiltration and fatty change in the liver. Hypolipidaemic, antioxidant, and hepatoprotective effects of *P. americana* were comparable to Reducdyn®. Qualitative screening of the extracts indicated the presence of alkaloids, flavonoids, saponins, steroids, tannins and triterpenoids.

Leaf extracts of *P. americana* possess hypolipidaemic, antioxidant, and hepatoprotective effects which may be attributed to individual or combined action of the phytoconstituents. This may account for its use in traditional medicine and could be further exploited in the management of diseases associated with hyperlipidaemia.

Keywords: P. americana, Leaf extracts, Hyperlipidaemia, Hepatotoxicity, Rats.

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CERTIFICATION

I certify that this work was carried out by Mr. Brai Bartholomew I. C. at the Department of Biochemistry, University of Ibadan, Ibadan, Nigeria.

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Supervisor

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DEDICATION

This research work is dedicated to the Omnipotent, Omniscient and Omnipresent God who is my "All-in- All".

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TABLE OF CONTENTS

Content	Page
Title page	i
Abstract	ii
Acknowledgement	iv
Certification	vi
Dedication	vii
Table of contents	viii
List of tables	xiv
List of figures	xvi
List of plates	xviii
List of abbreviations	xix
CHAPTER ONE	
1.0 Introduction	1
1.1 Aims and objectives	4
CHAPTER TWO	
2.0 Literature review	5
2.1 Lipids	5
2.2 Free radicals	9
2.3 Reactive oxygen species	10
2.4 Examples of free radicals	11
2.4.1 Superoxide anion ($O_2^{\bullet-}$)	11
2.4.2 Hydroxyl radical ($^{\bullet}OH$)	12

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UNIVERSITY OF IBADAN LIBRARY

2.4.3 Hydroperoxyl radical ($HO_2^{\bullet}$) 13

2.4.4 Nitric oxide radical ($NO^{\bullet}$) 13

2.4.5 Peroxynitrite anion ($ONOO^{-}$) 14

2.5 Sources of free radicals 14

2.6 Oxidative stress 17

2.7 Lipid peroxidation 18

2.7.1 Mechanism of lipid peroxidation 19

2.7.2 Enzymatic lipid peroxidation 22

2.8 Carbon tetrachloride-induced hepatotoxicity 23

2.8.1 Cellular sites of CCl_4-induced damage 24

2.8.2 Mechanism of CCl_4-induced liver damage 26

2.8.3 Inhibition of lipoprotein secretion with functional impairment of the Golgi apparatus 27

2.8.4 Effects of CCl_4 on lipid homeostasis 28

2.9 Defences against free radicals 30

2.9.1 Superoxide dismutase 30

2.9.2 Catalase 32

2.9.3 Glutathione peroxidase 34

2.9.4 Glutathione S-transferases 37

2.9.5 Glutathione 38

2.9.6 Selenium 42

2.9.7 Vitamin E 45

2.9.8 Vitamin C (Ascorbic acid) 49

UNIVERSITY OF IBADAN LIBRARY

AFRICAN DIGITAL HEALTH REPOSITORY PROJECT

2.10 Avocado (*Persea americana*) 53

CHAPTER THREE

3.0 MATERIALS AND METHODS 57

3.1 Materials and equipment 57

3.2 Reagents 58

3.3 Materials 61

3.3.1 Animals 61

3.3.2 Plant materials 61

3.3.3 High lipid (modified) diet composition 61

3.4 Methods 62

3.4.1 Phytochemical screening 62

3.4.1.1 Test for alkaloids 62

3.4.1.2 Test for tannins 62

3.4.1.3 Test for saponins 62

3.4.1.4 Test for flavonoids 63

3.4.1.5 Test for terpenoids (Salkowski's test) 63

3.4.2 Feeding of rats with cholesterol-enriched diet to induce hyperlipidaemia 63

3.4.3 Treatment of rats with CCl_4 to induce hepatotoxicity 64

3.4.4 Extraction of lipids 65

3.4.5 Preparation of tissue homogenate 66

3.4.6 Determination of total cholesterol 67

3.4.7 Determination of high density lipoprotein cholesterol 68

3.4.8 Determination of triglycerides 69

UNIVERSITY OF IBADAN LIBRARY

AFRICAN DIGITAL HEALTH REPOSITORY PROJECT

3.4.9 Determination of Low density lipoprotein cholesterol 70
3.4.10 Determination of glucose 70
3.4.11 Determination of total protein 71
3.4.12 Determination of aspartate aminotransferase 72
3.4.13 Determination of alanine aminotransferase 73
3.4.14 Determination of alkaline phosphatase 74
3.4.15 Determination of total bilirubin 75
3.4.16 Determination of malondialdehyde 76
3.4.17 Determination of conjugated dienes 77
3.4.18 Determination of protein carbonyls 78
3.4.19 Determination of glutathione 79
3.4.20 Determination of catalase activity 81
3.4.21 Determination of glutathione peroxidase activity 82
3.4.22 Determination of superoxide dismutase activity 83
3.4.23 Determination of glutathione S-transferase activity 85
3.4.24 Histopathological examination of liver 86
3.4.25 Statistical Analysis 86
CHAPTER FOUR
4.0 EXPERIMENTS AND RESULTS 87
4.1 Experiment 1: Lipid lowering activity of aqueous and methanolic leaf extracts of *P. americana* on diet-induced hyperlipidaemia in rats 87
4.2 Experiment 2: Antilipoperoxidative and antioxidant properties of aqueous and methanolic leaf extracts of *P. americana* in rats fed high lipid diet 110

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AFRICAN DIGITAL HEALTH REPOSITORY PROJECT

4.3 Experiment 3: Hepatoprotective activity of aqueous leaf extract of *P. americana* on CCl_4-induced hepatotoxicity in rats. 127

CHAPTER FIVE

5.0 Discussion and Conclusion 148
5.1 Discussion 148
5.2 Conclusion 165
5.3 Contribution to knowledge 166
References 167

UNIVERSITY OF IBADAN LIBRARY

AFRICAN DIGITAL HEALTH REPOSITORY PROJECT

LIST OF TABLES

Table 1. Mean weekly body weights of rats fed high lipid diet 94

Table 2. Mean weight of kidney, lung, heart and brain of rats fed high lipid diet 95

Table 3. Effect of aqueous and methanolic leaf extracts of *P. americana* on liver total cholesterol, high density lipoprotein cholesterol and triglycerides in rats fed high lipid diet 96

Table 4. Effect of aqueous and methanolic leaf extracts of *P. americana* on tissue malondialdehyde concentration in rats fed high lipid diet 117

Table 5. Effect of aqueous and methanolic leaf extracts of *P. americana* on reduced glutathione levels in rats fed high lipid diet 118

Table 6. Plasma and tissue catalase concentrations in rats fed high lipid diet 119

Table 7. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma and tissue superoxide dismutase concentrations in rats fed high lipid diet 120

Table 8. Effect of pre-treatment with aqueous extract of *P. americana* on CCl_4-induced liver damage in rats 134

Table 9. Hepatoprotective activity of aqueous extract of *P. americana* against CCl_4-induced hepatotoxicity in rats 135

Table 10. Effect of pre-treatment with aqueous leaf extract of *P. americana* on liver catalase, gluthathione peroxidase, superoxide dismutase and glutathione S-transferase activities in CCl_4-induced hepatotoxicity in rats 136

Table 11. Effect of pre-treatment with aqueous leaf extract of *P. americana* on

UNIVERSITY OF IBADAN LIBRARY

serum total protein, total cholesterol and triglycerides in CCl_4-induced hepatotoxicity in rats 137

Table 12. Effect of pre-treatment with aqueous leaf extract of *P. americana* on liver total protein, total cholesterol and triglycerides in CCl_4-induced hepatotoxicity in rats 138

Table 13. Effect of pre-treatment with aqueous leaf extract of *P. americana* on lipid peroxidation in CCl_4-intoxicated rats 139

Table 14. Effect of pre-treatment with aqueous leaf extract of *P. americana* on peripheral blood smears in CCl_4- intoxicated rats 140

UNIVERSITY OF IBADAN LIBRARY

AFRICAN DIGITAL HEALTH REPOSITORY PROJECT

LIST OF FIGURES

Fig. 1. Effect of aqueous and methanolic leaf extracts of *P. americana* on liver weight of rats fed high lipid diet 97

Fig. 2. Effect of aqueous and methanolic leaf extracts of *P. americana* on total cholesterol in rats fed high lipid diet 98

Fig. 3. Effect of aqueous and methanolic leaf extracts of *P. americana* on high density lipoprotein cholesterol in rats fed high lipid diet 99

Fig. 4. Effect of aqueous and methanolic leaf extracts of *P. americana* on triglycerides in rats fed high lipid diet 100

Fig. 5. Effect of aqueous and methanolic leaf extracts of *P. americana* on low density lipoprotein cholesterol in rats fed high lipid diet 101

Fig. 6. Effect of aqueous and methanolic leaf extracts of *P. americana* on index of atherogenicity (LDL/HDL ratio) in rats fed high lipid diet 102

Fig. 7. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma glucose in rats fed high lipid diet 103

Fig. 8. Effect of aqueous and methanolic leaf extracts of *P. americana* on aspartate aminotransferase activity in rats fed high lipid diet 104

Fig. 9. Effect of aqueous and methanolic leaf extracts of *P. americana* on alanine aminotransferase activity in rats fed high lipid diet 105

Fig. 10. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma malondialdehyde concentration in rats fed high lipid diet 121

Fig. 11. Effect of aqueous and methanolic leaf extracts of *P. americana* on liver, kidney and heart conjugated dienes in rats fed high lipid diet 122

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AFRICAN DIGITAL HEALTH REPOSITORY PROJECT

Fig. 12. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma, liver, kidney and heart protein carbonyl in rats fed high lipid diet 123
Fig. 13. Effect of aqueous and methanolic leaf extracts of *P. americana* on reduced glutathione concentration in rats fed high lipid diet 124
Fig. 14. Effect of aqueous and methanolic leaf extracts of *P. americana* on red blood cell glutathione peroxidase activity in rats fed high lipid diet 125
Fig. 15. Effect of aqueous and methanolic leaf extracts of *P. americana* on liver glutathione peroxidase activity in rats fed high lipid diet 126
Fig. 16. Effect of aqueous leaf extract of *P. americana* on reduced glutathione content in the serum of CCl_4-intoxicated rats 141
Fig. 17. Effect of aqueous leaf extract of *P. americana* on reduced glutathione content in the liver of CCl_4-intoxicated rats 142

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AFRICAN DIGITAL HEALTH REPOSITORY PROJECT

LIST OF PLATES

Plate 1: Liver section from normal rat showing preserved lobular architecture with normal erythrocytes 106

Plate 2. Liver section from hyperlipidaemic rat showing severe fatty change 107

Plate 3: Liver section from hyperlipidaemic rat treated with 10 mg kg^{-1} b. wt AEPA 108

Plate 4: Liver section from hyperlipidaemic rat treated with 10 mg kg^{-1} b. wt MEPA 109

Plate 5: Liver section from normal rat showing preserved lobular architecture and hepatocytes with numerous portal tracts dividing into lobules 143

Plate 6: Liver section from CCl_4-treated rat pre-treated with 100 mg kg^{-1} b. wt AEPA showing mild, diffuse fatty change, periportal necrosis and mononuclear cell infiltration 144

Plate 7. Liver section from CCl_4-treated rat pre-treated with 200 mg kg^{-1} b. wt AEPA showing mild, diffuse fatty change with less periportal necrosis 145

Plate 8. Liver section from rat pre-treated with 100 mg kg^{-1} b. wt Reducdyn® showing diffuse fatty change and mild mononuclear infiltration 146

Plate 9. Liver section from CCl_4-control rat showing extensive necrosis of hepatocytes with areas of fatty change and diffuse mononuclear infiltration 147

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LIST OF ABBREVIATIONS

AEPA	Aqueous extract of *P. americana*
AIDS	Acquired immunodeficiency syndrome
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
Apo B	Apoprotein B
ARDS	Acute respiratory distress syndrome
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
b. wt	Body weight
CAT	Catalase
CD	Conjugated diene
CDNB	1 - Chloro - 2, 4 - Dinitrobenzene
cm	Centimetre
COPD	Chronic obstructive pulmonary disease
COX	Cycloxygenase
CVD	Cardiovascular disease
CYP	Cytochrome P
D	Dilution factor
DCNB	1, 2 - Dichloro - 4 - Nitrobenzene
DHA	Dehydroascorbic acid
dl	decilitre

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DNA	Deoxyribonucleic acid
DNPH	2, 4 - Dinitrophenylhydrazine
DTNB	5, 5′ - Dithio - bis -2 - Nitrobenzoic acid
EDRF	Endothelium-derived relaxation factor
EDTA	Ethylenediamine tetraacetic acid
ELAM	Endothelium leukocytes adhesion molecule
FA	Fatty acid
G6PD	Glucose - 6 - phosphate dehydrogenase
GK	Glycerol kinase
GOD	Glucose oxidase
GPO	Glycerol - 3 - phosphate peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced)
GSHPx	Glutathione peroxidase
GSSG	Glutathione (oxidized)
GST	Glutathione S-transferase
Hb	Haemoglobin
HDL	High density lipoprotein
H & E	Haematoxylin-eosin
HIV	Human immunodeficiency virus
HMG-CoA	β-Hydroxy-β-methylglutaryl-CoA
HNE	4-Hydroxyl-2, 3-trans-nonenal
ICAM-1	Intercellular adhesion molecule 1

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IL-1	Interleukin-1
i. p.	Intraperitoneal
kDa	KiloDalton
kg	Kilogramme
LDL	Low density lipoprotein
LOX	Lipoxygenase
M-CSF	Macrophage colony-stimulating factor
MDA	Malondialdehyde
MEPA	Methanolic extract of *P. americana*
mg	Milligramme
min	Minutes
µM	Micromoles
mM	Millimoles
NEFA	Non-esterified fatty acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
Nm	Nanometre
NOS	Nitric oxide synthase
OD	Absorbance
PCV	Packed Cell Volume
PIPES	1, 4-piperazinediethanesulfonic acid
PKC	Protein kinase C
POD	Peroxidase

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PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
rpm	Revolutions per minute
sc	Subcutaneous
SEM	Standard error of mean
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reacting species
TBL	Total bilirubin
TCA	Trichloroacetic acid
T-CHOL	Total cholesterol
TG	Triglycerides
TNF-α	Tumour necrotic factor α
tRNA	Transfer ribonucleic acid
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volume
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very low density lipoprotein
v/v	Volume per volume
WBC	White blood cell
w/v	Weight per volume

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CHAPTER ONE

1.0 INTRODUCTION

Globalization has beneficial and harmful effects on the health of populations (Woodward *et al.*, 2001). The direct negative effects of globalization are shown by the increasingly globalised production and marketing of tobacco and alcohol, and salty, sugary, and fatty foods (Beaglehole and Yach, 2003). Diets of Western societies have been shown to be too high in calories, with low fibre, high animal fat, sugar and alcohol content. Dietary fat intake is higher than recommended in most Western countries and it is associated with the prevalence of cardiovascular disease, obesity and cancer (Velthuis te Wierik *et al.*, 1996). Developing countries have adapted agricultural production and food processing practices, dietary habits and lifestyle of the Western countries without any appraisal of the health implications. Also, global trade and marketing developments are driving the nutrition transition towards diets with a high proportion of saturated fat and sugars. This diet, in combination with tobacco use and little physical activity, leads to population-wide atherosclerosis and the widespread distribution of non-communicable diseases (Beaglehole and Yach, 2003). It is now known that non-communicable disease risk factor levels have increased during the past decade and this signifies an increase in the rate of non-communicable diseases in the next two decades.

Analyses of available aggregate data sources indicate that a shift towards "Western diets" is occurring in developing countries (Reddy and Yusuf, 1998; Popkin 2002). In Nigeria, there appears to be a cultural transition toward a more Westernized lifestyle. The traditional foods consisting mainly of roots, cereals, beans, tubers and vegetables are giving way to fatty foods, sweet snacks and drinks which are too calorie dense. These

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changes in dietary pattern among Nigerians, coupled with changes in physical activity patterns, increase use of tobacco products and alcohol are possible causes of hyperlipidaemia which is an important risk factor in the pathogenesis of chronic degenerative diseases such as cardiovascular disease, diabetes and cancer. It has been reported that the intakes of meat, eggs and milk were high in people with higher socioeconomic status and total fatty-acids in Nigerians were shown to be positively associated with cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides (Yeh *et al.*, 1996). The final report on the national survey on non-communicable diseases in Nigerian estimated the prevalence of hypertension to be 11.2% representing not less than 4.33 million Nigerians over 15 years of age. The prevalence of diabetes was put at 2.2% representing about 1.05 million Nigerians over 15 years of age (FMOH, 1997).

High systolic blood pressure levels were recently observed in some developing countries, including Nigeria that had low mean cholesterol (Ezzati *et al.*, 2005). Demographic and technological changes are increasingly modifying the income patterns of cardiovascular risk factors and shifting their burden to the developing world. As a result, low-income and middle-income countries increasingly face the double burden of infectious diseases and cardiovascular risk factors (Ezzati *et al.*, 2005). In Nigeria, communicable diseases are still present but non-communicable diseases are on the increase thus creating a double burden of disease.

The use of alternative medicine and the consumption of plant materials have been on the increase in many countries of the world, mostly because plant-derived drugs and herbal formulations are commonly considered to be less toxic and freer from side effects than synthetic ones (Mitra *et al.*, 1996; Bhattacharya *et al.*, 1997; Annapurna *et al.*, 2001). At

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present, a number of botanicals are still being used in ethnomedicine for the treatment of different diseases. Natural substances that can inhibit lipid oxidation are obtained from many different sources, including plants (Marcia *et al*., 2001).

Avocado (*Persea americana* Mill.) is one of the plants that have been widely used in ethnomedicine. The bark, fruit and leaf are used in traditional medicine in South America, West Indies and Africa to provide remedy for various ailments. The fruit is employed as a vermifuge and remedy for dysentery; the leaf juice has antibiotic activity; the aqueous extract of the leaves has a prolonged antihypertensive effect while the leaf decoction is taken as a remedy for diarrhoea, sore throat, haemorrhage and allegedly stimulates and regulates menstruation (Morton, 1987).

The leaf extracts from *P. americana* have been shown to have antiviral activity against *Herpes simplex* 1 virus (De Almeida *et al*., 1998); human immunodeficiency virus (HIV) 1 (Wigg *et al*., 1996) and adenovirus (De Almeida *et al*., 1998). It has anti-inflammatory activity (Guevarra *et al*., 1998; Adeyemi *et al*., 2002) and antihypertensive/hypotensive activity (De A Ribeiro *et al*., 1986; Girow *et al*., 1991; Adeboye *et al*., 1999). Recently, the aqueous leaf extract of *P. americana* was reported to possess hypoglycemic activity (Antia *et al*., 2005), vasorelaxant action (Owolabi *et al*., 2005), and anticonvulsant effect (Ojewole and Amabeoku, 2006).

Since hyperlipidaemia has been implicated in the pathogenesis of atherosclerosis, it is necessary to investigate the possible effect of *P. americana* on hyperlipidaemia. This study examined the effects of *P. americana* leaf extracts on hyperlipidaemia and lipid peroxidation. In addition, the hepatoprotective effects of the aqueous leaf extract of *P. americana* was investigated.

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1.1 AIMS AND OBJECTIVES

1. To determine the effect of the leaf extracts of *P. americana* on blood glucose and lipid profile in hyperlipidaemic rats
2. To assess the effect of the leaf extracts of *P. americana* on lipid peroxidation in rat tissues
3. To determine the effect of the leaf extracts of *P. americana* on antioxidant status in rats
4. To evaluate the protective effect of the leaf extracts of *P. americana* on CCl_4-induced hepatotoxicity in rats.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Lipids

Lipids are biological molecules comprising a diverse class of organic compounds that are insoluble in aqueous solutions but are soluble in organic solvents. They are functionally important in biological systems where they serve as structural components of membranes, function as energy reserves, vitamins and hormones, and lipophilic bile acids in lipid solubilisation. Lipids are divided into three principal groups viz. fatty acids (FA), triglycerides and phospholipids. Fatty acids which are straight chain organic acids are subdivided into three according to their degree of saturation or unsaturation _ saturated, monounsaturated and polyunsaturated. Polyunsaturated fatty acids (PUFAs) have two or more *cis* double bonds separated by a single methylene group (-CH=CH-CH_2-CH=CH-). All lipids are hydrophobic and mostly insoluble in blood, so they are transported within hydrophilic, spherical structures called lipoproteins, which possess surface proteins (apoproteins) that are cofactors and ligands for lipid metabolizing enzymes. Lipoproteins can be distinguished according to the type of protein they contain and their density. Low-density lipoproteins (LDLs) are the most cholesterol-rich of all lipoproteins. The LDL particle is a sphere with a single hydrophobic protein called apoprotein B (apo B) embedded in a non-polar core of cholesterol, which is linked to long-chain fatty acids to form cholesterol esters. LDL is a major cause of injury to the endothelium and underlying smooth muscle (Griendling and Alexander, 1997). When LDL particles become trapped in an artery, they can undergo progressive oxidation and be internalized by macrophages by means of the scavenger receptors on the surfaces of the cells. The internalization leads

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to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters, resulting in the formation of foam cells (Diaz *et al.*, 1997; Han *et al.*, 1997; Steinberg, 1997). Removal and sequestration of modified LDL are important parts of the initial, protective role of macrophage in the inflammatory response and minimize the effects of modified LDL on endothelial and smooth-muscle cells (Diaz *et al.*, 1997; Han *et al.*, 1997).

In addition to promoting the formation of foam cells, oxidized LDL has a direct chemotactic activity for monocytes and stimulates the binding of monocytes to the endothelium (Quinn *et al.*, 1988; Frostegard *et al.*, 1991). Once monocytes cross the endothelial layer, they become trapped in the subendothelial space, partly because oxidized LDL inhibits their exit from the arterial wall (Quinn *et al.*, 1987). Oxidized LDL is also cytotoxic to vascular cells thus promoting the release of lipids and lysosomal enzymes into the intimal extracellular space and enhancing progression of atherosclerotic lesions (Schwartz *et al.*, 1991). Modified LDL can induce the expression of adhesion molecules, chemokines, proinflammatory cytokines, and other mediators of inflammation in macrophages and vascular wall cells (Libby *et al.*, 2002). The inflammatory response itself can have a profound effect on lipoprotein movement within the artery. Mediators of inflammation such as tumour necrosis factor α (TNF- α), interleukin-1 (IL-1), and macrophage colony-stimulating factor (M-CSF) increase binding of LDL to endothelium and smooth muscle and increase the transcription of the LDL-receptor gene (Stopeck *et al.*, 1993; Hajjar and Haberland, 1997).

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The oxidative modification of LDL is a plausible link between lipids, inflammation and atherosclerosis and this link provides a convenient and simple rationale for the beneficial effect of antioxidants on coronary artery disease.

Reports indicate that abnormal lipid levels predispose individuals to atherosclerosis and cardiovascular disease (Glew *et al.*, 2002; Chrysohoou *et al.*, 2004).

Cardiovascular disease is associated with elevated blood levels of LDL, increase oxidation of LDL, raised levels of total cholesterol and triglycerides whereas a low level of high-density lipoprotein (HDL) is a risk factor for mortality from cardiovascular disease (Criqui *et al.*, 1993; Rahman and Lowe, 2006).

According to the guidelines of the American Heart Association, the optimal levels of lipids and lipoproteins are: total cholesterol <200 mg/dl, triglycerides <200 mg/dl, LDL <130 mg/dl and HDL >40 mg/dl.

LDL-cholesterol is a primary target of treatment of hyperlipidaemia. Cholesterol lowering agents have demonstrated great efficacy in prevention and cessation of the progression of atherosclerosis and statins are the main stay of LDL-cholesterol lowering treatment (Ballantyne, 1998). Statins are a class of drugs which are potent 3-Hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) redutase inhibitors. HMG-CoA reductase acts on the rate limiting step in cholesterol biosynthesis to inhibit HMG-CoA conversion to malevonate and thereby reducing LDL, very low-density lipoprotein (VLDL) and triglyceride levels (Al-Shaer *et al.*, 2004; Lutgens and Daemen, 2004). Examples of statins include lovastatin, pravastatin, simvastatin and atorvastatin.

The fibrates are another class of drugs that have been used in lipid-lowering therapy in the primary prevention of cardiovascular disease. Fibrates are known to be most effective

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in reducing plasma concentrations of triglyceride-rich lipoproteins (i.e. VLDL and chylomicrons (Gaw and Shepherd, 1999). Fibrates used as lipid-lowering agents include clofibrate, gemfibrozil, fenofibrate, ciprofibrate and bezafibrate.

Dietary modification is an integral part of the management of patients with hypercholesterolemia and the major focus of these dietary changes is to reduce intakes of high-fat dairy products, red meats and eggs with a concurrent increase in intakes of fish, fruits, grains and legumes which are known to reduce LDL cholesterol concentrations and are also associated with stabilization or reversal of coronary atherosclerosis (Ornish *et al.*, 1990; Watts *et al.*, 1996). Studies in Nigeria have shown that consumption of fruits and vegetables lowers levels of total cholesterol and triglycerides as well as reduce incidence of cardiovascular risk factors of major chronic diseases (Famodu *et al.*, 1998, Hung *et al.*, 2004; Odetola *et al.*, 2004; Adebawo *et al.*, 2006; Odetola *et al.*, 2006).

Other dietary modifications in the management of hyperlidaemia include the use of probiotics, soy, and soy isoflavones (Ali *et al.*, 2004; McVeigh *et al.*, 2006). The beneficial effects attributed to probiotics and probiotic-containing food products include the reduction of blood cholesterol and the primary probiotic bacteria associated with cholesterol lowering have been lactobacilli and bifidobacteria (Agerholm-Larsen *et al.*, 2000; Ali *et al.*, 2004; Cavallini *et al.*, 2009). Studies in animals and humans have shown that soy and soy isoflavones may protect against CVD through improvement on serum lipid profiles (Ali *et al.*, 2004; McVeigh *et al.*, 2006) and increased resistance of LDL to oxidation (Damasceno *et al.*, 2007).

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2.2 Free radicals

A free radical can be defined as a chemical species possessing an unpaired electron and is capable of independent existence. Free radicals and other reactive oxygen species in the human body are derived either from normal, essential metabolic processes such as phagocytosis and electron transport chain or from external sources such as cigarette smoke, radiation and certain drugs, pesticides, anaesthetics, and industrial solvents (Cheeseman and Slater, 1993).

Free radicals can be formed in three ways:

i) by the hemolytic cleavage of a covalent bond of a normal molecule with each fragment retaining one of the unpaired electrons. This generally requires high energy input from either high temperatures UV light or ionising radiation;

ii) by the loss of a single electron from a normal molecule;

iii) by the addition of a single electron to a normal molecule i.e, electron transfer. This is the more common process in biological systems (Cheeseman and Slater, 1993).

If free radicals are not inactivated, their chemical reactivity can damage all types of cellular macromolecules, including proteins, carbohydrates, lipids, and nucleic acids.

Free radicals can be positively charged, negatively charged or electrically neutral. They are generally more reactive than non-radicals due to their unpaired electron but different types of free radicals vary widely in their reactivity (Slater, 1984; Halliwell and Chirico, 1993; Rice-Evans and Burdon, 1993). One of the most important molecules in free radical biochemistry is the oxygen molecule (O_2). The oxygen molecule qualifies as a free radical because it contains two paired electrons, but is not particularly reactive due to

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a special electron arrangement that makes the reactions with oxygen spin restricted (Halliwell and Gutteridge, 1990). However, when oxygen is partly reduced, several different reactive oxygen species, both radicals and non-radicals may be produced (Cheeseman and Slater, 1993).

2.3 Reactive oxygen species

Reactive oxygen species (ROS) is a collective term which includes both oxygen radicals and certain non-radicals that are oxidising agents and/or are easily converted into radicals. Examples of ROS include radicals such as superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$); and non-radicals such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$).

Reactive oxygen species are produced continuously in the human body as a consequence of normal metabolic processes. Examples of reactions that lead to free radical formation are shown below:

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{\text{Xanthine oxidase}} \text{Urate} + O_2^{\bullet-} + 2H^+$$

$$NADPH + 2O_2 \xrightarrow{\text{NADPH oxidase}} NADP^+ + 2O_2^{\bullet-} + H^+$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

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2.4 Examples of Free Radicals

2.4.1 Superoxide anion ($O_2^{\bullet -}$)

Superoxide ($O_2^{\bullet -}$) is a one-electron reduction product of molecular oxygen that is formed during normal respiration in mitochondria and auto-oxidation reactions.

$$O_2 + e^- \longrightarrow O_2^{\bullet -}$$

This free radical derivative of oxygen is found in almost all aerobic cells owing to electron "leakage" from the electron transport chain. It is also formed by activated phagocytes (monocytes, macrophages, eosinophils and neutrophils) and the production of $O_2^{\bullet -}$ is an important factor in the killing of bacteria by these cells. Excessive production and/or inadequate removal of reactive oxygen species, especially superoxide anion ($O_2^{\bullet -}$), results in oxidative stress which has been implicated in the pathogenesis of many cardiovascular diseases, including atherosclerosis, hypertension, diabetes, and in endothelial dysfunction by decreasing nitric oxide (NO) bioactivity (Fukai *et al*., 2002).

$O_2^{\bullet -}$ is removed *in vivo* by the action of specific enzymes – the superoxide dismutases (SOD) which catalyze the dismutation of superoxide anion into oxygen and hydrogen peroxide according to the following equation:

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

The hydrogen peroxide formed is not a free radical but a weak oxidizing agent that can inactivate some enzymes such as superoxide dismutase, myeloperoxidase, aconitase and α-ketoglutarate dehydrogenase. Also, H_2O_2 can cross cell membranes where it may react with transition metals particularly iron to produce the most reactive and damaging of the oxygen free radical, the hydroxyl radical ($OH^{\bullet}$) (Cheeseman and Slater, 1993).

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In activated phagocytes the enzyme myeloperoxidase is expressed. This enzyme catalyzes the formation of the highly reactive oxidant hypochlorous acid (HOCl) from hydrogen peroxide and chloride (Heinecke *et al*., 1994; Van De Berg and Winterbourne, 1994). Hypochlorous acid is an important antimicrobial agent implicated to mediate in the oxidative modification of proteins and lipids (Van de Berg and Winterbourne, 1994). It is also reported to oxidize cholesterol with the formation of sterol epoxides and chlrohydrins which may be both cytotoxic and mutagenic (Heinecke *et al*., 1994).

2.4.2 Hydroxyl radical ($^{\cdot}OH$)

The hydroxyl ($^{\cdot}OH$) radical is an extremely powerful oxidant formed by the Haber-Weiss transition metals (Cheeseman and Slater, 1993).

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{\text{metal catalyst}} {}^{\cdot}OH + OH^- + O_2$$

Many types of metal ions such as chromium, nickel, copper and iron are theoretically able to catalyze the Haber-Weiss reaction. The ion (Fe^{2+}) - dependent decomposition of H_2O_2 is known as the Fenton reaction. $^{\cdot}OH$ can also be formed from water by high energy ionization radiation (Packer and Glazer, 1990). It is also generated via the oxidant peroxynitrite with $^{\cdot}OH$ being produced either as a decompositinn product (Van der Vliet *et al*., 1994) or consequent to the Haber-Weiss reaction following peroxynitrite release of copper from ceruloplasmin (Swain *et al*., 1994). $^{\cdot}OH$ is an extremely reactive oxidizing radical that will react with most biological molecules at diffusion-controlled rates (Cheeseman and Slater, 1993). It can cause DNA damage and initiate lipid peroxidation. However, the damage by this radical is non-selective because it does not survive long enough to diffuse away from its site of production (Packer and Glazer, 1990). $^{\cdot}OH$ causes

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strand breaks and base modification in DNA leading to changes in gene expression, mutation and apoptosis. Oxidation of protein side chains can result in enzyme, receptor and carrier dysfunction (Evans and Halliwell, 2001).

2.4.3 Hydroperoxyl radical ($HO_2^{\bullet}$)

The hydroperoxyl radical ($HO_2^{\bullet}$) is formed by protonation of the superoxide radical. $O_2^{\bullet-}$ generated in or diffusing into an area of low pH such as beneath activated macrophages adhering to surfaces would therefore cause an increase in the amount of $HO_2^{\bullet}$. This radical is thought to be able to cross biological membranes which $O_2^{\bullet-}$ cannot cross. $HO_2^{\bullet}$ is more reactive than $O_2^{\bullet-}$ and is repeatedly able to attack fatty acids directly leading to lipid peroxidation (Packer and Glazer, 1990).

2.4.4 Nitric oxide radical (NO•)

Nitric oxide (also known as endothelium-derived relaxation factor, EDRF) is a powerful vasodilator and decreases platelet aggregability, both effects being induced through activation of guanylate cylase (Wennmalm, 1994). Nitric oxide ($NO^{\bullet}$) is produced from arginine and molecular oxygen by an enzyme catalyzed reaction in the vascular endothelial cells, in nervous tissue and in activated phagocytes (Wennmalm, 1994). $NO^{\bullet}$ performs useful physiological functions, such as regulation of vascular smooth muscle tone (hence controlling blood pressure) and neurotransmitter action (Moncada *et al*., 1991).

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2.4.5 Peroxynitrite anion ($ONOO^-$)

Peroxynitrite anion is an oxidant with reactivity similar to that of $^{\bullet}OH$. It is the product of the reaction between $O_2^{\bullet -}$ and $NO^{\bullet}$ (Hogg *et al.*, 1993; Beckman *et al.*, 1994). At sites of inflammation, $O_2^{\bullet -}$ and $NO^{\bullet}$ will be found together in increased amounts, leading to increased formation of $ONOO^-$. $ONOO^-$ in mildly acidic surrounding becomes protonated and decomposes rapidly to form an intermediate with the same fierce reactivity as the hydroxyl radical (Van der Vliet *et al.*, 1994). $ONOO^-$ can induce the same type of oxidative damage as the $^{\bullet}OH$ such as the initiation of lipid peroxidation (Hogg *et al.*, 1993; Beckman *et al.*, 1994). Also, because of its greater ability to diffuse away from the site of production, the damage caused by $ONOO^-$ may be more selective and ultimately harmful.

2.5 Sources of free radicals

Free radicals are generally produced in cells by electron transfer reactions. These can be mediated by the action of enzymes or non-enzymatically, often through the redox chemistry of transition metal ions (Cheeseman and Slater, 1993). Free radicals and various reactive species are continuously produced in the body (Halliwell *et al.*, 1995). The major sources of free radicals and ROS produced in the body occur via the leakage of electrons from mitochondrial and microsomal electron transport chains, to molecular oxygen, generating superoxide (Cheeseman and Slater, 1993). They may also be derived from external sources such as cigarette smoke, radiation, UV light, pollution and from the metabolism of certain drugs (Halliwell and Chrico, 1993; Rice-Evans and Burdon, 1993).

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Enzymes can also produce superoxide or hydrogen peroxide such as the range of flavin oxidases located in peroxisomes. Another source of $O_2^{\bullet-}$ in animal cells is the so called auto-oxidation reactions in which certain compounds such as catecholamines, ascorbic acid, thiols, adrenalin and reduced flavins are alleged to react directly with O_2 to form $O_2^{\bullet-}$ (Fridovich, 1989). These auto-oxidation reactions can be greatly enhanced by the involvement of transition metal ions (Cheeseman and Slater, 1993).

Free radical production in cells can be greatly increased by certain toxic foreign compounds. The classical example is carbon tetrachloride which was the first such compound to be shown to exert its toxicity through a free radical mechanism, being metabolized to the trichloromethyl free radical by the action of cytochrome P-450 in the liver (Slater, 1966; Cheeseman *et al.*, 1985). Most of the H_2O_2 and other ROS generated during the normal metabolism of a typical eukaryotic cell is derived from $O_2^{\bullet-}$ that is formed from reduction of O_2 by components of the mitochondrial electron transport chain, primarily ubisemiquinone (QH') in complex III and secondarily NADH dehydrogenase (complex I), in what are believed to be side reactions of electron transport (Richter and Schweizer, 1997).

There also exist specialised systems whose primary purpose is to generate ROS for use in defence systems that protect against pathogens. A well-known example is when activated phagocytic cells (neutrophils, monocytes, macrophages and eosinophils) produce $O_2^{\bullet-}$ and H_2O_2 as one mechanism to kill bacteria and fungi and to inactivate viruses (Curnutte and Babior, 1987; Babior and Woodman, 1990). In addition, free radicals are also produced by an array of endogenous enzyme systems such as pyruvate metabolizing

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enzymes, oxidases, carboxylases, hydroxylases, peroxidases. fruit ripening enzymes and radical enzymes (Halliwell and Gutteridge, 1999). For example, H_2O_2 is additionally generated *in vivo* by several oxidase enzymes, such as glycolate oxidase, xanthine oxidase and D-amino acid oxidase (Chance *et al.*, 1979; McCord, 1987). There is also evidence that $O_2^{\bullet-}$ is also produced by several cell types other than phagocytes, including lymphocytes (Maly, 1990), fibroblasts (Meier *et al.*, 1990; Murrel *et al.*, 1990), and vascular endothelial cells (Arroyo *et al.*, 1990; Britigan *et al.*, 1992).

Such $O_2^{\bullet-}$ might be involved in intercellular signaling and could serve important biological functions (Halliwell and Cross, 1994).

Radical production is important in allowing phagocytes to kill some of the bacterial strains that they engulf. This can be illustrated by examining patients with chronic granulomatous disease, a series of inborn conditions in which the membrane-bound reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases system in phagocytes that makes the $O_2^{\bullet-}$ fails to work (Curnutte and Babior, 1987). Such patients have phagocytes that engulf and process bacteria normally but several bacterial strains are not killed and are released in viable form when the phagocytes die. Thus, patients suffer severe, persistent and multiple infections with such organisms such as *Staphylococcus aureus*.

Another killing mechanisms used by neutrophils is the enzyme myeloperoxidase (Weiss, 1989). It uses H_2O_2 produced by dismutation of $O_2^{\bullet-}$ to oxidize chloride ions into hypochlorous acid (HOCl), a powerful antibacterial agent.

$$H_2O_2 + Cl^- \longrightarrow HOCl + OH^-$$

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Thiol groups are easily oxidized by HOCl. Hence low molecular mass thiol compounds such as glutathione (GSH), N-acetylcysteine and mercaptopropionylglycine are very effective at protecting, for example, proteins against oxidative damage by HOCl (Aruoma *et al.*, 1989; Puppo *et al.*, 1990). Many molecules oxidize on contact with oxygen. For example several sugars including glucose, interact with proteins to produce radicals. It has been suggested that decades of exposure of body tissues to elevated blood glucose can result in diabetic patients suffering oxidative stress that may contribute to the side effects of hyperglycemia (Wolff and Dean, 1987).

2.6 Oxidative stress

While free radicals are produced as consequence of normal metabolism, efficient defence mechanisms exist *in vivo* to remove or inactivate them, thereby preventing or at least minimizing tissue damage. In health, the balance between ROS and the antioxidant defences lies slightly in favour of the ROS so that they are able to fulfill their biological roles (Evans and Halliwell, 2001). Oxidative stress is defined as a disturbance in the balance between antioxidants and prooxidants (free radicals and other reactive species), with increase levels of prooxidants leading to potential damage (Halliwell, 1997; Sies, 1997). This imbalance can be an effect of depletion of endogenous antioxidants, low dietary intake of antioxidants and/or increased formation of free radicals and other reactive species. Oxidant stress can be significant especially if the individual is exposed to environmental challenges which increase the production of reactive species above normal levels, for instance, infection. The incidence of kwashiorkor increases following measles epidemics and may be precipitated in severe malnutrition by a sudden intense

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oxidative stress caused by infection (Golden *et al.*, 1991). In these situations normal defence mechanisms are insufficient and tissue damage, which may be extensive, irreversible and fatal, develops (McCord, 1993; Gutteridge, 1994).

Oxidative damage to DNA, proteins and lipids can ultimately result in disorganization, dysfunction and destruction of membranes, enzymes and proteins (Slater, 1984, Halliwell, 1994; 1997). Specifically, peroxidation of membrane lipids may cause impairment of membrane function, decreased fluidity, inactivation of membranebound receptors and enzymes, increased permeability of ions and possibly eventual membrane rupture (Gutteridge and Halliwell, 1990; Gutteridge, 1995). Free-radical induced oxidative damage has been implicated in the development of various conditions such as diabetes, cancer, cataract, rheumatoid arthritis and atherosclerosis (Steinberg *et al.*, 1989; Parthasarathy *et al.*, 1992; Cheeseman and Slater, 1993; Cerutti, 1994). Oxidative stress has also been implicated in the pathogenesis of several viral infections including hepatitis, influenza and acquired immunodeficiency syndrome (AIDS) (Semba and Tang, 1999).

2.7 Lipid peroxidation

All biological membranes are characterized by the large amounts of PUFAs associated with amphipathic lipids and a variety of membrane proteins. One striking feature of PUFAs is that they can undergo oxidation in biological systems by a process known as lipid peroxidation (Porter *et al.*, 1995).

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2.7.1 Mechanism of lipid peroxidation

In peroxide-free system, lipid peroxidation is initiated when a hydrogen atom is abstracted from a methylene group (>CH_2 group) of an unsaturated fatty acid (Gutteridge and Halliwell, 1990; Halliwell and Chirico, 1993). PUFAs are particularly susceptible to peroxidation and once the process is initiated it proceeds as a free radical-mediated chain reaction involving initiation, propagation and termination (Gutteridge, 1995). Initiation of lipid peroxidation is caused by attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group upon a PUFA (Gutteridge and Halliwell, 1990; Halliwell and Chirico, 1993; Gutteridge, 1995). Removal of a hydrogen atom leaves behind an unpaired electron on the carbon atom to which it was originally attached.

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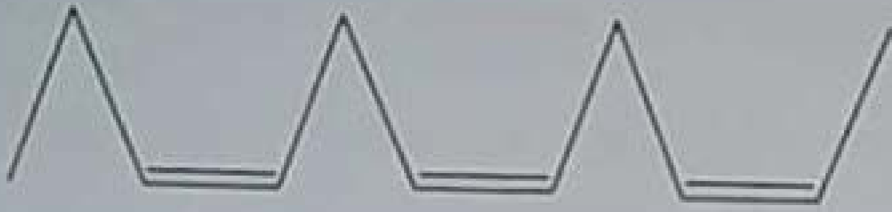
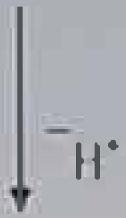
PUFA

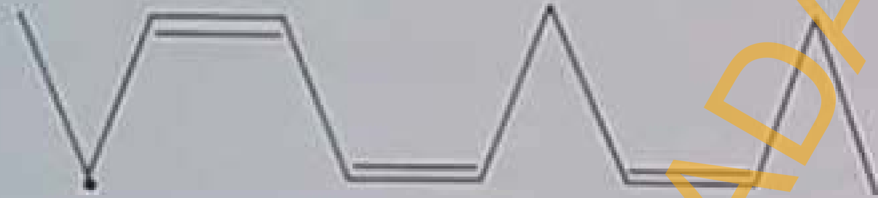




Loss of $H^{\bullet}$ to a free radical

$R^{\bullet}$
Carbon-centred radical

Molecular rearrangement



$R^{\bullet}$
Conjugated diene

$+O_2$

Uptake of oxygen

$ROO^{\bullet}$
Peroxyl radical

Abstraction of a $H^{\bullet}$ from an adjacent fatty acid

$+H^{\bullet}$

ROOH
Hydro-peroxide

Mechanism of non-enzymatic lipid peroxidation (modified from Gutteridge, 1995)

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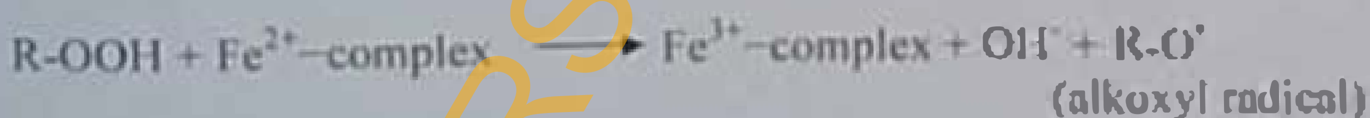
The carbon centred radical is stabilized by a molecular rearrangement to form a conjugated diene, followed by reaction with oxygen to give a peroxyl radical. Peroxyl radicals are capable of abstracting a hydrogen atom from another adjacent fatty acid side-chain to form a lipid hydroperoxide, but can also combine with each other or attack membrane proteins. When the peroxyl radical abstracts a hydrogen atom from a fatty acid, the new carbon-centred radical can react with oxygen to form another peroxyl radical and so the propagation of the chain reaction of lipid peroxidation can continue. Hence, a single substrate radical may result in conversion of multiple fatty acid side chains into lipid hydroperoxides. The length of the propagation chain before termination depends on several factors such as the oxygen concentration and the amount of chain-breaking antioxidants present. Pure lipid peroxides are reported to be stable at physiological temperatures, but their decomposition is stimulated by high temperatures or by exposure to transition metal complexes, especially iron salts (Packer and Glazer, 1990).

Ferrous ions reduce lipid peroxides to alkoxyl radicals while ferric ions can form both alkoxyl and peroxyl radicals with lipid peroxide:



$$R\text{-}OOH + Fe^{2+}\text{–complex} \longrightarrow Fe^{3+}\text{–complex} + OH^{-} + R\text{-}O^{\bullet}$$

(alkoxyl radical)

$$R\text{-}OOH + Fe^{3+}\text{–complex} \longrightarrow Fe^{2+}\text{–complex} + OH^{+} + R\text{-}O_2^{\bullet}$$

(peroxyl radical)

The redox recycling of these iron complexes perpetuates the decomposition. Final decomposition products of the reaction between lipid peroxides and iron or copper complexes include the hydrocarbon gases pentane, ethane and ethylene, carboxyl compounds and aldehydes, such as malondialdehyde (MDA) and 4-hydroxyl-2, 3-trans,

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nonenal (HNE) (Esterbauer *et al.*, 1991). The number of double bonds determines the susceptibility of a fatty acid to peroxidation (Wagner *et al.*, 1994; Porter *et al.*, 1995). An adjacent double bond weakens the energy of attachment of the hydrogen atoms present on the next carbon atom. Therefore, the greater the number of double bonds present on the next carbon atom in a fatty acid chain, the easier the removal of a hydrogen atom, that is why PUFAs are more susceptible to peroxidation.

2.7.2 Enzymatic lipid peroxidation

The peroxidation of PUFAs can proceed not only through non-enzymatic free radical-induced pathways, but also through processes that are enzymatically catalysed (Halliwell and Gutteridge, 1990; Gutteridge, 1995; Brash, 1999). Free radicals are probably important intermediates in the enzymatically-catalysed reaction but are localized to the active site of the enzyme. Cycloxygenase (COX) and lipoxygenase (LOX) fulfill the definition for enzymatic lipid peroxidation when they catalyze the controlled peroxidation of various fatty acid substrates. For example, the ferric form of lipoxygenase (LOX- Fe^{3+}) catalyses the oxidation of PUFA (RH) to form a pentadienyl radical ($R^{\bullet}$). The $R^{\bullet}$ reacts with O_2 to form $ROO^{\bullet}$ which abstracts a $^{\bullet}H$ to form ROOH.

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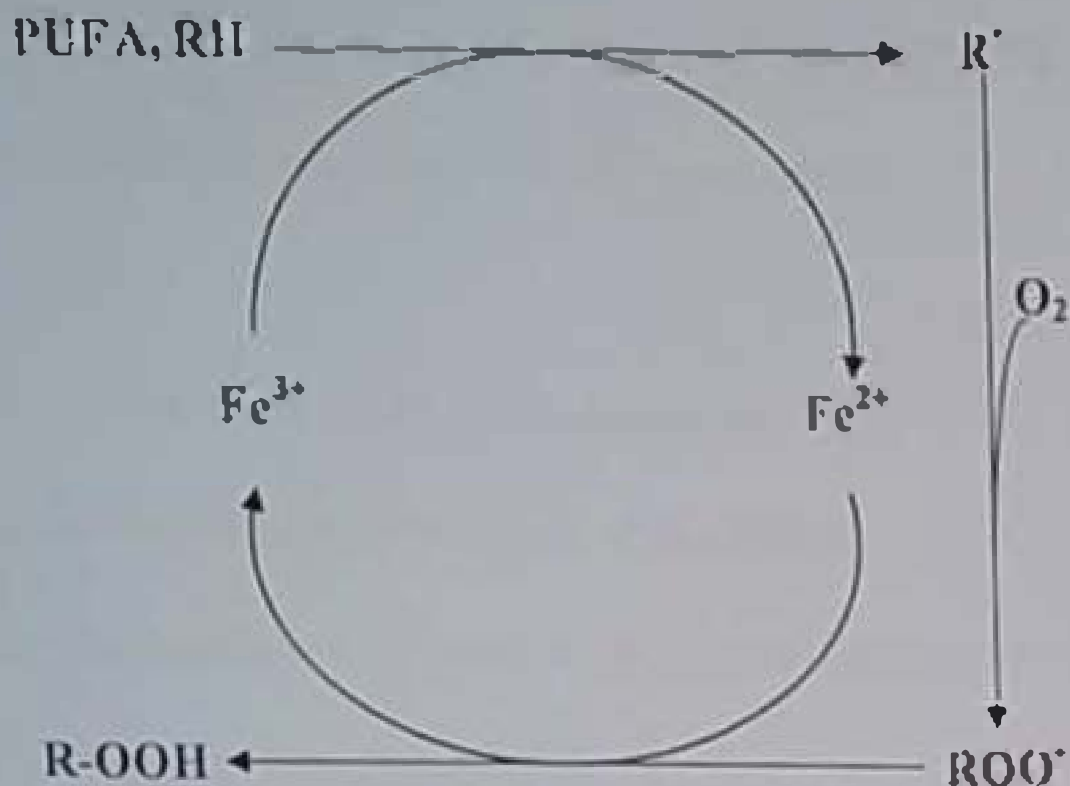

Lipoxygenase action (Adapted from Brash, 1999)

The hydroperoxides and endoperoxides produced from enzymatic lipid peroxidation become stereospecific and have important biological functions upon conversion to stable active compounds.

2.8 Carbon tetrachloride-induced hepatotoxicity

Carbon tetrachloride (CCl_4) belongs to a group of compounds called haloalkanes. It was once used as a solvent, cleaner, and degreaser both for industrial and home use. Over the years CCl_4 has proved highly useful as an experimental model for the study of certain hepatotoxic effects (Slater, 1981). It consistently produces liver injury in many species, including non-human primates and man (Kumar *et al.*, 1972; Yoshida *et al.*, 1999).

CCl_4-induced toxicity is an amalgamated term that, depending on dose and duration of exposure, or time of observation, covers a variety of effects that in general may be termed toxic. At low doses transient effects prevail, such as loss of Ca^{2+} sequestration, impairment of lipid homeostasis, release of noxious or beneficial cytokines, and apoptotic events, followed by regeneration (Weber *et al.*, 2003). Higher doses or longer exposure

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over a long period of time may lead to more serious and permanent effects such as fatty degeneration, fibrosis, cirrhosis, and even cancer.

2.8.1 Cellular sites of CCl_4-induced damage

The toxicity of many chemicals is thought to originate from destruction of membrane-bound polyunsaturated fatty acids during lipid peroxidation processes (Gilette *et al.*, 1974; Plaa and Witschi, 1976).

CCl_4 belongs to the first group of hepatotoxins, which is usually further subdivided into substances that are metabolized by the mixed function oxidases, and substances that are substrates for other enzyme systems (Monks and Lau, 1988; Nelson and Pearson, 1990).

The toxicity of reactive compounds or their metabolites may result from covalent (primary) interactions with critical target molecules such as DNA, lipids, proteins, or carbohydrates, or from the alteration of target molecules via secondary bond formation (lipid peroxidation, generation of reactive oxygen species, alteration of reduced or oxidized glutathione, GSH/GSSG). CCl_4 is known to assume a special role in that, once activated, it affects cellular homeostasis through both primary and secondary bond formation (Weber *et al.*, 2003).

The principal site for the manifestation of the effects of CCl_4 poiosoning is the liver and the pathological changes following CCl_4 poisoning have been identified at the biochemical and ultrastructural levels. Endoplasmic reticulum, plasma membrane, mitochondria, and Golgi apparatus are the main sub-cellular structures of hepatocytes affected by CCl_4 exposure, suggesting the hypothesis that primary lipid-containing structures of the cell are affected by CCl_4 (Reynolds, 1963). Following oral

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administration. CCl_4 is concentrated in the liver and reaches a maximum level 1 to 2 h of dosing (Reynolds, 1963). The earliest histological evidence of tissue derangement occurs 5 to 6 h after administration when necrosis begins (Lockard *et al.*, 1983). Central zone necrosis and massive necrosis are reported to occur after 12 h and 24 to 48 h post administration respectively (Zimmermann, 1976).

Liver injury induced by CCl_4 is characterized by the impairment of a number of cellular functions. An increase in microsomal lipids, reduced secretion of triglycerides from the endoplasmic reticulum into the plasma and a decline in several microsomal enzymes appear 2 to 3 h after administration (Plaa, 2000; Boll *et al.*, 2001; Weber *et al.*, 2003).

Increased radical formation from sustained CCl_4 exposure, combined with the presence of prooxidants such as high Fe^{2+} concentrations ("iron overload") is suggested to overwhelm cellular repair mechanisms and cause permanent or even fatal liver damage. Thus, the sequence of reactions of the liver cell in response to CCl_4 metabolites consists of initial derangements of sub-cellular structures, followed by reversible alterations of cellular metabolism, causing secondary damage that ultimately may lead to pathological consequences (Brattin *et al.*, 1985).

The metabolism of CCl_4 starts with the formation of the trichloromethyl free radical, $CCl_3^{\cdot}$ (McCay *et al.*, 1984) through the action of the mixed function cytochrome P450 oxygenase system of the endoplasmic reticulum (Slater, 1984; Nelson and Harrison, 1987 and Recknagel *et al.*, 1989). This process involves reductive cleavage of carbon-chlorine bond without the introduction of oxygen into the molecule during the reaction. Free radical activation of CCl_4, which may contribute significantly to its toxicity, has also been observed in mitochondria (Tomasi *et al.*, 1987; Boll *et al.*, 2001).

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The major cytochrome isozyme implicated in the biotransformation of CCl_4 is cytochrome P450 (CYP) 2E1 (CYP 2E1), but CYP 2B1 and CYP 2B2 are also capable of attacking CCl_4 (Raucy *et al.*, 1993; Gruebele *et al.*, 1996).

The $CCl_3^{\bullet}$ reacts with various biologically important substances such as amino acids, nucleotides and fatty acids, as well as proteins, nucleic acids and lipids by abstracting a hydrogen , mostly from unsaturated fatty acids to form chloroform (Castro, 1984).

In the presence of oxygen, the $CCl_3^{\bullet}$ radical is converted to the trichloromethyl peroxy radical, $CCl_3OO^{\bullet}$ which is more reactive and thus more short-lived than the $CCl_3^{\bullet}$ radical (Mico and Pohl, 1983). $CCl_3OO^{\bullet}$ is far more likely than $CCl_3^{\bullet}$ to abstract a hydrogen from PUFA thereby initiating the process of lipid peroxidation, a complex series of reactions that terminate in the complete disintegration of the PUFA molecule with the formation of aldehydes, other carbonyls and alkanes (Forni *et al.*, 1983; Cheeseman *et al.*, 1985; Comporti, 1985; Tribble *et al.*, 1987).

2.8.2 Mechanism of CCl_4-induced liver damage

Haloalkylation and lipid peroxidation represent two distinct mechanisms for CCl_4-induced liver damage (Dianzani, 1984). It is not clear, however, which of them plays a leading role in liver cell necrosis (Recknagel, 1983; Recknagel *et al.*, 1989).

The two mechanisms are capable of producing cell damage, both in a reversible fashion initially, as long as dose, duration of exposure, and exacerbating factors do not overwhelm regenerative capacity (Weber *et al.*, 2003).

Liver damage by CCl_4 is apparently a complicated process that may turn into a vicious circle where potentially reversible changes, when sustained by high doses of, or long-

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term exposure to the toxicant deprive the hepatocyte of its regenerative capacity (Weber *et al*., 2003).

Mehendale (1991) proposed a two-step process for chemical-induced hepatotoxicity. Firstly, the reactive metabolites of CCl_4 initiate hepatocyte injury. This would lead to catastrophic cell necrosis, but the toxicant attack also initiates a number of cellular responses some of which will cause the tissue to recover provided there is sufficient energy supply left and the toxicant action subsides within 24 h. One important prerequisite to initiate recovery appears to be the energy state of the cell ; since high doses of CCl_4 deplete cellular ATP, any treatment that helps to sustain or replenish ATP levels will allow recovery from an otherwise necrotizing dose (Soni and Mehendale, 1994).

Recovery from fatty degeneration, fibrosis and cirrhosis can be achieved mostly by antioxidants or agents that counteract collagen deposition (Ohishi *et al*., 2001). It has been suggested that prevention of haloalkane-induced cancer may be possible with prophylactic use of herbal remedies (Guyton and Kensler, 2002).

2.8.3 Inhibition of lipoprotein secretion with functional impairment of the Golgi apparatus

The initial damage in CCl_4-induced steatosis begins with the inhibition or blockage of lipoprotein secretion from hepatocytes into the circulation (Dianzani and Poli, 1984). The extent of CCl_4-induced fatty liver formation is related to the amount of reactive CCl_4 metabolites produced and persistent CCl_4 intoxication of the liver affects its capacity to synthesize lipids (Pencil *et al*., 1984). The CCl_4-induced accumulation of fat is paralleled

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by changes in plasma membrane function. These changes are considered to occur early during CCl_4 intoxication and have been shown to affect membrane-bound enzymes such as adenylate cyclase, 5′-nucleotidase and $Na^+ K^+$-ATPase due to a detergent-like effect of changed membrane lipids and subsequent solubilization of membrane proteins (Paradisi *et al.*, 1985). The secretion of VLDL from hepatocytes is highly decreased by CCl_4 (Boll *et al.*, 2001). It has been shown that in the early phase of CCl_4 poisoning the Golgi apparatus of the liver becomes functionally impaired (Poli *et al.*, 1985). The Golgi apparatus plays a fundamental role in synthesis, maturation and secretion of VLDL. CCl_4 induces accumulation of labeled lipids and reduces the activities of glucosyl- and galactosyl- transferases in the Golgi apparatus (Marinari *et al.*, 1985). Analysis of the different fractions purified from microsomes shows that CCl_4 treatment impairs the secretory side (fractions F_1 and F_2) as well as the formative side (fraction F_3) of the apparatus (Marinari *et al.*, 1985). F_1 and F_2 fractions are involved in the sequestration of lipoprotein micelles into secretory vesicles whereas F_3 and endoplasmic reticulum are involved in the coupling of apoprotein and lipid to form the final lipoprotein. Thus, CCl_4 affects all steps of lipoprotein formation. Through its effect on assembly and composition of lipoproteins, CCl_4 drastically impairs their ability to act as structural components of transport vehicles for lipids (Weber *et al.*, 2003).

2.8.4 Effect of CCl_4 on lipid homeostasis

Fatty degeneration of the liver (steatosis) following CCl_4 poisoning is in part due to an imbalance between lipid synthesis and degradation and in part a consequence of the failure of triglycerides to move as VLDL from liver to the circulation (Boll *et al.*, 2001).

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The rate of synthesis of triglycerides depends on the availability of substrates for *de novo* synthesis and the activity of enzymes involved in the synthesis. Endogenous and exogenous fatty acids are mainly metabolized by two processes in the liver: (1) esterification to form triglycerides, phospholipids, and other fatty acid esters, and (2) β-oxidation to form CO_2 and ketone bodies. Development of fatty liver can be the result of excessive fatty acid and/or triglyceride synthesis, or an inhibited oxidation process, or both (Weber *et al*., 2003).

CCl_4 increases cholesterol synthesis, the rate of lipid esterification and synthesis of fatty acids and triglycerides from acetate (Boll *et al*., 2001). The increased esterification of fatty acids is a response secondary to other CCl_4-induced effects – inhibition of β-oxidation and decreased cellular lipid secretion (Fromenty and Pessayre, 1995; Boll *et al*., 2001). It is postulated that CCl_4 positively affects the transport of acetate into the liver cell resulting in increased substrate availability (Weber *et al*., 2003).

CCl_4 lowers β-oxidation of fatty acids, hydrolysis of triglycerides, and also the content of unsaturated fatty acids, while *de novo* fatty acid synthesis and saturated fatty acids increase thus providing more fatty acids for esterification (Boll *et al*., 2001).

A major metabolic defect induced by CCl_4 intoxication to the rats appears to be inhibition of hepatic triglyceride release. This inhibition of outward transport would allow the accumulation of triglycerides within the liver and the occurrence of fatty liver associated with CCl_4 poisoning (Heimberg *et al*., 1962).

In summary, CCl_4-induced damage is characterized by hepatocyte membrane damage caused by lipid peroxidation, increased plasma levels of hepatic enzymes such as AST, ALP and ALT, fatty degeneration (steatosis i.e. accumulation of triglycerides in the

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liver), reduced β-oxidation of fatty acids and necrosis. Thus, quantitative measurements of plasma levels of liver enzymes, total cholesterol and hepatic triglyceride level, together with histopathological examination of hepatocytes provide a good assessment of the extent of liver damage or regeneration when challenged with CCl_4.

2.9 Defences against Free Radicals

The human body has several mechanisms for defence against free radicals and other reactive oxygen species. The various defences are complementary to one another because they act on different oxidants or in different cellular compartments (Langseth, 2000).

2.9.1 Superoxide dismutases

Superoxide dismutases (SOD) are a group of metalloenzymes present in all respiring cells that catalyse the dismutation of $O_2^{\bullet -}$ into O_2 and H_2O_2 (Fridovich, 1997).

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

There are three isoenzymes of SOD in mammals, the first of which was discovered by McCord and Fridovich (1969). This CuZnSOD was isolated from cytoplasm, nucleus and peroxisomes. It is a dimmer of 16 kDa. Immunohistochemical and cell fractionation procedures have supported a cytosolic location for CuZnSOD in many cells. In hepatocytes, for example, about 70% is cytosolic and 12% is in the nucleus (Chang *et al.*, 1988). More recent evidence based on immunofluorescence with monoclonal antibody markers now suggests that CuZnSOD in human fibroblasts, hepatoma cells and yeast cell is predominantly a peroxisomal enzyme. Its cytosolic location apparently arises from

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rupture of peroxisomes during homogenization (Keller *et al.*, 1991). The second isoenzyme is MnSOD, an 80-kDa tetramer, which is cytoplasmically synthesized and located in the mitochondria. The third isoform, EC-SOD (extracellular) was discovered by Marklund, 1982) and is a CuZnSOD with a positively charged binding domain optimized in the extracellular matrix. This isoenzyme has been shown to have particularly high expression in vascular tissue (Oury *et al.*, 1994) and umbilical cord tissue (Sandstrom *et al.*, 1993).

The structure of CuZnSOD in bovine erythrocytes has been determined as homodimer of 16 kDa with the active site located within a cylinder ß-structure (Richardson *et al.*, 1975), where it is well protected and is known to retain catalytic activity during isolation procedures (Forman and Fridovich, 1973). The mechanism of action of SOD is that the copper ion at the active site is reduced by one O_2^- molecule, then reoxidised by another in a continuing cycle. Thus, copper oscillates between the monovalent and divalent states. Mutant forms of the CuZnSOD appear to explain familial forms of a fatal neurological disease known as amyotrophic lateral sclerosis, or motor neuron disease (Hosler and Brown, 1995). In this condition, the motor degenerates over the course of a few years leading to weakness and eventually paralysis, with death from pneumonia caused by the inability of the patient to clear respiratory secretions. The mutant enzymes dismute superoxide in a normal fashion, but they have excess peroxidase activity, an activity present in normal CuZnSOD to only a very limited extent (Wiedau-Pazos *et al.*, 1996). It is presently thought that the oxidative damage inflicted by increased peroxidase activity of the mutant dismutase is responsible for the early death of those neurons (Babior, 1997).

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Although SOD is important, an excess of SOD in relation to peroxide metabolizing enzymes can be deleterious (Scott *et al*., 1989; Groner *et al*., 1990; Amstad *et al*., 1991; White *et al*., 1991). This has been shown by transfecting cells with human cDNA encoding SOD (Amstad *et al*., 1991). Transgenic mice overexpressing human CuZnSOD are resistant to elevated O_2 and to certain toxic agents (Groner *et al*., 1990; White *et al*., 1991) but they show certain neuromuscular abnormalities resembling those found in patients with Down's syndrome (Groner *et al*., 1990). The gene encoding CuZnSOD is located on chromosome 21 in humans and Down's syndrome is usually caused by trisomy of this gene, raising tissue CuZnSOD levels by about 50%. The data available at present are consistent with the view that the excess of CuZnSOD may contribute to at least some of the abnormalities in patients with Down's syndrome (Groner *et al*., 1990).

2.9.2 Catalase

Catalase (CAT) is present mostly in peroxisomes of nearly all aerobic cells. It serves to protect the cell from the toxic effects of H_2O_2 by catalyzing its decomposition into molecular oxygen and water. The overall reaction is as follows:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

CAT has a molecular weight of 240 kDa, and is composed of four identical subunits (tetramer), each containing a heme prosthetic group at the catalytic centre. CAT monomers from certain species (e.g. cow) also contain one tightly bound NADPH per subunit. The NADPH lies on the surface, whereas the heme is embedded in the middle of each monomer about 20Å below the molecular surface and 23Å from the centre of the tetramer (Murthy *et al*., 1981). This NADPH may serve to protect the enzyme from

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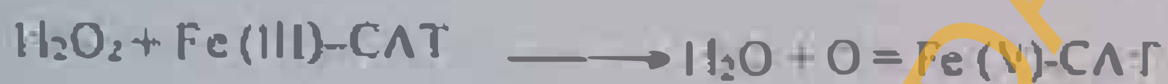
oxidation by its H_2O_2 substrate. Heme consists of a protoporphyrin ring and a central iron atom. The iron can either be in the ferrous (Fe^{2+}) or the ferric (Fe^{3+}) oxidation state. Each heme is exposed through a funnel-shaped channel 30Å long and 15Å wide (Murthy *et al.*, 1981). The channel is lined with hydrophilic residues as the channel descends, constricting toward the heme (Belal *et al.*, 1989). CAT is an enzyme that can function in two distinct modes. The catalytic mode is responsible for H_2O_2 breakdown which is thought to occur in two stages (Sichak and Dounce, 1986; Halliwell and Gutteridge, 1999):



$$H_2O_2 + Fe(III)\text{-}CAT \longrightarrow H_2O + O{=}Fe(V)\text{-}CAT$$

$$H_2O_2 + O{=}Fe(V)\text{-}CAT \longrightarrow Fe(III)\text{-}CAT + H_2O + O_2$$

Fe (III)-CAT represents the native catalase molecules and O=Fe (V)-CAT represents catalase compound I which was first described by Chance et al., (1979). Formation of compound leads to characterization changes in the absorbance spectrum of catalase. Also catalase compound I can react with a limited number of hydrogen donors such as ethanol or methanol and oxidize these substances by utilizing O_2 in the form of H_2O_2 molecule in a two-electron oxidation step. This is the peroxidatic mode of CAT action (Sichak and Dounce, 1986).

$$O{=}Fe(V)\text{-}CAT + RCH_2OH \longrightarrow Fe(III)\text{-}CAT + RC(=O)H + H_2O$$

CAT can also oxidize nitrite (NO_2^-) into nitrate (NO_3^-) *in vitro* (Chance *et al.*, 1979). The rate of H_2O_2 removal via CAT is 10^8 times faster than the dismutation of H_2O_2 to H_2O and O_2 (Forman and Fisher, 1981), meaning that it is virtually impossible to saturate CAT activity under normal biological conditions. Phagocytic cells contain CAT which can

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scavenge not only H_2O_2 produced within the neutrophil but also H_2O_2 added exogenously to cell preparations because H_2O_2 can freely move across the cell membrane (Voetman and Roos, 1980). CAT is erroneously said to work only at high concentrations of H_2O_2 and to serve principally as a backup for the glutathione dependent systems. However, the enzyme has a binding site for NADPH at H_2O_2 concentrations in the vicinity of those at which the glutathione-dependent systems operate (Gaetani *et al.*, 1996). It is therefore likely that some half the H_2O_2 produced in the cell is destroyed by CAT.

2.9.3 Glutathione peroxidase

Glutathione Peroxidase (GSHPx) is a tetramer with a molecular weight of between 76 and 105 kDa, with four selenium (Se) atoms per molecule. GSHPx helps prevent lipid peroxidation of cell membranes by consuming free peroxide in the cell. The summary of the reaction is:

$$GSH + H_2O_2 \xrightarrow{GSHPx} GSSG + H_2O$$

GSHPx uses glutathione (GSH) as the reducing agent. It differs not only from species to species but also from tissue to tissue. 76 ± 1 kDa for rat liver, 84 ± kDa for bovine erythrocyte and 95 ± 3 kDa human erythrocyte (Roger and William, 1980). It is present at high levels in kidneys, erythrocytes and livers.

GSHPx is a tetrameric protein composed of two dimmers and is almost flat with dimensions of 90.4 x 109.5 x 58.6Å. The four active sites are located on the surface and Se atoms (in form of selenocysteine) are found in each active site. The Se atoms in each dimer are 21Å apart (Roger and William, 1980). The selenocysteine is introduced into the protein by a special t-RNA that is initially charged with serine but undergoes a series of

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reactions to convert it to t-RNA selenocysteine. Selenocysteine is encoded by the triplet UGA, which ordinarily introduces a stop but in the context of the glutathione mRNA is recognized by the seleno-cysteine-linked t-RNA (Chambers and Harrison, 1987). The large distances of the Se atoms in tetramer allow the formation of intra-molecular diselenide bridges during catalysis, which plays an important role in the enzyme function. Se is important for the enzymatic activity of GSHPx. Release of Se from the enzyme can irreversibly lose the function of the GSHPx activity. X-ray photoelectron spectroscopic studies show that the protein-bound Se undergoes reversible substrate-induced redox-selenol derivatives. (R-SeH) in reduced form and a seleninyl (R-SeO-OH) or selenenyl (R-Se-OH) in oxidized form (Ladenstein *et al.*, 1979).

Animals having Se-deficient diets show rapid decrease in tissue GSHPx activity through Se re-supplementation. It has been found that a single large oral dose of selenite or selenomethionine given to Se-deficient rats resulted in significant increases in kidney, liver and stomach GSHPx activity after 48 hours (Roger and William, 1980). Iodoacetate selectively inhibits the enzyme by reacting with the selenocysteine residue in GSHPx. The inhibition can be reversed by addition of an amount of hydroperoxides stoichiometric with the selenocysteine residues (Roger and William, 1980). It has been demonstrated that a substrate-dependent reversible redox change of the selenocysteine residue is the basic catalytic process in the function of GSHPx. Substrate-GSHPx is readily reoxidized spontaneously and generally extreme precautions are required to preserve selenocysteine-containing enzymes in the physiological state. The first step of the catalytic cycle of GSHPx can be described as the oxidation of an ionized selenol by a hydroperoxide to produce a selenic acid derivation (Ursini *et al.*, 1985). The selenol can be regenerated

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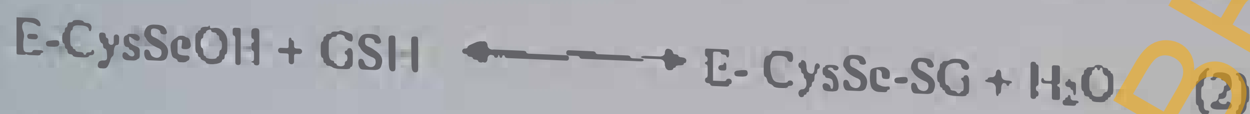
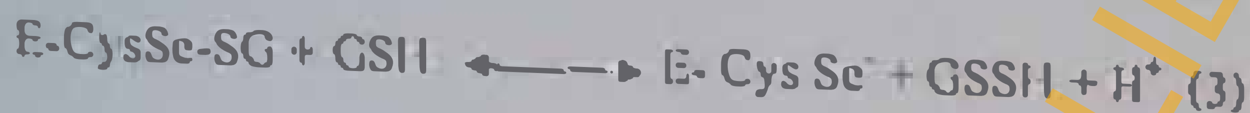
following two consecutive reactions involving the selenic acid residue and GSH. Intermediate of a selenosulfide is formed between the enzyme and GSH. The series of reactions is as follows:

$$E\text{-}CysSe^{-} + H^{+} + ROOH \longrightarrow E\text{-}CysSeOH + ROH \quad (1)$$



$$E\text{-}CysSeOH + GSH \longleftrightarrow E\text{-}CysSe\text{-}SG + H_2O \quad (2)$$



$$E\text{-}CysSe\text{-}SG + GSH \longleftrightarrow E\text{-}Cys\,Se^{-} + GSSH + H^{+} \quad (3)$$

GSHPx can scavenge both H_2O_2 as well as organic hydroperoxides and has a high affinity for such peroxides.

$$2GSH + H_2O_2 \xrightarrow{GSHPx} GSSH + 2H_2O$$

$$2GSH + ROOH \xrightarrow{GSHPx} GSSG + ROH + H_2O$$

The efficiency by which GSHPx can scavenge hydroperoxides increases with increasing GSH concentration (Paglia and Valentine, 1967; Ursini *et al*., 1985, Esworthy *et al*., 1993).

Lipid hydroperoxides which are formed during the peroxidation of lipids containing unsaturated fatty acids are reduced; not by the usual GSHPx but by a special enzyme known as phospholipids hydroperoxide glutathione, designed specifically to handle peroxidised fatty acids in phospholipids. This enzyme can reduce both H_2O_2 and lipid hydroperoxides to corresponding hydroxides (water and a lipid hydroxide respectively). In contrast to the phospholiid hydroperoxide glutathione peroxidase ordinary glutathione peroxidase is unable to act on lipid hydroperoxides. Families with inherited deficiencies of GSHPx have been reported (Anonymous, 1980; Cohen *et al*., 1985). Affected members manifest a mild to moderate severe haemolytic anaemia that is aggravated by infection and by oxidant drugs such as nitrofurantoin and certain sulfonamides.

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2.9.4 Glutathione S-transferases

Glutathione S-Transferases (GSTs; E.C. 2.5.1.18) are a multi-gene family of enzymes involved in the detoxification and activation of a wide variety of chemicals.

GSTs catalyse the nucleophilic attack of glutathione (GSH) on electrophilic substrates, thereby decreasing their reactivity with cellular macromolecules (Armstrong, 1997). Most GSTs exist as soluble enzymes, although a small family of microsomal GSTs has been characterized (Anderson *et al.*, 1994; Jakobsson *et al.*, 1996), and a mitochondrial GST (GST Kappa) has also been identified (Pemble *et al.*, 1996).

The soluble forms of GSTs exist as dimeric proteins, with subunit molecular weights of approximately 25 kDa. Each subunit of the dimeric enzyme has an active site composed of two distinct functional regions: a hydrophilic G-site, which binds the physiological substrate glutathione, and an adjacent H-site which provides a hydrophobic environment for the binding of structurally diverse electrophilic substrates (Armstrong, 1997). The G-site is highly conserved between all GSTs due to its high specificity for GSH, while the H-site can be quite divergent between different GSTs, and exhibits broad and variable substrate binding specificity. The mammalian soluble GSTs are divided into four main classes, alpha (A), mu (M), pi (P), and theta (T) (Mannervik *et al.*, 1992; Hayes and Pulford, 1995; Coggan *et al.*, 1998).

GSTs catalyze the general reaction:

$$GSH + R\text{–}X \xrightarrow{GST} GSR + HX$$

The function of the enzyme is to (1) bring the substrate into close proximity with GSH by binding both GSH and the electrophilic substrate to the active site of the protein, and (2) activate the sulfhydryl group on GSH, thereby allowing for nucleophilic attack of GSH

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on the electrophilic substrate (R–X) (Armstrong, 1997). The formation of a thioether bond between the cysteine residue of GSH and the electrophile usually results in a less reactive and more water-soluble product, and thus GSTs are usually detoxification reactions (Armstrong, 1997). A large number of diverse chemicals such as 1-Chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), and cumene hydroperoxide serve as substrates for GSTs (Hayes and Pulford, 1995).

The ability of many phytochemicals to induce GSTs has generated much interest and research in the role of dietary GST induction as a mechanistic explanation for the anticarcinogenic effects of fruits and vegetables. Presently, there is substantial experimental animal evidence demonstrating that GST induction can reduce the effectiveness and potency of a variety of chemical carcinogenesis (Hayes and Pulford, 1995; Clapper and Szarka, 1998; Williamson *et al.*, 1998).

2.9.5 Glutathione

Reduced glutathione, most commonly called glutathione (GSH), is a relatively small molecule ubiquitous in living systems (Kidd, 1997; Sen, 1997). Occurring naturally in all human cells, GSH is a water-phase orthomolecule. Its intracellular depletion ultimately results in cell death and its clinical relevance has been researched for decades. GSH is the smallest intracellular thiol (-SH) molecule. Its high electron-donating capacity (high negative redox potential) with high intracellular concentration generate great reducing power (Kidd, 1997). GSH levels in human tissues normally range from 0.1 to 10 millimolar (mM), most concentrated in the liver (up to 10 mM), and in the spleen, kidney, lens, erythrocytes and leukocytes (Bremer *et al.*, 1981).

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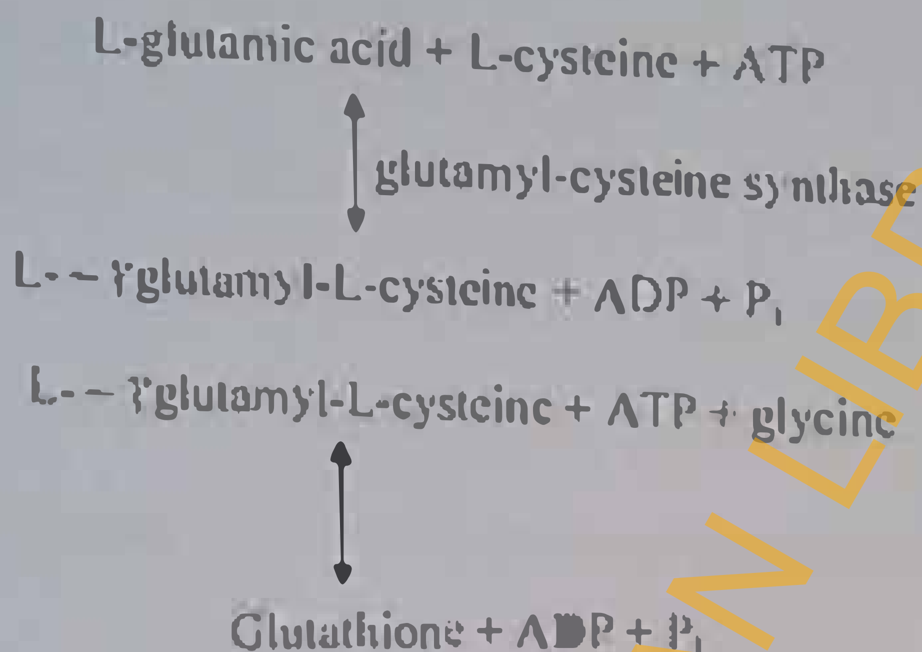
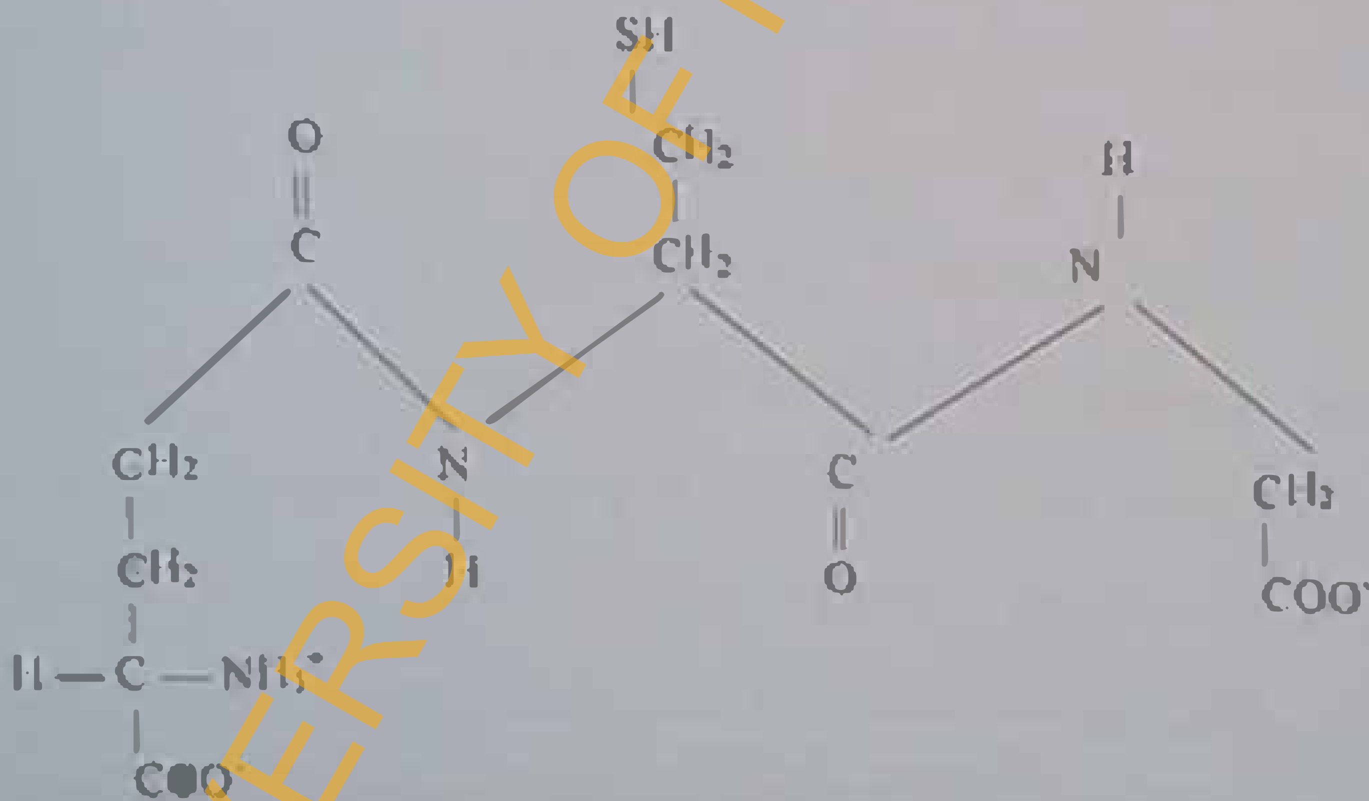
GSH is a linear tripeptide of L-glutamine, L-cysteine and glycine. It is synthesized according to the following two reactions:



Technically, N-L-gamma-glutamyl-cysteinyl-glycine or L-glutathione, molecule has a sulfhydryl (-SH) group on the cysteinyl portion, which accounts for its strong electron-donating character.



Glutathione (γ-glutamyl-cysteinyl-glycine)

As electrons are lost, the molecule becomes oxidized and two such molecules become linked (dimerised) by a disulfide bridge to form glutathione disulfide or oxidized glutathione (GSSG). This linkage is reversible upon reduction. GSH is under tight homeostatic control both intracellularly and extracellularly (Kidd, 1997). A dynamic

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balance is maintained between GSH synthesis, its recycling from GSSG and its utilization. GSH synthesis involves two closely linked, enzymatically controlled reactions that utilize ATP (Anderson, 1997). Cysteine and glutamate are combined by γ-glutamyl cysteinyl synthetase, and then GSH synthetase combines γ-glutamylcysteine with glycine to generate GSH. If GSH levels rise, they self-limit further GSH synthesis, otherwise, cysteine availability is usually rate-limiting. Fasting, protein-energy malnutrition, or other dietary amino acid deficiencies limit GSH synthesis (Verjee and Behal, 1976; Whitcomb and Block, 1994).

GSH can be depleted by oxidative stressors such as ultraviolet and other radiation, viral infections, environmental toxins, household chemicals and heavy metals, surgery inflammation, burns, septic shock and dietary deficiencies of GSH precursors and enzyme cofactors (Spies *et al.*, 1994, Whitcomb and Block, 1994, Kidd, 1997; Look *et al.*, 1997; Luo *et al.*, 1998). Direct attack by free radicals and other oxidative agents can also deplete GSH as it is being consumed (Sen, 1998). The liver is the largest GSH reservoir. The parenchymal cells synthesize GSH for P-450 conjugation and numerous other metabolic requirements, then export GSH as a systemic source of -SH/reducing power (Anderson, 1997). GSH equivalents circulate in the blood predominantly as cystine, the oxidized and more stable form of cysteine. Cells import cystine from the blood, reconvert it to cysteine and from it synthesize GSH. Conversely, inside the cell GSH helps re-reduce oxidized forms of other antioxidants such as ascorbate and alpha (α) -tocopherol (Meister, 1994).

GSH is an extremely important cell protectant. It directly quenches reactive hydroxyl free radicals, other oxygen-centred free radicals, and radical centres on DNA and other

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biomolecules (Kidd, 1997). GSH is an essential cofactor for many enzymes which require thiol-reducing equivalents and helps keep redox- sensitive active sites on enzymes in the necessary reduced state (Weber, 1999). GSH/GSSG balance is crucial to homeostasis, stabilizing the cellular biomolecular spectrum and facilitating cellular performances and survival (Kidd, 1997; Weber, 1999). Glutathione status is a highly sensitive indicator of cell functionality and viability. As intracellular GSH becomes reduced, the cells functionality is progressively reduced until it dies. In humans, GSH depletion is linked to a number of disease states (Kidd, 1997; Sen, 1997; Gul *et al.*, 2000). Individual with inherited deficiencies of the GSH-synthesizing enzymes exhibit limited or generalized GSH deficiency with haemolytic anaemia, spinocerebellar degeneration, peripheral neuropathy, myopathy and aminoaciduria and often develop severe neurological complications in the fourth decade of life. Low erythrocyte GSH also manifests in hereditary non-spherocytic lymphocytic leukemia and glucose-6-phosphate dehydrogenase (G6PD) deficiency (Meister and Larsson, 1995; Sen, 1997; Gul *et al.*, 2000).

Immune cell functionality and proliferation rely on adequate intracellular GSH and healthy humans with low lymphocyte GSH can have low CD4 counts. Chronic viral infections may trigger GSH depletion in circulating immune cells or GSH/GSSG imbalance. Patients with chronic hepatitis C virus were found to have low GSH in their circulating monoctyes (Anderson, 1997). Human immunodeficiency virus (HIV) infection and sequelae feature systemic GSH depletion (Anderson, 1997). Oxidative stress is elevated at all stages of HIV disease; HIV infection lowers GSH in the plasma, erythrocytes, T-cells and other lymphocytes and monocytes (Pace and Leaf, 1995).

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Children with HIV also demonstrate low GSH (Look *et al*., 1997; Gul *et al*., 2000). Plasma and erythrocyte GSH can be low in patients with cirrhosis or result from acute or chronic alcohol intake (Gul *et al*., 2000). In non-alcoholic liver disease, liver GSH can be abnormally low and GSSG high (Altomare *et al*., 1998. GSH deficiency has been linked to various pulmonary diseases including chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), neonatal lung damage and asthma (Sen, 1997; Anderson, 1997; Gul *et al*., 2000). Patients with gastritis and /or duodenal ulcer linked to *Helicobacter pylori* infection can have low GSH (Gul *et al*., 2000). In diabetics, the erythrocytes and platelets can be low in GSH (Yoshida *et al*., 1995; Gul *et al*., 2000). A variety of neurodegenerative diseases manifest abnormally low GSH. In Alzheimer's, a decrease in lymphoblast GSH has been reported (Kidd, 1997; Sen, 1997; Gul *et al*., 2000).

2.9.6 Selenium

Selenium is a trace mineral that is essential to good health but required only in small amounts (Thomson, 2004). Selenium was firmly established as an essential nutrient in 1973 when it was shown to be a constituent of the enzyme glutathione peroxidase (Rotruck *et al*., 1973). Clinical evidence indicating that selenium is essential for human beings appeared in 1979 (Keshan Disease Research Group, 1979). Chinese scientists carried out a selenium supplementation study in children living in a Se-deficient region. Selenium supplementation essentially abolished the occurrence of Keshan disease, a childhood cardiomyopathy that was often fatal. It then became clear that selenium was an essential nutrient for humans.

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Selenium is an essential micronutrient of major metabolic significance. It is incorporated as selenocysteine at the active site of a wide range of proteins. Under physiological conditions, the selenium in selenocyteine is almost fully ionized and consequently is an extremely efficient biological catalyst (Arthur *et al.*, 1997). Many selenoproteins may exist in mammalian systems and up to 30 have been identified by ^{75}Se labeling in vivo (Evenson and Sunde, 1988; Burk and Hill, 1993). Of these, 15 selenoproteins have been purified or cloned including the glutathione peroxidase enzymes which represent a major class of functionally important selenoproteins (Burk and Hill, 1993). Selenium is present in soil and enters the food chain through plants. We obtain most of our dietary selenium from bread, cereal, meat and poultry. Tissue levels of selenium are readily influenced by dietary intake which itself is governed by geographical differences in available selenium in soil (Brown and Arthur, 2001).

Selenium has a number of biochemical functions as evidenced by the list of selenoproteins. For example, the glutathione peroxidases remove hydrogen peroxide and lipid hydroperoxides at the expense of reduced glutathione (Burk, 2002). The antioxidant nature of selenium is different from that of other antioxidant nutrients. Selenium functions as a component of antioxidant enzymes e.g glutathione peroxidase and thioredoxin reductase (Burk, 2002). Part of the evidence that selenium is an antioxidant is the observation that the nonselenium-dependent antioxidant enzymes heme-oxygenase-1 and glutathione-S-transferase are induced in selenium deficiency (Burk, 1983). The induction is postulated to compensate for the decrease that occurs in antioxidant selenoenzymes. It has been reported that there is no increase in products of lipid peroxidation in animals with selenium deficiency alone, supporting the compensation

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hypothesis (Burk *et al.*, 1995). Oxidative injury does occur, however, in selenium-deficient animals that are stressed in certain ways. The induction of vitamin E deficiency in the selenium-deficient animal causes oxidative injury with severe organ damage and death (Schwarz and Foltz, 1957).

There is evidence that selenium deficiency may contribute to development of a form of heart disease, hypothyroidism and a weakened immune system (Combs, 2000; Zimmerman and Kohrle, 2002). It is well established that dietary selenium is important for a healthy immune response. The effects of selenium can include reduced T-cell counts, impaired lymphocytes proliferation and responsiveness (Kiremidjian–Schumacher *et al.*, 1994). Dietary supplementation of humans with 200 µg of sodium selenite enhances T-lymphocyte immune responses (Roy *et al.*, 1994). Selenium has been suggested to be a cancer chemo-preventive agent. This proposal is based on studies showing that supra-nutritional intakes of selenium often delay or reduce the development of cancer in animal modes of the disease (Ip, 1998). It has also been reported in humans that secondary endpoint cancers were apparently prevented by selenium supplementation (Clark *et al.*, 1996). Selenium may also prevent or slow tumour growth. Certain breakdown products of selenium are believed to prevent tumour growth by enhancing immune cell activity and suppressing development of blood vessels to the tumour (Combs *et al.*, 2001).

Low blood selenium concentrations have been associated with increased cardiovascular disease mortality and rheumatoid arthritis (Brown and Arthur, 2001; Kose *et al.*, 1996). Selenium may help to relieve symptoms of arthritis by controlling levels of free radicals (Aaseth *et al.*, 1998). HIV/AIDS malabsorption can deplete levels of many nutrients

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including selenium. Selenium deficiency is associated with decreased immune cell counts, increased disease progression and high risk of death (Look *et al.*, 1997; Singhal and Austin, 2002). HIV/AIDS gradually destroys the immune system and oxidative stress may contribute to further damage of immune cells. Antioxidant nutrients such as selenium help protect cells from oxidative stress, thus potentially slowing progression of the disease (Romero-Alvira and Roche, 1998). It has been suggested that selenium status may be a significant predictor of survival for those infected with HIV (Baum and Shor-Posner, 1998).

2.9.7 Vitamin E

Vitamin E is a fat-soluble vitamin. Eight different naturally occurring substances have vitamin E activity in animals: α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols. The four tocopherol and tocotrienol isomers structurally consist of a chromatin head group and a phytyl side chain giving vitamin E compounds amphipathic character (Kamal-Eldin and Appelqvist, 1996). α-tocopherol is the most active form of vitamin E. It is a highly lipophilic molecule and is the chief antioxidant in biological membranes. Vitamin E is a chain-breaking antioxidant preventing the chain propagation step during lipid auto-oxidation (Serbinova and Packer, 1994). It reacts with alkoxy radicals (LO˙), lipid peroxyl radicals (LOO˙) and with alkyl radicals (L˙) derived from PUFA oxidation (Buettner, 1993; Kamal-Eldin and Appel-qvist, 1996). It has been reported that α-tocopherol-depleted LDL is able to undergo rapid lipid peroxidation, whereas LDL isolated from α-tocopherol-supplemented subjects exhibits increased

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resistance to *ex-vivo* copper-induced oxidation (Esterbauer *et al.*, 1987; Dieber-Rotheneder *et al.*, 1991).

The reaction between vitamin E and lipid radical occurs in the membrane-water interphase where vitamin E donates a hydrogen ion to lipid radical with consequent tocopheroxyl radical (TO˙) formation (Beuttner, 1993). Regeneration of the tocopheroxyl radical back to its reduced form can be achieved by ascorbate, GSH or co-enzyme Q. α-tocopherol tends to localize in membranes and lipoproteins and is quantitatively and qualitatively the major antioxidant in extracts prepared from LDL and central to the control of radical-induced peroxidation. In addition to scavenging peroxyl radicals α-tocopherol can also react with sunlight oxygen ($^1O_2$) and the 2e⁻ oxidants HOCl and ONOO⁻. LDL is a key carrier of vitamin E in the circulation and it is estimated that, for individuals who are receiving any supplement, the average LDL particle contains 7 molecules of α- and 0.5 molecules of γ-tocopherol. It has been sufficiently demonstrated that as the vitamin E content in LDL or endothelial cells is increased, there is an overall protection against LDL oxidation (Steinbrecher *et al.*, 1984; Dieber-Rotheneder *et al.*, 1991). Some studies have indicated that α-tocopherol can act as a pro-oxidant in LDL via α-tocopheroxyl radical-mediated formation of lipid radicals (Bowry and Stocker, 1993; Kamal-Eldin and Appelqvist, 1996; Neuzil *et al.*, 1997). Also, *in vitro* and *in vivo* enrichment of LDL with α-tocopherol accelerates rather than inhibits the initial stages of LDL oxidation (Bowry and Stocker, 1993). It has been clearly shown that prooxidant function of α-tocopherol on LDL was clearly inhibited in vitro by antioxidants such as ascorbate and ubiquinol (Stocker *et al.*, 1991; Suarna *et al.*, 1995; Upston *et al.*, 1999).

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It has also been suggested that it is the lack of availability of co-antioxidants rather than depletion of vitamin E that explains why lipids become increasingly oxidized as lesions develop (Terentis *et al.*, 2002). The fact that advanced human atherosclerotic lesions contain ascorbate suggests that vitamin C may be localized within cells, whereas lipoprotein lipid oxidation may occur outside cells (Terentis *et al.*, 2002). Increased adherence of monocytes to the endothelium constitutes one of the early visible changes in experimental atherosclerosis (Chan, 1998). Exposure of oxidized LDL to endothelial cells stimulates the expression of endothelium derived adhesion molecules: endothelium leukocytes adhesion molecule (ELAM), intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1). These proteins promote monocyte adhesion and subsequent migration into the intima where monocytes diffrentiatiate into macrophages (Holvoet and Collen, 1994).

Enrichment of human endothelial cells in culture with vitamin E causes a down-regulation in the expression of VCAM-1 and a functional change in the reduction of monocyte adhesion to endothelial cells, presumably due to decreased expression of adhesive molecules (Devaraj *et al.*, 1996; Cominacini *et al.*, 1997; Martin *et al.*, 1997). Results from animal models show that vitamin E supplementation significantly inhibited the accumulation of macrophages in the aortas. It was also effective in the reduction of atherosclerosis, expression of VCAM-1 and reduction of smooth muscle proliferation in rabbits (Sirikci *et al.*, 1996; Meydani, 2004). These observations in animal models support the concept that down-regulation of adhesion molecule expression, suppression of monocyte/macrophage activation, and inhibition of smooth muscle proliferation by vitamin E are some of the potential mechanisms by which vitamin E may suppress the

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development of atherosclerosis. Inhibition of smooth muscle cell proliferation by vitamin E could be directly scavenging ROS or by an inhibiting protein kinase C (PKC) activation and associated ROS production (Boscoboinik *et al.*, 1991, Keaney *et al.*, 1996).

Animal studies have provided constituent evidence for a beneficial effect of α-tocopherol on vasodilation as well as insight into underlying mechanisms. Supplementation of cholesterol-fed rabbits with α-tocopherol increased both the resistance of LDL to oxidation and agonist-induced relaxation of thoracic aortas (Keaney *et al.*, 1993). It has been proposed that α-tocopherol acts in the vascular wall by inhibiting PKC-mediated Phosporylation of endothelial cell muscarinic receptors and enhancing agonist-induced nitric oxide synthase (NOS) activation (Keaney *et al.*, 1996).

The vitamin E status of an organism is determined by factors other than the level of vitamin intake and it has been suggested that a high degree of interaction exists among antioxidant nutrients (Chan, 1993). For example, vitamin C reinforces the antioxidant effect of vitamin E by regenerating the active form of the vitamin after it has reacted with a free radical.

In intact animals, a sparing effect of vitamin C on vitamin E status has been noted in the guinea pigs and fish (Bedich *et al.*, 1984; Hamre *et al.*, 1997). In human platelet homogenate, oxidized vitamin E was shown to be regenerated by vitamin C or reduced glutathione (Chan *et al.*, 1991). Recycling of oxidized tocopherol has also been shown to be afforded by lipoate and ubiquinol (Podda *et al.*, 1994; Stoyanovsky *et al.*, 1995; Packer *et al.*, 1997). The regeneration of vitamin E by other antioxidants is one part of

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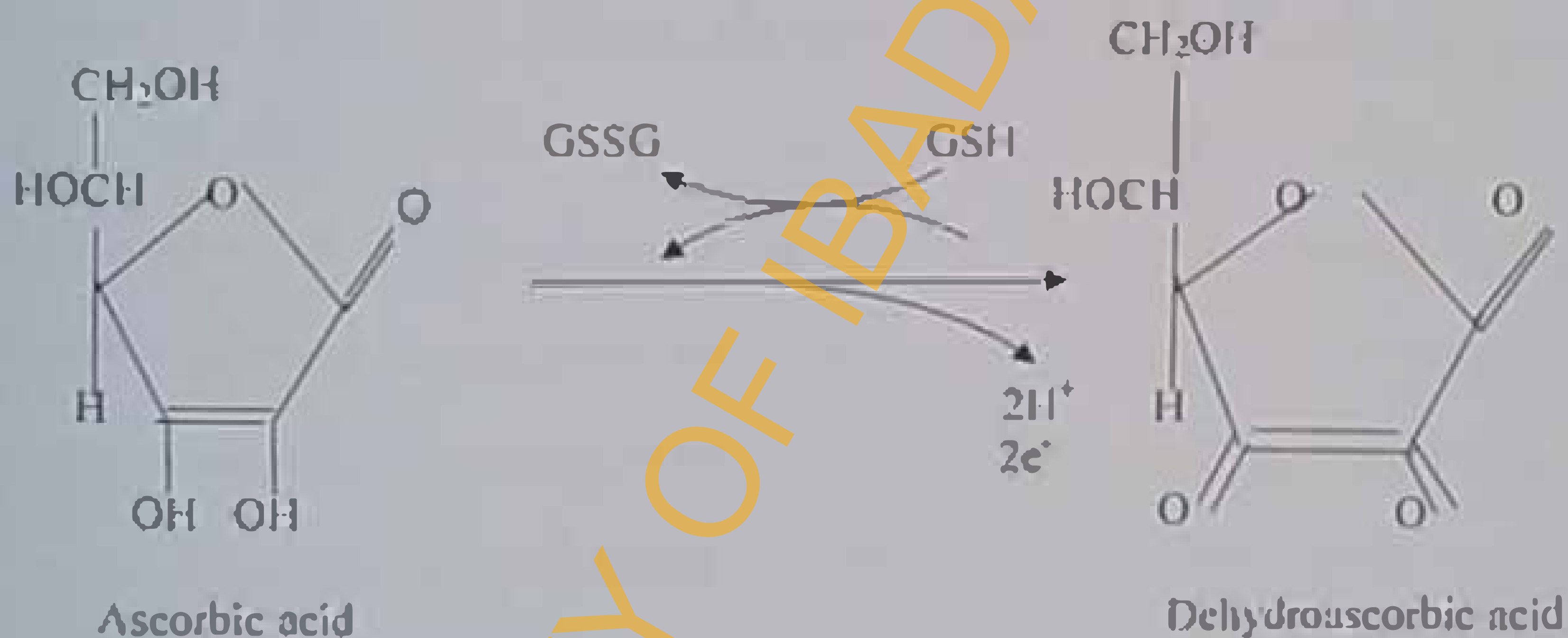
the intricate co-operation that exists between different antioxidants in the antioxidant defence system.

2.9.8 Vitamin C (Ascorbic acid)

Vitamin C is a water-soluble vitamin that exists in the body primarily in its reduced form, ascorbic acid. The oxidized form of the vitamin, dehydroascorbic acid (DHA) is easily reduced intracellularly to ascorbic acid. Ascorbic acid recycling process is as shown below:



Vitamin C is an electron donor (reducing agent) and probably all of its biochemical and molecular functions can be accounted for by this function. The ability of the vitamin to provide electrons and be readily converted back to its reduced form by GSH accounts for its particular effectiveness as an *in vivo* antioxidant (Jacob and Sotoudeh, 2002). The concentration of vitamin C in body tissues and fluids varies greatly, with high levels maintained in leukocytes, eye, adrenals, pituitary and brain, whereas low levels are found in plasma and saliva (Jacob and Sotoudeh, 2002). Vitamin C readily undergoes reversible oxidation and reduction and plays an important role as a redox agent in biological

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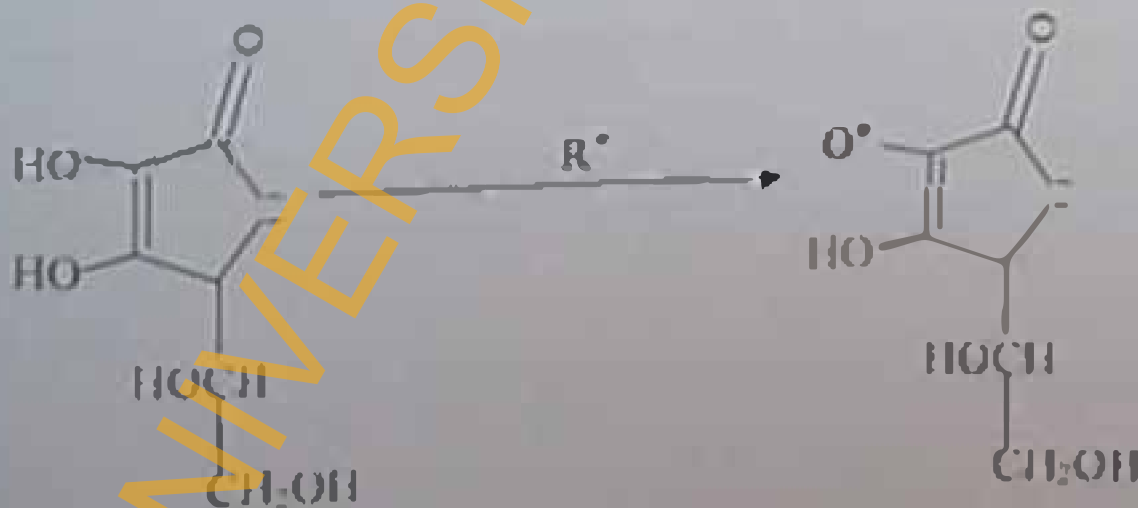
systems (Kuroyanagi *et al*., 2002). Its best understood function is in the synthesis of collagen which promotes the formation of hydroxyproline (Peterkofsky, 1991).

Unhydroxylated collagen is unstable and cannot form the triple helix required for normal structure of subcutaneous tissue, cartilage, bone and teeth. The failure of cells to deposit collagen fibrils and intracellular cement substance leads to delayed wound healing (Bsoul and Terezhalmy, 2004). Vitamin C is specifically required for the activity of eight human enzymes involved in collagen, hormone, amino acid and carnitine synthesis or metabolism (England and Seatter, 1986). As a co-factor for propyl and lysyl hydroxylases, ascorbate is an essential part of the molecular cross-linking that gives collagen its elasticity. Ascorbic acid is also involved in the synthesis or modulation of some components of the nervous system, the microsomal drug-metabolizing system, synthesis of corticosteroids and conversion of cholesterol to bile acids (Katsuki, 1996).

Ascorbic acid reacts with free radicals that arise in the aqueous compartments of tissues forming the innocuous ascorbate semiquinone (Koyama *et al*., 1994; Roginsky and Stegmann, 1994).



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The semiquinone is consumed in a dismutation reaction in which two semiquinone molecules react to produce a molecule of ascorbate and a molecule of dehydroascorbate

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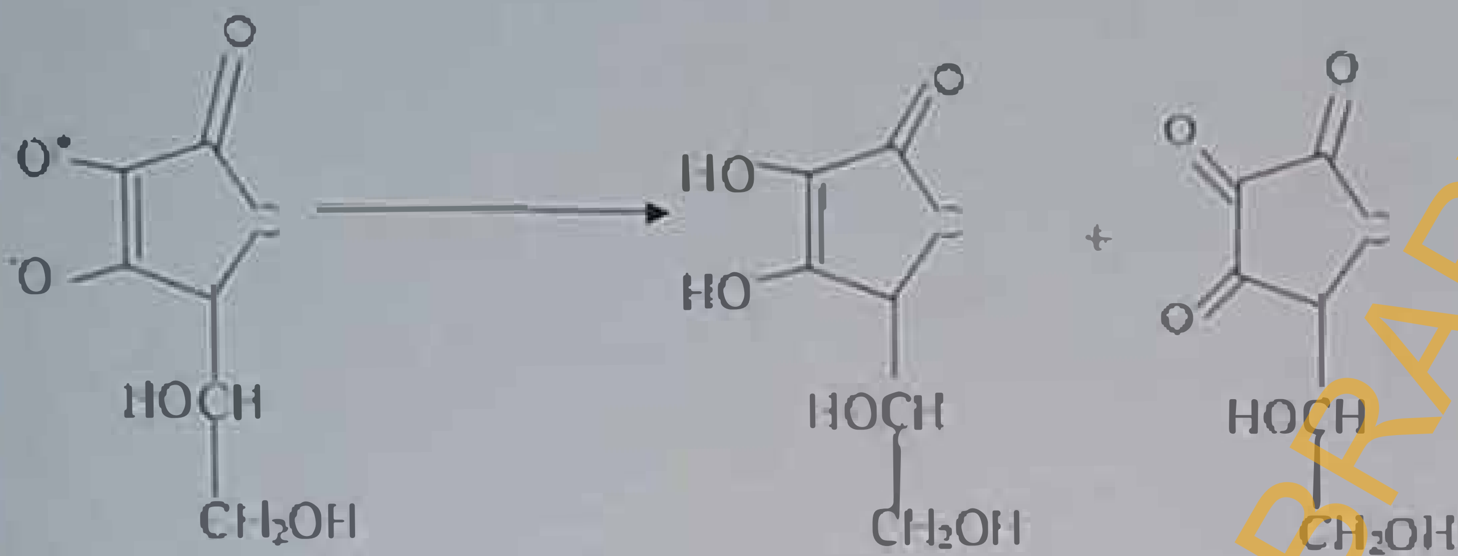

The dehydroascorbate is then enzymatically reduced back to ascorbate by dehydroascorbate reductase. Ascorbic acid is a powerful antioxidant because it can donate a hydrogen atom and form a relatively stable ascorbyl free radical. As a scavenger of ROS, ascorbate has been shown to be effective against $O_2^{\cdot-}$, H_2O_2, OH^- and $^1O_2$ (Weber *et al.*, 1996). The ascorbyl free radical can be converted back to reduced ascorbate by accepting another hydrogen atom or it can undergo further oxidation to dehydroascorbate. Dehydroascorbate is unstable but is more fat soluble than ascorbate and is taken up 10 – 20 times more rapidly by erythrocytes, where it will be reduced back to ascorbate by GSH or NADPH from the hexose monophosphate shunt (Hornig, 1975). Dehydroascorbic acid is reported to be undetectable in plasma (Levine *et al.*, 1993). This suggests that the fate of oxidized ascorbic acid is either immediate redox recycling to the reduced form, or immediate further oxidation with consequent destruction of the vitamin.

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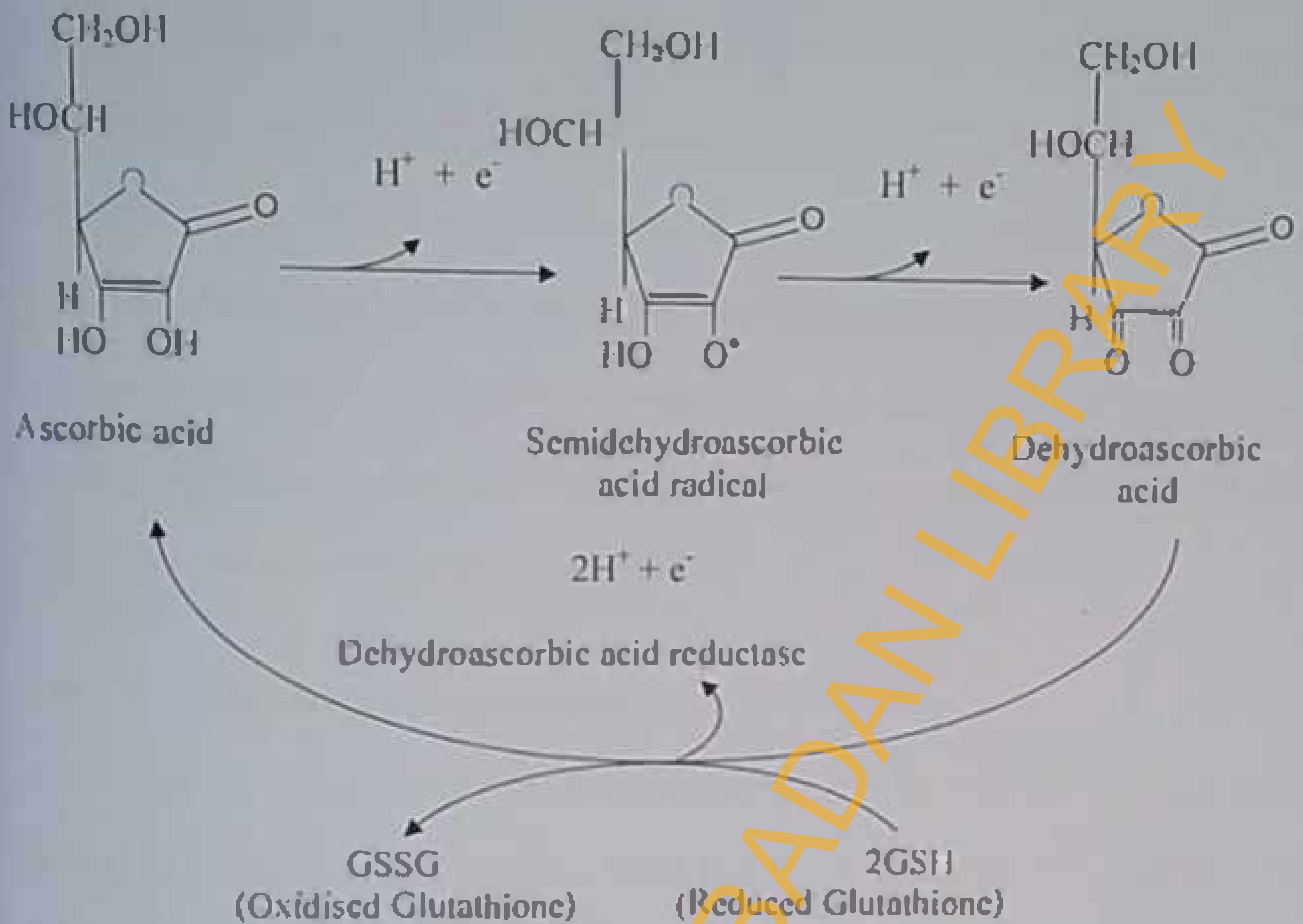

The existence of a mechanism to maintain plasma ascorbate in the reduced state means that the level of vitamin C necessary for optimal activity is not absolute because the turnover will change in response to oxidant pressure. Recycling of vitamin C will depend on the reducing environment which exists in metabolically active cells.

It has been suggested that vitamin C can protect circulating and membrane lipids from free radicals. Vitamin C is also believed to protect lipids indirectly by sparing or reconstituting the active forms of vitamin E (Tappel, 1962). Atherosclerotic plaques impair endothelium dependent vasodilation in human coronary and peripheral blood vessels and acute administration of vitamin C may reverse this endothelial dysfunction (Ting *et al*., 1997; Hamabe *et al*., 2001). There is evidence linking high intake of vitamin C with reduced mortality from heart disease (Enstrom *et al*., 1992; Sahyoun *et al*., 1996).

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In vivo, vitamins C and E have been shown to reduce oxidative stress in HIV infected patients and to reduce the viral load (Allard *et al*., 1998).

Antioxidant action of vitamin C inhibits the formation of carcinogenic N-nitroso compounds that are implicated in gastric and lung cancer (Carr and Frei, 1999).

The possible anticarcinogenic effect of vitamin C appears to be related to its ability to detoxify carcinogens or block carcinogenic processes through its action as an antioxidant or as a free radical scavenger (Rock *et al*., 1996).

2.10 AVOCADO (*Persea americana*)

Plants were the major source of materials which the ancient man resorted to for combating various ailments and thus preserving his health (Akah and Ekekwe, 1995; Calixto, 2000).

The use of alternative medicine and the consumption of plant materials have been on the increase in many countries in the world, mostly because plant-derived drugs and herbal formulations are commonly considered to be less toxic and freer from side effects than synthetic ones (Bailey and Day, 1989; Mitra *et al*., 1996; Bhattacharya *et al*., 1997; Annapurna *et al*., 2001). It is estimated that about 65-80% of the world's population which lives in developing countries depends essentially on plants for primary health care because of poverty and lack of access to modern medicine (Akerele, 1993).

At present, a number of botanicals are still being used in folk-medicine for treatment of different diseases. It is now known that a series of phytochemicals innate in food systems or that can be incorporated into food-delivery systems or dietary supplements hold considerable promise in combating disease.

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A large number of herbal drugs which have been evaluated in clinical trials are currently being used in herbal medicine. They include the extract of *Ginkgo biloba* for the treatment of CNS and cardiovascular disorders (Brautigam *et al.*, 1998), *Hypericum perforatum* (St. John's wort) used as an antidepressant (Viticllo, 1999), *Panax ginseng* (ginseng) herbs used as a tonic (Tylor, 1994), *Tanacetum parthenium* (feverfew) used to treat migraine headache, *Allium sativum* (garlic) used to lower low-density lipoprotein cholesterol (Aouadi *et al.*, 2000), *Silybium marianum* (milk thistle) used for repairing liver function including cirrhosis, *Valeriana officinalis* (valerian) used as a sedative and sleeping aid (Wagner *et al.*, 1998), *Cassia acutifolia* (Senna) and *Rhamnus purshiana* (cascara sagrada) which are used as laxatives (Calixto, 2000), *Echinacea purpura* (Echinacea) used as an anti-inflammatory and immunostimulant (Calixto, 2000), *Arnica montana* (arnica) used to treat post-traumatic and postoperative conditions (Karow *et al.*, 2008), and *Serenoa repens* (saw palmetto) used for the treatment of benign prostatic hyperplasia (Gerber and Fitzpatrick, 2004).

Avocado (*Persea americana*) is an important edible fruit belonging to the Laurel family, Lauraceae. The avocado tree may be erect, usually up to 9 m but sometimes up to 18 m or more with a trunk 30 – 60 cm in diameter or it may be short and spreading with branches beginning close to the ground (Morton, 1987). Almost evergreen, being shed briefly in dry seasons at blooming time, the leaves are alternate, dark green and glossy on the upper surface, whitish on the underside; variable in shape (lanceolate, elliptic, oval, ovate or obovate) 7.5 – 40 cm long. The fruit (commonly known as avocado pear) is pear-shaped,

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oval or nearly round and may be 7.5 – 33 cm long and up to 15 cm wide. The skin may be yellow-green, deep-green or very dark-green (Morton, 1987).

Persea americana is indigenous to Central and South America. Although concentrated in the Latin American countries, the tree is now cultivated in the United States, Asia, parts of Europe and tropical Africa. It grows well in soils that are loose, well-drained, slightly acid and rich in organic matter (Tokura *et al*., 1996). The tree grows at elevation from sea level to 2400 m with average temperatures of 16 to 24°C.

Avocado is one of the plants that have been widely used in ethnomedicine. The bark, fruit and leaf are used in traditional medicine in South America, West Indies and Africa to provide remedy for ailments such as hypertension, haemorrhage and menstrual disorder (Morton, 1987). The fruit skin is antibiotic and is employed as a vermifuge and remedy for dysentery. In Nigeria, the leaf has various local names such as Ewé pia (Yoruba), Akwukwo Ube oyibo (Igbo) and Ganyen piya (Hausa). The leaves are chewed as a remedy for pyorrhea and the aqueous extract of the leaves has a prolonged antihypertensive effect. The leaf decoction is taken as a remedy for diarrhoea, sore throat and haemorrhage and it allegedly stimulates and regulates menstruation (Morton, 1987). The aqueous leaf extracts from *P. americana* have been shown to have antiviral activity against *Herpes simplex* 1 virus (De Almeida *et al*., 1998); human immunodeficiency virus (HIV) 1 (Wigg *et al*., 1996) and adenovirus (De Almeida *et al*., 1998). It has anti-inflammatory activity (Guevarra *et al*., 1998; Adeyemi *et al*., 2002) and antihypertensive/ hypotensive activity (De A Ribeiro *et al*., 1986; Girow *et al*., 1991; Adeboye *et al*., 1999). Recently, the aqueous leaf extract of *P. americana* was reported to possess

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hypoglycemic activity (Antia *et al.*, 2005), vasorelaxant action (Owolabi *et al.*, 2005), and anticonvulsant effect (Ojewole and Amabeoku, 2006).

There are no documented reports on the hypolipidaemic, antioxidative and hepatoprotective effects of *P. americana*. This study investigates the effects of *P. americana* on lipids, indices of oxidative stress and antioxidant status in diet-induced hyperlipidaemia. In addition, the protective effects of *P. americana* on CCl_4-induced liver damage were investigated.

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials and Equipment

Glasswares

Cotton wool

Whatman filter paper

Syringes & needles

Surgical gloves

Potassium oxalate bottles

Lithium heparin bottles

Ethylenediaminetetraacetic acid (EDTA) bottles

Paper tape

Parafilm

Screw-cap tubes

Eppendorf tubes

Permanent markers

Petri dishes (disposable)

Micropippettes

Mortar & pestle

Waring blender

Dissecting kit

Thermometer

Refrigerator

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Freezers (- 20°C, - 80°C)

Hot air oven

Weighing balance

Vortex mixer

Incubator

Water bath

Sohxlet Extractor

pH meter

Biofuge (refrigerated)

UV spectrophotometer

Groundnut oil	Grand, Nigeria
Olive oil	Goya En Espana, Spain

3.2 Reagents

5, 5' - Dithio-bis 2-nitrobenzoic acid (DTNB)	Sigma-Aldrich, Germany
1- Chloro-2, 4-dinitrobenzene (CDNB)	Sigma-Aldrich, Germany
Trichloroacetic acid	BDH, England
Reducdyn®	Knoll AG, Germany
2-Thiobarbituric acid (TBA)	Sigma-Aldrich, Germany
Potassium chloride	Merck, Germany
Ethylenediamine tetraacetic acid (EDTA)	Sigma-Aldrich, Germany
Tris [hydroxymethyl] aminomethane hydrochloride	Sigma-Aldrich, Germany
Sodium hydroxide	BDH, England

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Sucrose	BDH, England
Sodium chloride	Avondale, England
Sodium carbonate	Fisher Scientific, U. S. A.
2-Dinitrophenylhydrazine	BDH, England
Potassium dihydrogen orthophosphate	Merck, Germany
Di-sodium hydrogen orthophosphate	Merck, Germany
Cholesterol	BDH, England
Cholic acid	BDH, England
Glutathione (reduced)	Roche, Germany
Methanol (Analar)	BDH, England
Hydrochloric acid	BDH, England
Coomassie Brilliant Blue	Merck, Germany
Phosphoric acid	M & B, Germany
Cyclohexane	Fisons Scientific, England
Carbon tetrachloride	BDH, England
Ethyl acetate	BDH, England
95% Ethanol	Fisher Scientific, U. K
Sodium citrate	M & B, Germany
Hydrogen peroxide	Sigma-Aldrich, Germany
Triton X-100	BDH, England
Epinephrine	BDH, England
Sodium dihydrogen phosphate	Sigma-Aldrich, Germany
Potassium cyanide	BDH, England

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Di-potassium phosphate	Merck, Germany
Glacial metaphosphoric acid	Sigma-Aldrich, Germany
Sodium hydrogen carbonate	Sigma-Aldrich, Germany
Chloroform	BDH, England
Glutathione peroxidase assay kit (RANSEL)	Randox, U. K.
Bilirubin assay kit	Human, Germany
Cholesterol assay kit	Randox, U. K.
Glucose assay kit	Randox, U. K.
Triglyceride assay kit	Randox, U. K.
Alkaline phosphatase assay kit	TECO, U. S. A.
Aspartate aminotransferase assay kit	Randok, U. K.
Alanine aminotransferase assay kit	Randox, U. K.

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3.3 Materials

3.3.1 Animals

Four-week old male albino rats were obtained from the animal colony of the Nigerian Institute of Medical Research, Yaba. Rats were housed in cages and allowed to acclimatize for one week with a 12-h light: dark cycle and had free access to water and standard rat chow purchased from Ladokun Feeds, Ibadan.

3.3.2 Plant Materials

Fresh leaves of *P. americana* were obtained from a cultivated plant in Lagos and were sent for authentication at the Department of Botany & Microbiology, University of Ibadan. The leaves were air-dried and stored until needed. Dried leaves were pulverized in a Waring blender and the aqueous and methanolic extracts prepared by means of Soxhlet extraction. The extracts were evaporated to dryness and stored in clean sterile vials until required.

3.3.3 High Lipid (Modified) Diet Composition

The high lipid diet was prepared following a modified method of Yuan and Kitts (2003).

The composition of the diet is as follows:

Standard rat chow	79.25 %
Groundnut oil	20 %
Cholesterol	0.5 %
Cholic acid	0.25 %

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3.4 Methods

3.4.1 Phytochemical Screening

Qualitative analysis of the extracts was carried out using standard procedures as described by Harborne (1973), Trease and Evans (1989) and Sofowora (1993).

3.4.1.1 Test for alkaloids

0.5 g of extract was diluted to 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia and 5 ml of chloroform and then shaken gently to extract the alkoloidol base. The chloroform layer was extracted with 10 ml of acetic acid and divided into two portions. Mayer's reagent was added to one portion and Draggendorf's reagent to the other. The formation of a cream and a reddish brown precipitate with Mayer's and Draggendorf's reagents respectively indicate the presence of alkaloids.

3.4.1.2 Test for tannins

0.5 g of extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or blue-black colouration

3.4.1.3 Test for saponins

0.5 g of the extract was dissolved in 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a persistent froth. The frothing was mixed with 3

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drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

3.4.1.4 Test for flavonoids

Three methods were used to test for the presence of flavonoids in the extract.

5 ml of dilute ammonia was added to a portion of an aqueous filtrate of the extract followed by addition of 1 ml concentrated H_2SO_4. A yellow colouration that disappears on standing indicates the presence of flavonoids.

A few drops of 1% aluminium solution were added to a portion of the filtrate. A yellow colouration indicates the presence of flavonoids.

A portion of the extract was heated with 10 ml of ethyl acetate over a steam water bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml dilute ammonia solution. A yellow colouration indicates the presence of flavonoids.

3.4.1.5 Test for terpenoids (Salkowski test)

0.5 g of extract was mixed in 2 ml of chloroform. 3 ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

3.4.2 Feeding of rats with cholesterol-enriched diet to induce hyperlipidaemia

24 male albino rats were divided into four feeding groups (A, B, C and D) of six rats in each. Hyperlipidaemia was induced by feeding the rats with the high lipid diet according to the following regimen:

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Group A (normal control): standard rat chow + water only.

Group B (negative control): high lipid diet + water.

Group C: high lipid diet + 10 mg kg^{-1} b.wt aqueous extract of *P. americana* (AEPA).

Group D: high lipid diet + 10 mg kg^{-1} b.wt methanolic extract of *P. americana* (MEPA).

The animals were observed daily and weighed weekly for eight weeks. At the end of the 8 weeks feeding period, rats were anaesthetized with sodium pentobarbital, 100 mg kg^{-1} b.wt (Wang *et al.*, 2004). Blood was withdrawn via cardiac puncture when animals were rendered unconscious under pentobarbital anaesthesia. The blood was collected in heparinised tubes followed by centrifugation at 3,000 rpm for 5 minutes at 4°C to separate the plasma. The plasma was stored in clean tubes at -20°C pending analysis. 0.5ml aliquot of the whole blood was also collected in heparinised tubes for GSHPx assay.

After sacrificing the rats, the livers, hearts, brains, kidneys and lungs were quickly excised and perfused with chilled 1.15 % (w/v) KCl solution in order to remove all traces of haemoglobin. The tissues were blotted dry, weighed and stored at -80°C pending analysis. Some portions of the livers were preserved in 10 % Formol saline for histopathological analysis.

3.4.3 Treatment of rats with CCl_4 to induce hepatotoxicity

To evaluate the hepatoprotective action of *P. americana*, thirty (30) albino rats were randomly divided into five treatment groups of six (6) rats each.

Group I (normal control): given distilled water orally for 7 days

Group II (CCl_4-treated control): given distilled water for 7 days.

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Group III: pre-treated with the standard drug Reducdyn at a dose of 100mg/kg/day orally for 7 days.

Group IV: pre-treated with AEPA at a dose of 100mg kg/day orally for 7 days.

Group V: pre-treated with AEPA at a dose of 200mg/kg/day orally for 7 days.

On the seventh day, animals in groups II – V were injected with a fresh mixture of equal volumes of CCl_4 and olive oil (3ml/kg, sc) half an hour after the administration of the last dose of the pre-treatment drug/extract. Rats in group I were injected with olive oil (3ml/kg, sc). All animals were starved overnight and sacrificed by cervical dislocation. Blood samples were collected by cardiac puncture into plain sterile tubes and allowed to coagulate. The serum was separated by centrifugation at 3,000 rpm for 10 min at 4°C. A portion of the blood was placed in heparinized tubes for determination of some haematological parameters.

After sacrificing the rats the livers were quickly excised and perfused with chilled 1.15 % (w/v) KCl solution in order to remove all traces of haemoglobin. The livers were blotted dry, weighed and stored at -80°C pending analysis. Some portions of the livers were preserved in 10 % Formol saline for histopathological analysis.

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3.4.4 Extraction of Liver Lipids

Liver lipids were extracted according to the method of Folch *et al.* (1957).

Principle

The extraction is based on the solubility of lipids in organic solvents and the immiscibility of polar and non-polar solvents.

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Procedure

1 g of liver was homogenized with 17 ml of 2:1 chloroform-methanol mixture (v/v) for a few minutes, and then diluted to a final volume of 20 ml. The homogenate was filtered through a fat-free filter paper into a glass centrifuge tube. 10 ml of the crude extract was mixed thoroughly with 2 ml of water and the mixture was allowed to separate into 2 phases without interfacial fluff by standing it for few hours. As much of the upper phase as possible was removed by siphoning and removal of its solutes was completed by rinsing the interface three times with small amounts of pure solvents upper phase in such a way as not to disturb the lower phase. The extract was stored at 4°C.

3.4.5 Preparation of Tissue Homogenate

A 10 % (w/v) homogenate was prepared from liver, kidney, heart and lung according to the method of Yuan and Kitts (2003). Briefly, 1 g of tissue was homogenized in 10 ml of ice-cold homogenizing buffer (8 mM Na_2HPO_4, 12 mM NaH_2PO_4, 1.15 % KCl, pH 7.4) and centrifuged at 12,000 rpm for 20 min at 4°C. Portions of the homogenate were used for measuring the levels of oxidation products (malondialdehyde, conjugated dienes and protein carbonyls). Another portion was immediately stored at -80°C for analysis of SOD, CAT and GSHPx.

To prepare the brain homogenate, 1g of tissue was subjected to homogenization in 10-fold volume of ice-cold 0.25M sucrose solution. The homogenate was centrifuged at 7,000 rpm for 10 min at 0°C.

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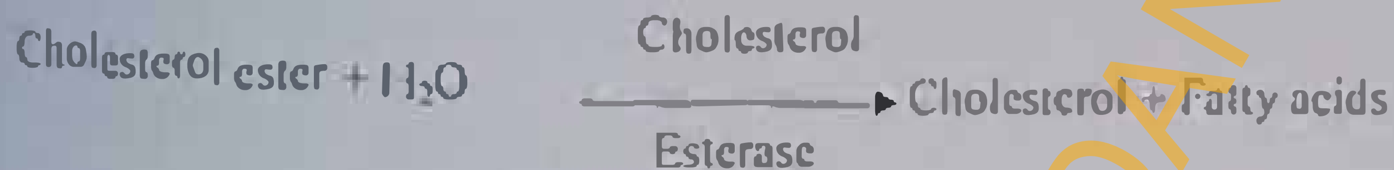
3.4.6 Determination of total cholesterol

Total cholesterol (T-CHOL) was determined enzymatically using the method of Trinder (1969).

Principle

Cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.



The reagent used contained 80mM pipes buffer, pH 6.8, 0.3mM 4-aminoantipyrine, 6mM phenol, 0.5U peroxidase, 0.15U cholesterol esterase and 0.1U cholesterol oxidase. The sample cuvette contained 0.01 ml of test sample to which was added 1 ml of reagent while the blank had only 1 ml of reagent. Similarly, 1 ml of reagent was added to 0.01 ml of standard cholesterol (200 mg/dl) in another cuvette.

The contents of the cuvettes were mixed thoroughly and allowed to incubate for 10 minutes at room temperature. The absorbance of the sample was measured against the reagent blank at 500 nm with Spectronic® Helios Gamma & Delta spectrophotometer.

Calculation

Using a standard,

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$$\text{Concentration of Cholesterol in sample} = \frac{A_{sample}}{A_{standard}} \times \text{Conc. of standard}$$

where,

A_{sample} = absorbance of sample

$A_{standard}$ = absorbance of standard

3.4.7 Determination of high density lipoprotein cholesterol

High density lipoprotein cholesterol (HDL-CHOL) was determined according to the method of Lopes-Virella *et al*. (1977).

Principle

Low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in HDL fraction which remains in the supernatant is determined.

Procedure

The reagent used is made up of 0.55 mM phosphotungstic acid and 25 mM magnesium chloride. 0.5 ml of diluted precipitant was added to 0.2 ml of sample in a tube. After mixing thoroughly the tube was allowed to stand for 10 minutes at room temperature followed by centrifugation for 2 minutes at 12,000 rpm. The resulting clear supernatant was separated and used for determination of cholesterol content as outlined in section 3.4.6.

Calculation

$$\text{Concentration of HDL-CHOL} = \frac{A_{sample}}{A_{standard}} \times \text{Conc. of standard}$$

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where,

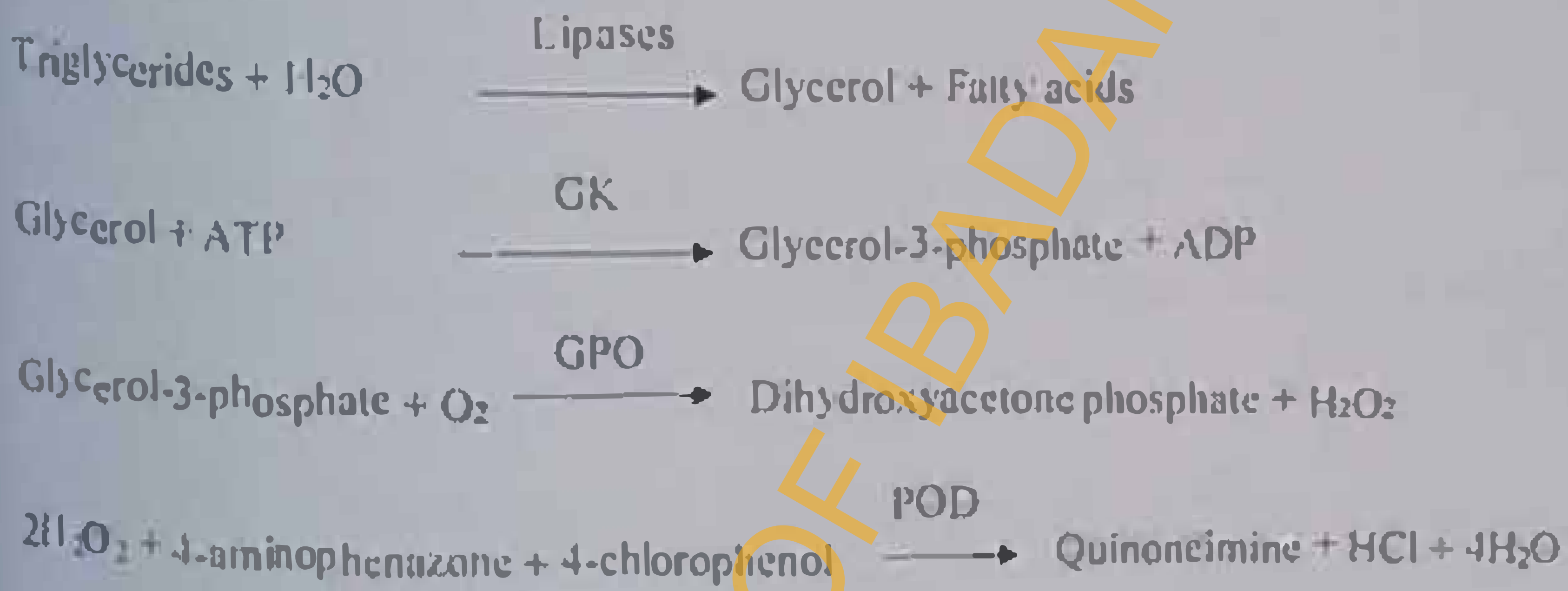
A_{sample} = absorbance of sample

$A_{standard}$ = absorbance of standard

3.4.8 Determination of triglycerides

Triglycerides were determined according to the colorimetric method of Tietz (1990). This method involves the measurement of triglycerides after enzymatic hydrolysis with lipases.

Principle



$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipases}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + HCl + 4H_2O$$

The indicator is quinoneimine formed from H_2O_2, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

Procedure

The RANDOX kit procedure was used. The reagent used contained 40 mM PIPES buffer, pH 7.6, 5.5 mM 4-chlorophenol, 17.5 mM magnesium ions, 0.5 mM 4-aminophenazone, 1 mM ATP, 150 U lipases, 0.4 U glycerol kinase, 1.5 U glycerol-3-phosphate oxidase and 0.5 U peroxidase. The sample cuvette contained 0.01 ml of test sample, to which was added 1 ml of reagent. Another cuvette contained 0.01 ml of the standard triglyceride solution and 1 ml of the reagent. The blank had only 1 ml of the reagent. Thorough mixing was done and the reaction mixture was incubated for 10 minutes at room

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temperature. The absorbances of the sample and standard were measured against the reagent blank at 500 nm with Spectronic® Helios Gamma & Delta spectrophotometer.

Calculation

$$\text{Triglyceride concentration} = \frac{A_{sample}}{A_{standard}} \times \text{Conc. of standard}$$

where,

A_{sample} = absorbance of sample

$A_{standard}$ = absorbance of standard

3.4.9 Determination of Low density lipoprotein cholesterol

LDL-CHOL concentration was estimated according to the method of Friedewald *et al.* (1972) using the formula:

$$\text{LDL-CHOL} = \text{Total Cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL-CHOL}$$

3.4.10 Determination of glucose

Glucose was determined according to the method of Barham and Trinder (1972) using commercial kit manufactured by RANDOX Laboratories Ltd., Crumlin, United Kingdom. The RANDOX reagent is composed of: Buffer (100 mmol/L phosphate buffer, pH 7.0; phenol 11 mmol/L); enzyme reagent (0.77 mmol/L 4-aminophenazone; 1500 U/L glucose oxidase; 1500 U/L peroxidase); and glucose standard (100 mg/L)

Principle

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase with phenol and 4-

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aminophenazone to form a red violet quinoneimine which can be colorimetrically determined.

$$Glucose + O_2 + H_2O \xrightarrow{GOD} Gluconic\ acid + H_2O_2$$

$$2H_2O_2 + 4\text{-aminophenazone} + phenol \xrightarrow{POD} quinoneimine + 4H_2O$$

Procedure

1 ml of the working reagent was added to 0.01 ml of the plasma and 0.01 ml of glucose standard. 1 ml of the working reagent was taken in another tube to serve as blank. The contents of the tubes were mixed thoroughly and then incubated for 25 minutes at room temperature. The absorbances of the standard and the plasma sample were read against reagent blank at 500nm with a Spectronic* Helios Gamma & Delta spectrophotometer.

Plasma glucose was calculated thus:

$$\frac{A_{sample}}{A_{standard}} \times \frac{\text{Concentration of Standard}}{1}$$

where,

A_{sample} = absorbance of sample
$A_{standard}$ = absorbance of standard

3.4.11 Determination of total protein

Total protein was determined by the Bradford assay (Braford, 1976).

Principle

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding

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to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

Procedure

100 µl of sample was taken in a clean test tube. To this was added 5 ml of Bradford reagent and incubated at room temperature for 5 mins. The absorbance was measured at 595 nm with a Spectronic® Helios Gamma & Delta spectrophotometer. A standard protein solution was similarly treated.

Calculation

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

3.4.12 Determination of aspartate aminotransferase

Aspartate aminotransferase (AST) was determined by the method of Reitman and Frankel (1957) using commercial kits manufactured by RANDOX Laboratories Ltd., Crumlin, United Kingdom. The kit contained two reagents:

Reagent 1:- 100 mM Phosphate buffer, pH 7.4, 100 mM L-aspartate, 2 mM α–oxoglutarate

Reagent 2:- 2 mM 2, 4-dinitrophenylhydrazine.

Principle

The oxaloacetate that is formed is reacted with 2, 4-dinitrophenylhydrazine. The resulting hydrazone of oxaloacetate is highly coloured. The AST is measured by monitoring the concentration of oxaloacetate hydrazone formed.

Procedure

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0.1 ml of sample was added to 0.5 ml of reagent 1 (100 mM phosphate buffer, pH 7.4, 100 mM L-aspartate, 2 mM α-oxoglutarate), mixed and allowed to incubate for 30 minutes at 37°C. 0.5ml of 2, 4-dinitrophenylhydrazine was added, mixed and allowed to stand for 20 minutes at room temperature. 5 ml of 0.4 M NaOH was added and after mixing thoroughly, the absorbance of sample was read against reagent blank after 5 minutes at 546 nm in a Spectronic* Helios Gamma & Delta spectrophotometer. The reagent blank was made up of 0.5 ml of reagent 1, 0.1 ml distilled water and 5 ml NaOH.

Calculation

The activity of AST was obtained from the table provided in the instruction manual for the kit.

3.4.13 Determination of alanine aminotransferase

The method of Reitman and Frankel (1957) for the determination of alanine aminotransferase (ALT) was adopted.

Commercial kit from RANDOX Laboratories Ltd., Crumlin, United Kingdom was used. The kit contained two solutions:

1. Buffer - 100 mM phosphate buffer, pH 7.4, 200 mM L-alanine, 2 mM α-oxoglutarate
2. 2 mM 2, 4-dinitrophenylhydrazine

Principle

$$\alpha\text{-oxoglutarate} + \text{L-alanine} \xrightarrow{ALT} \text{L-glutamate} + \text{pyruvate}$$

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The pyruvate formed is reacted with 2, 4-dinitrophenylhydrazine. The resulting pyruvate hydrazone is highly coloured and its absorbance at 530 – 550 nm is proportional to the concentration of ALT.

Procedure

0.1 ml of sample was added to 0.5 ml of solution 1, mixed and allowed to stand for 30 minutes at 37°C. Then 0.5ml of 2, 4-dinitrophenylhydrazine solution was added, mixed thoroughly and incubated at room temperature for 20minutes. 5ml of 0.4M NaOH was added and the absorbance of the sample was measured after 5 minutes against the reagent blank (0.5 ml of solution 1, 0.1 ml distilled H_2O and 0.5 ml NaOH), at 546 nm in Spectronic® Helios Gamma & Delta spectrophotometer.

Calculation

The activity of ALT in the sample was obtained from the table provided in the kit manual.

3.4.14 Determination of alkaline phosphatase

Alkaline phosphatase (ALP) was determined using commercial kit manufactured by TECO Diagnostics, Anaheim, U. S. A. The kit contained:

1. Alkaline phosphatase substrate: 3.6 mM Sodium thymolpthalein monophosphate in 0.2 M 2-Amino-2-methyl-1-propanol buffer; 1.0 mM Magnesium chloride; pH 10.2
2. Alkaline phosphatase colour developer: 0.1 M Sodium hydroxide, 0.1 M Sodium carbonate
3. Alkaline phosphatase standard: 0.5 mM/L Thymolphthalein in n-Propanol

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Reaction Principle

The alkaline phosphatase acts upon the AMP-buffered sodium thymolphthalein monophosphate. The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen, which is measured photometrically.

Procedure

0.5 ml of alkaline phosphatase substrate was dispensed into clean dry test tubes and equilibrated to 37°C for 3 min.

At timed intervals, 0.5 ml of standard, control, and sample was added to its respective test tube followed by gentle mixing. 0.5 ml of deionized water was placed in another test tube for reagent blank. The contents of the tubes were then allowed to incubate for 10 min at 37°C.

At the end of the incubation, 2.5 ml of alkaline phosphatase colour developer was added with thorough mixing. The absorbance of the colour developed was read at 590 nm using Spectronic® Helios Gamma & Delta spectrophotometer.

Alkaline phosphatase activity was calculated as follows:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

3.4.15 Determination of total bilirubin

Total bilirubin (TBL) was determined using commercial kits prepared by Human, Wiesbaden, Germany. The test kit contained:

1. Total bilirubin reagent (TBR): 14 mM Sulphanilic acid, 300 mM Hydrochloric acid, 200 mM Caffeine, 420 mM Sodium benzoate
2. T-Nitrite reagent (TNR): 390 mM Sodium nitrite.

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Principle

Bilirubin reacts with diazotized sulphanilic acid to form a red azo dye. The absorbance of this dye at 546 nm is directly proportional to the bilirubin concentration in the sample.

Procedure

40 µl of TNR was added to 1000 µl of TBR, mixed thoroughly and incubated for 5 min at room temperature. 100 µl of sample was added to the mixture and allowed to stand for 20 minutes at room temperature. A sample blank was prepared but without TNR. Absorbance of sample was measured against sample blank at 546 nm with Spectronic® Helios Gamma & Delta spectrophotometer.

Concentration of total bilirubin was calculated as follows:

Calculation

Bilirubin concentration (mg/dl) = $A_{546} \times 13.0$

(mg/dl) x 17.1 = (µmol/L)

3.4.16 Determination of malondialdehyde

Malondialdehyde was determined by the method of Buege and Aust (1978) for thiobarbituric acid reactive substances (TBARS). Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of the peroxidation reaction. The thiobarbituric acid assay is the most frequently used method for determining the extent of lipid peroxidation *in-vitro*. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red species absorbing at 535nm.

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Reagent

The reagent stock is TCA-TBA-HCl (15 % (w/v) trichloroacetic acid, 0.375 % (w/v) thiobarbituric acid, 0.25 N hydrochloric acid.

Procedure

1 ml of biological sample (plasma or tissue homogenate) was combined with 2 ml of TCA-TBA-HCl reagent and mixed thoroughly. The solution was heated for 15 minutes in a boiling waterbath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 x g for 10 minutes. The absorbance of the clear supernatant was measured against a blank that contains all the reagents minus the sample in a Spectronic® Helios Gamma & Delta spectrophotometer.

Calculation

The malondialdehyde was calculated using extinction co-efficient of $1.56 \times 10^5 M^{-1} cm^{-1}$.

3.4.17 Determination of conjugated dienes

Conjugated dienes were quantified by the diene conjugated assay described by Buege and Aust (1978).

Principle

Lipid peroxidation is accompanied by a rearrangement of the PUFA double bonds, leading to the formation of conjugated dienes, which absorb at 233 nm. Therefore, lipid peroxidation can be assayed by recording the increase in absorbance of extracted membrane lipids at 233 nm.

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Reagent

The reagent stock is TCA-TBA-HCl (15 % (w/v) trichloroacetic acid, 0.375 % (w/v) thiobarbituric acid, 0.25 N hydrochloric acid.

Procedure

1 ml of biological sample (plasma or tissue homogenate) was combined with 2 ml of TCA-TBA-HCl reagent and mixed thoroughly. The solution was heated for 15 minutes in a boiling waterbath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 x g for 10 minutes. The absorbance of the clear supernatant was measured against a blank that contains all the reagents minus the sample in a Spectronic® Helios Gamma & Delta spectrophotometer.

Calculation

The malondialdehyde was calculated using extinction co-efficient of 1.56×10^5 M^{-1} cm^{-1}.

3.4.17 Determination of conjugated dienes

Conjugated dienes were quantified by the diene conjugated assay described by Buege and Aust (1978).

Principle

Lipid peroxidation is accompanied by a rearrangement of the PUFA double bonds, leading to the formation of conjugated dienes, which absorb at 233 nm. Therefore, lipid peroxidation can be assayed by recording the increase in absorbance of extracted membrane lipids at 233 nm.

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Procedure

1 ml of tissue homogenate was mixed thoroughly with 5 ml of chloroform: methanol (2:1), followed by centrifugation at 1000 x g for 5 minutes to separate the phases. Most of the upper phase was removed by suction and 3 ml of the lower, chloroform phase are recovered. The chloroform layer was placed in a test tube and taken to dryness in a water bath at 4°C. The lipid residue in the test tube was dissolved in 1.5 ml cyclohexane, and the absorbance was determined at 233 nm against a cyclohexane blank in a Spectronic® Helios Gamma & Delta Sepctrophotometer.

Calculation

The amount of hydroperoxides produced was calculated using a molar extinction coefficient of $2.52 \times 10^4\ M^{-1}\ cm^{-1}$.

3.4.18 Determination of protein carbonyls

Protein carbonyl content was determined by the reaction with 2,4-dinitrophenylhydrazine (DNPH) as described by Levine *et al.* (1990).

Principle

This assay is used as an indicator of protein damage by free radical reactions.

The carbonyl group reacts with DNPH to form the 2, 4-dinitrophenylhydrazone which can be measured spectrophotometrically at 370 nm.

Procedure

500 µl of tissue homogenate was taken in a clean dry eppendorf tube and 500 µl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added. The mixture was allowed to stand at room temperature for 10 minutes. The samples were precipitated with 500 µl of

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20 % trichloroacetic acid (TCA), centrifuged at 11,000 rbm for 3 minutes. The pellet was washed with 1 ml of ethanol-ethyl acetate (1:1; v/v) to remove free DNPH reagent, allowed to stand for 10 minutes. The sample was centrifuged for 5 minutes at 11,000 rpm and the supernatant was discarded. The washing procedure was repeated two times for a total of three washes. The resulting protein pellet was resuspended in 600 µl of 100 mM sodium hydroxide solution. The samples were incubated at 37°C for 15 minutes to aid dissolution of protein. Samples were spectrophotometrically analyzed against complementary blank treated with 2 M HCl instead of DNPH. The protein carbonyls levels were calculated using a molar absorption co-efficient of 22, 000 $M^{-1}cm^{-1}$.

3.4.19 Determination of glutathione

The thiol group in glutathione (GSH) is a potent reducing agent, rendering GSH the most abundant intracellular small molecule thiol. GSH plays a pivotal defensive role against oxidative insults as an endogenous scavenger of free radicals and its level in the blood is a sensitive indicator of antioxidant status in circulation (Cooper and Kristal, 1997; Piemonte *et al*., 2001). It plays its antioxidant role in the detoxification of a variety of electrophilic compounds and peroxides via catalysis by GST and GSHPx.

Red blood cell glutathione was determined according to the method of Beutler *et al.* (1963).

Principle

Virtually all non-Protein sulfhydryl of red blood cells is in the form of GSH. 5, 5'-Dithiobis-2-nitrobenzoic acid (DTNB) is a disulfide compound which is readily reduced by sulfhydryl compounds forming a highly coloured yellow anion. The absorbance of the

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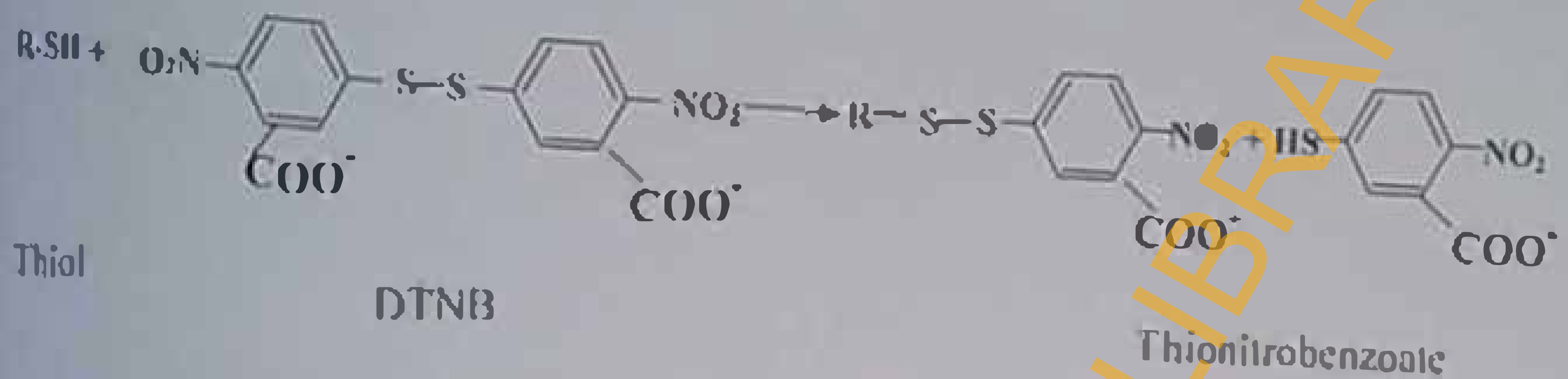
yellow anion is measured colorimetrically at 412 nm and is directly proportional to the GSH concentration.



Procedure

0.9 ml of distilled water and 1.5 ml precipitating solution (1.67 g glacial metaphosphoric acid, 0.20 g Na_2EDTA, 30 g NaCl, 100 ml distilled H_2O) were added to 0.1 ml of blood and mixed thoroughly. After 15 minutes of incubation at room temperature the reaction mixture was centrifuged at 3000 g for 15 minutes at 4°C.

2 ml of 0.3 M phosphate solution and 250 µL DTNB solution (200 mg in 100 ml of 1 % sodium citrate solution) were added to 500 µL of the clear supernatant. A blank was prepared with 1 ml phosphate solution, 1 ml distilled H_2O, 0.5 ml precipitating solution, and 250 µL DTNB solution. Both the blank and sample reaction mixtures were read against distilled water at 412 nm in Spectronic® Helios Gamma & Delta spectrophotometer.

Total GSH was estimated in other tissues (liver, kidney, heart, lung and brain) by the method of Sedlak and Lindsay (1968).

100 µL of the homogenate was added to 1 ml of 0.2 M Tris.EDTA buffer, pH 8.2, 0.9 ml 20 mM EDTA, pH 4.7 followed by 20 µL 10 mM DTNB. After 30 minutes of incubation at room temperature, the mixture was centrifuged and absorbance of the supernatant read

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against distilled water at 412 nm. The blank contained 1 ml Tris-EDTA buffer, 0.9 ml EDTA, 100 µL distilled water and 20 µL DNTB.

Calculation

$$\text{GSH concentration} = \frac{A_{sample}}{\varepsilon_{412nm}} \times \frac{V}{v}$$

where

A_{sample} = absorbance of sample

ε_{412nm} = extinction co-efficient (13,600 $M^{-1}cm^{-1}$)

V = total volume of reaction mixture

v = volume of sample in the reaction mixture

3.4.20 Determination of catalase activity

Catalase (CAT) was determined according to the *in vitro* method of Aebi (1984).

Principle

In the ultraviolet range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm.

$$2H_2O \xrightarrow{CAT} 2H_2O + O_2$$

The difference in absorbance (ΔA_{240}) per unit time is a measure of the catalase activity.

Procedure

0.99 ml of 1 % Triton X-100 was added to 0.01 ml of tissue homogenate. The mixture was diluted with 1.9 ml of 50 mM phosphate buffer and mixed thoroughly.

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1 ml of 30 mM H_2O_2 was added to the final mixture in a cuvette, mixed and the change in absorbance read at 240 nm for 3 minutes in a Spectronic® Helios Gamma & Delta spectrophotometer.

The activity of catalase was calculated using a molar extinction co-efficient of 40 $M^{-1}cm^{-1}$.

3.4.21 Determination of glutathione peroxidase activity

Glutathione peroxidase (GSHPx) was determined according to the UV method of Paglia and Valentine (1967) using the RANSEL kit, a commercial kit for *in vitro* determination of GSHPx in whole blood manufactured by RANDOX Laboratories, Crumlin, United Kingdom.

Principle

GSHPx catalyses the oxidation of GSH by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form (GSH) with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured.

$$2GSH + ROOH \xrightarrow{GSHPx} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

Procedure

0.05 ml of heparinised whole blood was diluted (1:40 dilution) with 2 ml of the diluting agent provided in the kit. 0.02 ml of the diluted sample was used for the assay. The assay mixture contained 50 mM phosphate buffer, pH 7.2, 4.3 mM EDTA; 4 mM GSH; 0.5 U GR; 0.34 mM NADPH and 0.18 mM cumene hydroperoxide.

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The content of the cuvette was thoroughly mixed and the initial absorbance of the sample against reagent blank was measured at 340 nm with a Spectronic® Helios Gamma & Delta spectrophotometer. The absorbance was read again after 1 and 2 minutes.

Calculation

GSHPx concentration was calculated using the formula:

U/L of Haemolysate = 8412 X $\Delta O.D._{340nm}$/min

where,

$\Delta O.D._{340nm}$/min = change in absorbance at 340 nm per minute

3.4.22 Determination of superoxide dismutase activity

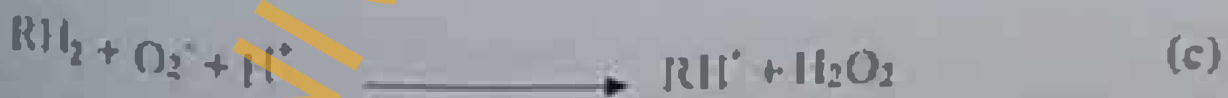
Superoxide dismutase (SOD) activity was determined in the plasma and tissue homogenates according to the method of Misra and Fridovich (1972).

Principle

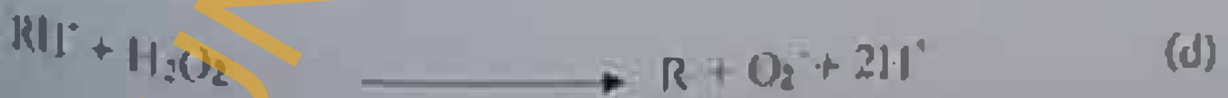
The assay of SOD is an indirect method which is based on the inhibitory effect of SOD in the initial rate of epinephrine auto-oxidation at elevated pH. The oxidation of epinephrine is followed in terms of the production of adrenochrome, which exhibits an absorption maximum at 480 nm. If RH_3^- represents epinephrine and R represents adrenochrome, the following reactions represent the chain reaction as it might occur at high pH:

$RH_3^- + Me^n \longrightarrow RH_3^{\cdot} + Me^{n-1}$ (a)

$RH_3^{\cdot} + O_2 \longrightarrow RH_2 + O_2^- + H^+$ (b)



$RH_2 + O_2^- + H^+ \longrightarrow RH^{\cdot} + H_2O_2$ (c)



$RH^{\cdot} + H_2O_2 \longrightarrow R + O_2^- + 2H^+$ (d)

$RH_3^- + O_2^- + 2H^+ \longrightarrow RH_3^{\cdot} + H_2O_2$ (e)

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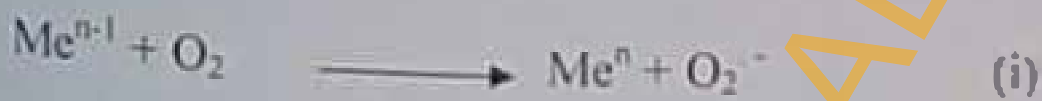
In this way, one initiating event, here shown as the univalent oxidation of an epinephrine anion by a metal cation (reaction a) or by a superoxide anion (reaction e), starts a chain reaction in which O_2^{-} is a propagating species. It is clear that SOD should strongly inhibit this mechanism. At lower pH, the organic radical generated by the initiating event could lead to adrenochrome formation by a series of dismutation reactions such as:

$$RH_3^{\cdot} + RH_3^{\cdot} \longrightarrow RH_2 + RH_4 \qquad (f)$$

$$RH_3^{\cdot} + RH_2 \longrightarrow RH^{\cdot} + RH_4 \qquad (g)$$

$$RH^{\cdot} + RH^{\cdot} \longrightarrow R + RH_2 \qquad (h)$$

In this case SOD could not inhibit adrenochrome formation. The reduced metal generated in reaction (a) would, in any case, be reoxidised by reaction with oxygen.



$$Me^{n-1} + O_2 \longrightarrow Me^{n} + O_2^{-} \qquad (i)$$

$O_2^{\cdot-}$ generated by reaction (i) could either dismutate or react with epinephrine as in reaction (e) (Misra and Fridovich, 1972).

Procedure

0.02 ml of sample was added to 3 ml of 50 mM sodium carbonate buffer, pH 10.2 to equilibrate. The reaction was initiated by the addition of 0.03 ml of freshly prepared 3 mM epinephrine as the substrate to the buffer sample mixture and quickly mixed by inversion. The reference cuvette contained 3 ml of buffer, 0.03 ml of 3 mM epinephrine and 0.02 ml of distilled water. The increase in absorbance at 480 nm due to the adrenochrome formed was monitored for 2-3 minutes in a Spectronic[a] Helios Gamma & Delta spectrophotometer.

Calculation

Enzyme activity can be expressed as:

% Inhibition = 100 - 100 x $\frac{\text{rate of epinephrine oxidation in the presence of SOD}}{\quad}$

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rate of epinephrine oxidation in the absence of SOD

(Goldberg and Stern, 1976)

1 unit of SOD activity = amount of SOD giving 50 % inhibition

$$\text{Units/g wet tissue} = \frac{\%\ \text{inhibition}}{A} \times \frac{1}{50} \times 1000$$

where;

A = mg of tissue in the reaction mixture

1/50 = converts to 50% inhibition

1000 = converts to g of wet tissue

3.4.23 Determination of glutathione S-transferase activity

Glutathione S-transferase (GST) activity was assayed by the method of Habig *et al.* (1974) with 1-Chloro-2, 4-dinitrobenzene (CDNB) as substrate. GST is involved in the detoxification of a wide variety of chemicals. It catalyzes the nucleophilic attack of glutathione on electrophilic substrates, thereby decreasing their reactivity with cellular macromolecules (Armstrong, 1997).

Principle

GST catalyzes the conjugation of 1-Chloro-2, 4-dinitrobenzene (CDNB) to form 2, 4-dinitrobenzene-S-glutathione which can be monitored spectrophotometrically at 340 nm.

Procedure

0.1 ml of 20 mM CDNB and 1.7 ml of distilled water were added to 1 ml of 0.2 M phosphate buffer (pH 6.5). The mixture was incubated at 37°C for 5 minutes. After incubation, 0.1 ml of tissue homogenate and 0.1 ml of 20 mM GSH were added and increase in absorbance was monitored for 5 minutes using Spectronic® Helios Gamma & Delta spectrophotometer. The enzyme activity was calculated using the extinction coefficient

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rate of epinephrine oxidation in the absence of SOD (Goldberg and Stern, 1976)

1 unit of SOD activity = amount of SOD giving 50 % inhibition

$$\text{Units/g wet tissue} = \frac{\%\ \text{inhibition}}{A} \times \frac{1}{50} \times 1000$$

where;

A = mg of tissue in the reaction mixture

1/50 = converts to 50% inhibition

1000 = converts to g of wet tissue

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9.6 $mM^{-1} cm^{-1}$. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the conjugation of 1 µmol CDNB per minute.

(Activity = µmoles of GSH-CDNB conjugate formed /min/mg protein).

3.4.24 Histopathological examination of liver

Livers of rats from different groups were perfused with 10% neutral formalin solution. Hepatic tissue was dehydrated and embedded in paraffin. Paraffin sections were made and stained using haematoxylin-eosin (H&E) dye. The stained sections were examined under a microscope for histopathological changes in liver architecture, and their photomicrographs were taken.

3.4.25 Statistical Analysis

Data were expressed as mean ± standard error of means (S. E. M) and analyzed by analysis of variance (ANOVA). Statistical significance of the difference of the means was evaluated by Student's t-test. Differences were considered statistically significant if the p value was < 0.05.

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CHAPTER FOUR

4.0 EXPERIMENTS AND RESULTS

4.1 Experiment 1: Lipid lowering activity of aqueous and methanolic leaf extracts of *P. americana* on diet-induced hyperlipidaemia in rats

Introduction

Hyperlipidaemia is an important risk factor in the pathogenesis of chronic degenerative diseases such as atherosclerosis, diabetes and cancer. Cardiovascular disease is associated with elevated blood levels of LDL, increase oxidation of LDL, raised levels of total cholesterol and triglycerides whereas a low level of high-density lipoprotein (HDL) is a risk factor for mortality from cardiovascular disease (Criqui *et al.*, 1993; Rahman and Lowe, 2006). A logical strategy to prevent or treat atherosclerosis and reduce the incidence of cardiovascular disease events is to target the hyperlipidaemia by diet and/or lipid-lowering drugs (La Rosa *et al.*, 1990). Cholesterol lowering agents such as statins and fibrates have demonstrated great efficacy in prevention and cessation of the progression of atherosclerosis.

Studies in Nigeria have shown that consumption of fruits and vegetables lowers levels of total cholesterol and triglycerides as well as reduce incidence of cardiovascular risk factors of major chronic diseases (Famodu *et al.*, 1998; Hung *et al.*, 2004; Odetola *et al.*, 2004; Adebawo *et al.*, 2006; Odetola *et al.*, 2006).

This experiment was aimed at evaluating the lipid-lowering properties of aqueous and methanolic extracts of *P. americana*.

Procedure

Hyperlipidaemia was induced in male albino rats as described in section 3.4.2.

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Male albino rats (24) were divided into four feeding groups (A, B, C and D) of six rats each. Group A was fed with standard rat chow and water. Groups B to D were fed with the modified diet and water to provoke hyperlipidaemia. In addition, experimental group C was treated orally with aqueous extract of *P. americana* (AEPA) at a daily dose of 10 mg kg^{-1} b.wt. Similarly, group D was treated orally with methanolic extract of *P. americana* (MEPA) at a daily dose of 10 mg kg^{-1} b.wt. Rats in group B received no treatment and served as negative control. The animals were observed daily and weighed weekly for eight weeks.

At the end of the 8 weeks feeding period, rats were anaesthetized with sodium pentobarbital, 100 mg kg^{-1} b.wt (Wang *et al*., 2004).

Blood was withdrawn via cardiac puncture when animals were rendered unconscious under pentobarbital anaesthesia. The blood was collected in heparinised tubes followed by centrifugation at 3,000 rpm for 5 minutes at 4°C to separate the plasma. The plasma was stored in clean tubes at -20°C pending analysis.

10 % (w/v) homogenate was prepared from liver, kidney, heart and lung. Briefly, 1 g of tissue was homogenized in 10 ml of ice-cold homogenizing buffer (8 mM Na_2HPO_4, 12 mM NaH_2PO_4, 1.15 % KCl, pH 7.4) and centrifuged at 12,000 rpm for 20 min at 4°C.

The various biochemical parameters were assayed as described in sections 3.4.6 to 3.4.13 and the histopathological examination of the liver sections was done as detailed in section 3.4.24.

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Results

Table 1 shows the mean weekly weights of rats in the four groups. The body weights of the rats in all the groups increased throughout the study period. However, body weight gain was higher ($p<0.05$) in the hyperlipidaemic rats compared to normal control during the first week. Also, body weight gain in the 3rd and 8th weeks was significantly ($p<0.05$) lower in AEPA and MEPA treated rats (Groups C and D) compared to hyperlipidaemic control (Group B). The overall body weight gain per cent for rats in groups A, B, C, and D were 134 %, 132 %, 80 % and 74 % respectively showing that there was a decrease in the overall body weight gain in AEPA and MEPA treated rats compared with hyperlipidaemic control.

The mean liver weight of rats in the three groups fed high lipid diet (Groups B – D) was significantly higher ($p<0.05$) compared with the mean liver weight of normal control rats, group A (Fig. 1). There was however no significant difference in the mean liver weight of rats fed high lipid diet (Groups B, 7.45 ± 0.97 g/8 weeks) and those treated with AEPA and MEPA (Groups C and D, 7.43 ± 1.57 and 6.89 ± 0.60 g/8 weeks respectively).

Table 2 shows the brain, kidney, heart, and lungs weights in the four experimental groups. Statistical analysis indicates that the mean brain weight of rats treated with AEPA and MEPA (Group C and D, 1.60 ± 0.08 and 1.57 ± 0.04 g respectively) are greater ($p<0.05$) than the mean brain weight of normal and hyperlipidaemic control rats (Groups A and B, 1.40 ± 0.12 and 1.42 ± 0.08 g respectively). The mean lungs weight of the rats treated with AEPA and MEPA (Groups C and D) was higher than mean lungs weight of normal and hyperlipidaemic control rats (Groups A and B). There were no significant ($p>0.05$) differences in the mean kidney weight (Groups A, B, C and D: 0.93 ± 0.13, 0.88

± 0.09, 0.83 ± 0.10 and 0.80 ± 0.14 g respectively) or heart weight (Groups A, B, C and D: 0.53 ± 0.14, 0.53 ± 0.07, 0.55 ± 0.07 and 0.55 ± 0.12 g respectively) among the groups.

Figure 2 shows plasma T-CHOL concentration in rats across the four groups. Plasma T-CHOL concentration was significantly ($p<0.05$) elevated in rats fed high lipid diet (Groups B, C and D: 95.01 ± 16.47, 87.03 ± 17.07 and 90.62 ± 20.72 mg/dl respectively) compared with rats fed standard rat chow (Group A, 50.72 ± 24.15 mg/dl). There was no significant ($p>0.05$) difference in the concentration of plasma T-CHOL among the rats fed high lipid diet (Groups B, C and D). However, the hyperlipidaemic control rats (Group B) showed remarkable increase in plasma T-CHOL than the rats treated with AEPA and MEPA (Groups C and D respectively).

Plasma HDL-CHOL concentration was significantly ($p<0.05$) lower in the hyperlipidaemic control rats (Group B, 7.72 ± 3.28 mg/dl) compared with normal control rats (Group A: 16.35 ± 7.72 mg/dl). Treatment with AEPA and MEPA resulted in elevation of HDL-CHOL to values significantly ($p<0.05$) higher than the hyperlipidaemic control (Groups C and D: 14.31 ± 2.29 and 12.94 ± 4.34 mg/dl respectively) but not significantly different from the values in the normal control (Fig. 3).

Figure 4 shows the plasma concentration of triglycerides in the four experimental groups. Plasma triglycerides concentration was increased ($p<0.05$) in rats fed high lipid diet compared to normal control. However, treatment with AEPA lowered ($p<0.05$) plasma triglycerides compared with the hyperlipidaemic control rats. Also, the rats treated with MEPA had higher ($p<0.05$) triglycerides concentration than normal control rats and rats treated with AEPA.

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Plasma LDL-CHOL concentration was increased ($p<0.05$) in the rats fed high lipid diet (Groups B, C and D: 80.01 ± 18.37, 64.54 ± 17.76 and 64.13 ± 19.41 mg/dl respectively) compared with normal control rats (Group A: 18.87 ± 15.59 mg/dl). LDL- CHOL concentration was not significantly ($p>0.05$) different among rats fed high lipid diet, although the hyperlipidaemic control rats (Group B) exhibited higher levels of plasma LDL-CHOL than the treated (Fig. 5).

LDL-CHOL: HDL-CHOL ratio (a useful index of atherogenicity) was highest in the hyperlipidaemic control rats (Group B, 11.81 ± 4.85) and least in the normal control rats (Group A, 1.50 ± 0.87). Inter-group comparison also shows that the LDL-CHOL: HDL-CHOL ratio in the hyperlipidaemic control rats was significantly higher ($p<0.05$) than in the treated groups (Fig. 6).

Table 3 shows the liver T-CHOL, HDL-CHOL and TG concentrations in rats across the four experimental groups. Liver T-CHOL concentration was raised ($p<0.05$) in rats fed high lipid diet (Groups B, C and D: 655.65 ± 27.38, 616.43 ± 23.19 and 610.30 ± 17.65 mg/dl respectively) compared with the normal control rats (Group A, 47.53 ± 3.03 mg/dl). Similar patterns were observed for liver LDL-CHOL and TG concentrations. However, hepatic HDL-CHOL concentration was higher ($p<0.05$) in the hyperlipidaemic control (Group B, 11.67 ± 1.52 mg/dl) and MEPA treated rats (Group D: 12.41 ± 1.76 mg/dl) compared to normal control rats (Group A, 5.11 ± 0.75 mg/dl).

The mean plasma glucose concentrations of rats in the four experimental groups are depicted in Figure 7. Plasma glucose was significantly ($p<0.05$) increased in rats fed high lipid diet compared with normal control. Treatment with AEPA and MEPA induced a

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reduction (16 % and 11 % respectively) in plasma glucose of treated rats compared to the hyperlipidaemic control.

The activity of aspartate aminotransferase (AST) in plasma of rats is shown in Figure 8. The decline observed in the activity of plasma AST in rats treated with AEPA and MEPA (Groups C and D: 31.20 ± 6.62 and 36.83 ± 10.54 U/l respectively) was not significant ($p>0.05$) compared to the hyperlipidaemic control rats (Group B, 42.48 ± 6.98 U/l) and normal control (Group A, 41.27 ± 9.52 U/l).

Plasma ALT activities in the four groups of rats are shown in Figure 9. ALT activity was lower ($p<0.05$) in the hyperlipidaemic control rats (17.13 ± 2.32) compared to AEPA and MEPA treated (42.04 ± 5.8; 44.25 ± 3.75 respectively) and normal control rats (30.20 ± 2.08). However, there was no significant difference ($p>0.05$) in ALT activity in AEPA and MEPA treated rats compared with normal control rats.

The photomicrographs of the liver sections stained with H & E dye are as shown in Plates 1 – 4. The liver of rats fed standard chow had preserved lobular architecture while fatty changes were observed in the liver of rats fed high lipid diet. The fatty changes were most severe in the hyperlipidaemic rat.

Conclusion

Administration of AEPA and MEPA resulted in a reduction in body weight gain (14 and 25 % respectively) compared with the hyperlipidaemic control. It could be that the extract increases the catabolism of lipids accumulated in adipose tissue thereby causing a decrease in body weight.

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However, administration of AEPA and MEPA lowered plama T-CHOL in the treated rats suggesting that the extracts possess hypocholesterolemic effect.

However, treatment of rats with AEPA and MEPA elicited significant restoration of HDL-CHOL in the treated animals compared with the hyperlipidaemic control. This may serve to protect against lipid peroxidation and development of atherosclerosis.

The aqueous extract alone was able to lower triglycerides in rats fed high lipid diet probably by suppressing synthesis thus suggesting that AEPA could be used in ameliorating hypertriglyceridemia.

The lowering of LDL-CHOL and the index of atherogenicity by AEPA and MEPA in this study could represent a protective mechanism against the development of atherosclerosis and this could account for its use in ethnomedicine for the treatment of hypertension.

It could be concluded that *P. americana* has hypoglycemic property in rats.

Treatment with AEPA and MEPA did not have significant effect on AST and ALT activities in the rats suggesting that the treatment was well tolerated by the animals.

Treatment with AEPA and MEPA ameliorated the severe fatty changes caused by the high lipid diet thus providing a supportive evidence for the lipid lowering effect of the extracts.

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TABLE 1: Mean weekly body weights (g) of rats fed high lipid diet.

Week	A	B	C	D
0	65.95 ± 3.46	65.88 ± 11.23	93.13 ± 9.62	87.37 ± 11.01
1	69.52 ± 7.80	77.95 ± 13.89[a]	101.99 ± 12.30[b]	95.64 ± 9.18[b]
2	81.45 ± 6.93	85.12 ± 14.61	114.16 ± 14.24	108.10 ± 10.40
3	90.13 ± 8.64	94.20 ± 17.34[a]	117.62 ± 15.12[b]	111.41 ± 10.40[b]
4	107.58 ± 9.59	97.19 ± 15.52	124.20 ± 16.19	112.89 ± 25.82
5	123.82 ± 9.46	115.24 ± 17.21	134.92 ± 19.99	124.45 ± 27.44
6	135.83 ± 6.84	125.27 ± 19.60	152.86 ± 24.72	141.53 ± 29.88
7	141.31 ± 7.37	135.60 ± 16.54	160.18 ± 24.65	150.55 ± 30.11
8	154.13 ± 9.50	152.59 ± 20.80[a]	167.56 ± 25.74[b]	152.35 ± 29.93[c]

Values are expressed as means ± SEM for six rats.
Values not sharing a common superscript differ significantly at $p < 0.05$.
A, fed standard chow; B, fed high lipid diet; C, fed high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, fed high lipid diet + 10 mg kg^{-1} b.wt MEPA daily.

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TABLE 2: Mean weight (g) of kidney, lungs, heart and brain of rats fed high lipid diet

	Group			
	A	B	C	D
Kidney	0.93 ± 0.05	0.88 ± 0.04	0.83 ± 0.04	0.80 ± 0.06
Lung	0.76 ± 0.03	0.72 ± 0.05[a]	0.87 ± 0.05[b]	0.90 ± 0.10[b]
Heart	0.53 ± 0.05	0.53 ± 0.03	0.55 ± 0.03	0.55 ± 0.05
Brain	1.40 ± 0.04	1.42 ± 0.05[a]	1.60 ± 0.03[b]	1.57 ± 0.02[b]

Values are expressed as means ± SEM for six rats.
Values not sharing a common superscript differ significantly at $p < 0.05$.
A, fed standard chow; B, fed high lipid diet; C, fed high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, fed high lipid diet + 10 mg kg^{-1} b.wt MEPA daily.

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TABLE 3. Effect of aqueous and methanolic leaf extracts of *P. americana* on liver total cholesterol, high density lipoprotein cholesterol and triglycerides (mg/dl) rats fed high lipid diet

	Group			
	A	B	C	D
T-CHOL	47.53 ± 3.03	655.65 ± 27.38[a]	616.43 ± 23.19[a]	610.30 ± 17.65[a]
LDL-CHOL	35.15 ± 3.89	529.04 ± 18.73[a]	415.54 ± 19.91[a]	416.28 ± 3.62[a]
HDL-CHOL	5.11 ± 0.75	11.67 ± 1.52[a]	9.68 ± 2.33[a]	12.41 ± 1.76[a]
TG	36.30 ± 3.55	1021.98 ± 59.13[a]	739.65 ± 65.52[a]	787.21 ± 76.79[a]

Values are expressed as means ± SEM for six rats.
[a] Significantly different from normal control ($p < 0.05$).
A, fed standard chow; B, fed high lipid diet; C, fed high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, fed high lipid diet + 10 mg kg^{-1} b.wt MEPA daily.

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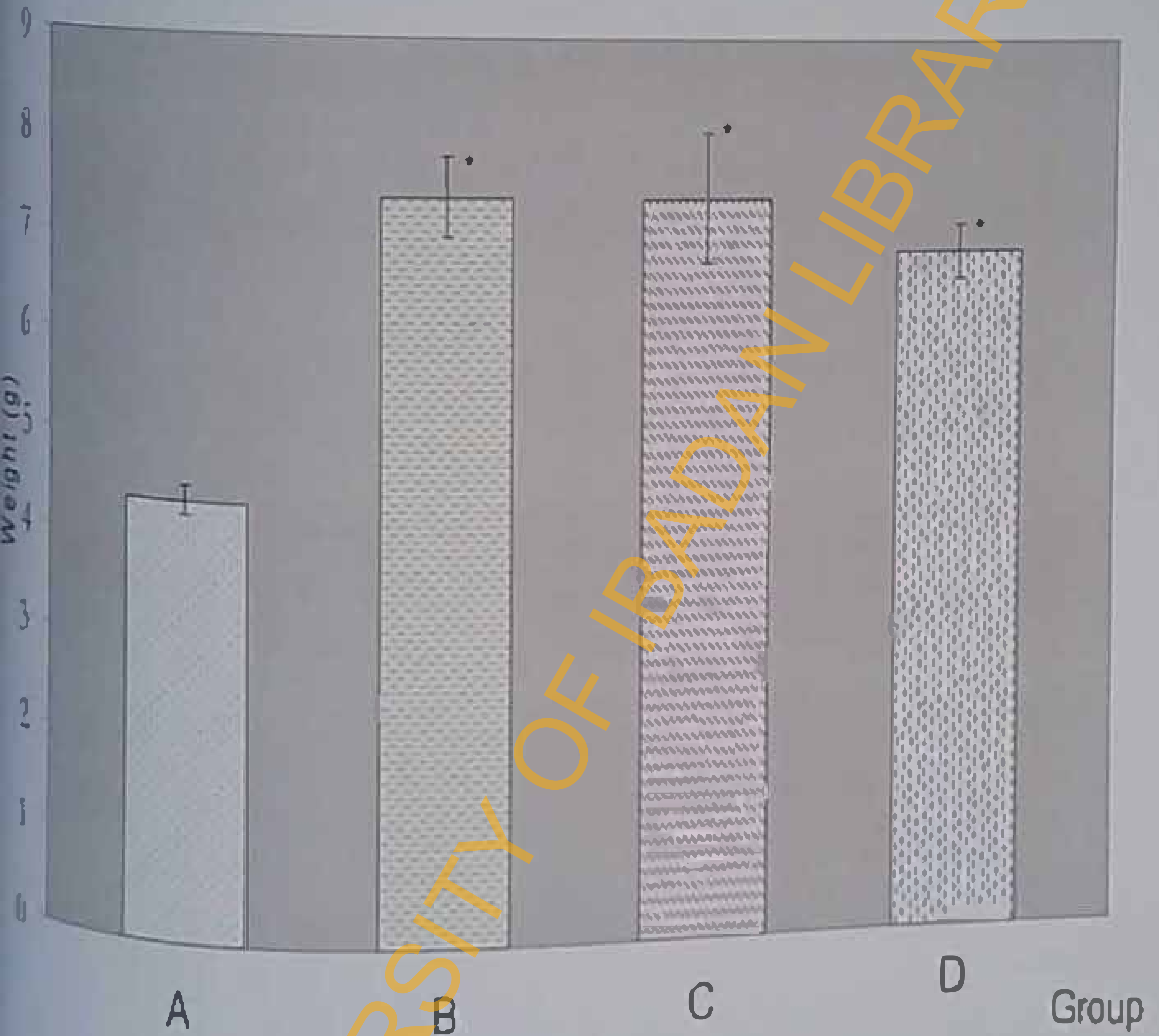

Fig.1. Effect of aqueous and methanolic leaf extracts of *P. americana* on liver weight of rats fed high lipid diet.

Values are means ± SEM (n = 6)
* Differ significantly from normal control ($p<0.05$)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b.wt MEPA.

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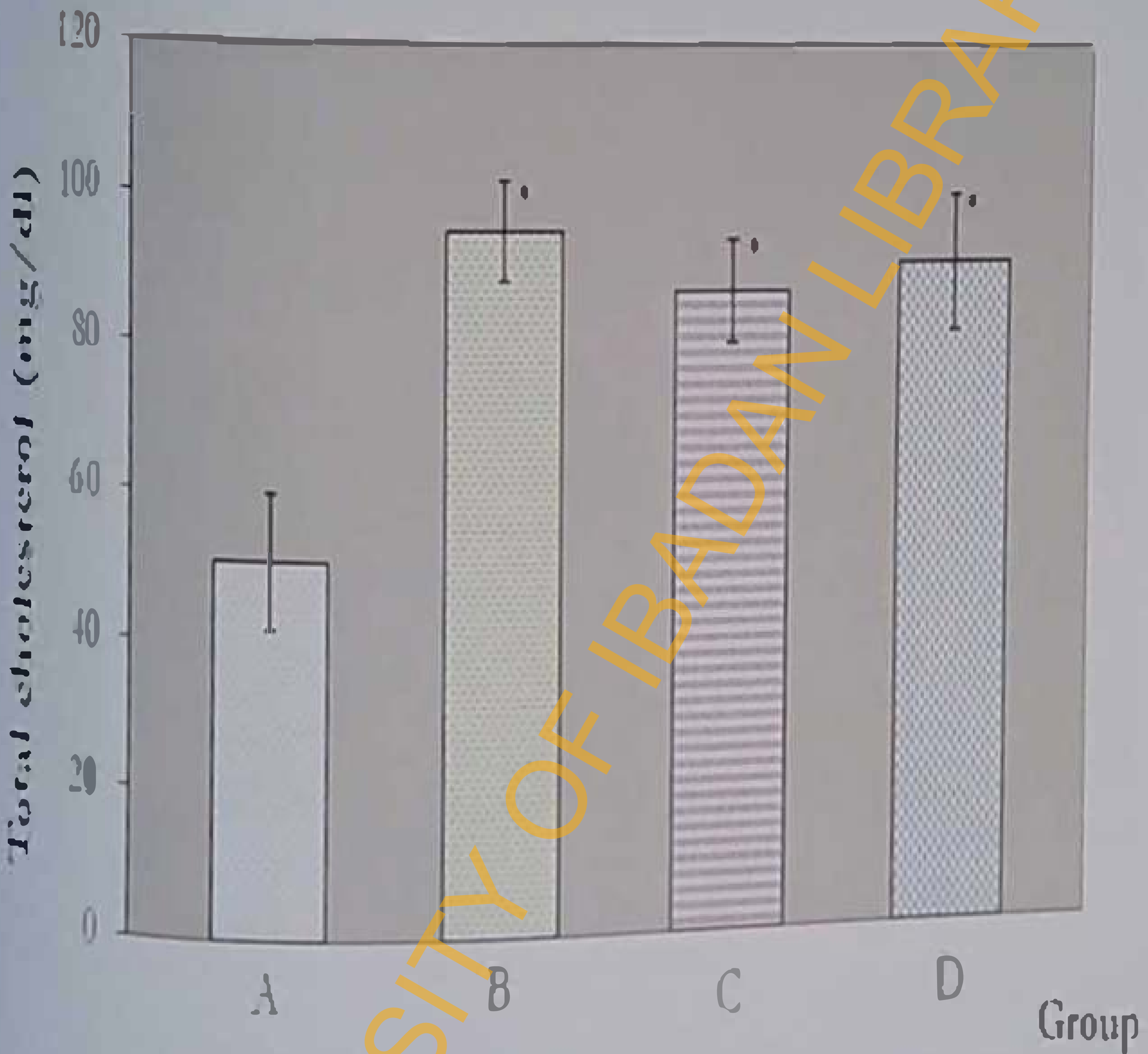

Fig.2. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma total cholesterol in rats fed high lipid diet.

Values are means ± SEM (n = 6)
* Significantly different from normal control ($p<0.05$)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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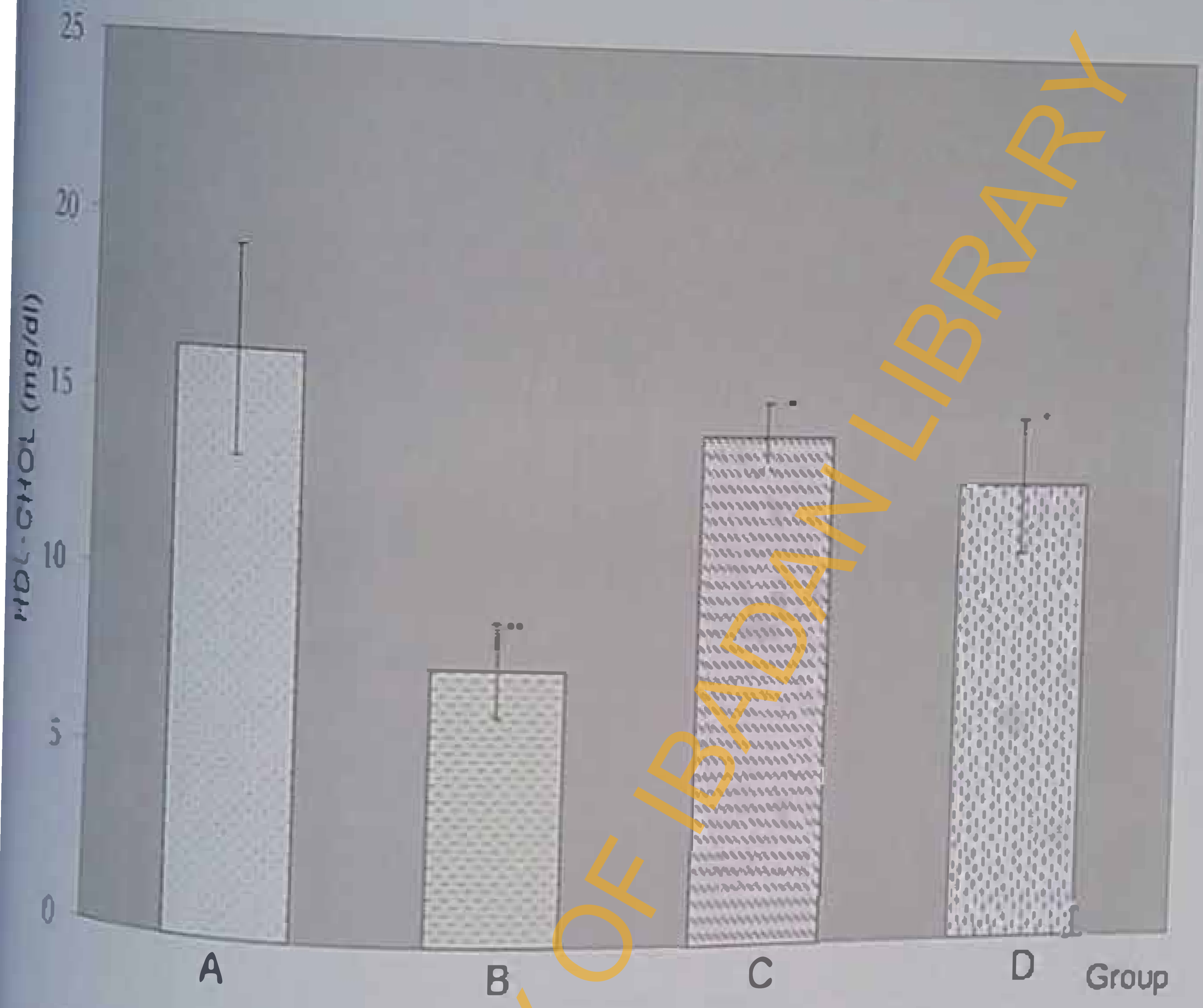

Fig.3. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma high density lipoprotein cholesterol in rats fed high lipid diet.

Values are means ± SEM (n = 6)
** Significantly different ($p<0.05$)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b.wt MEPA.

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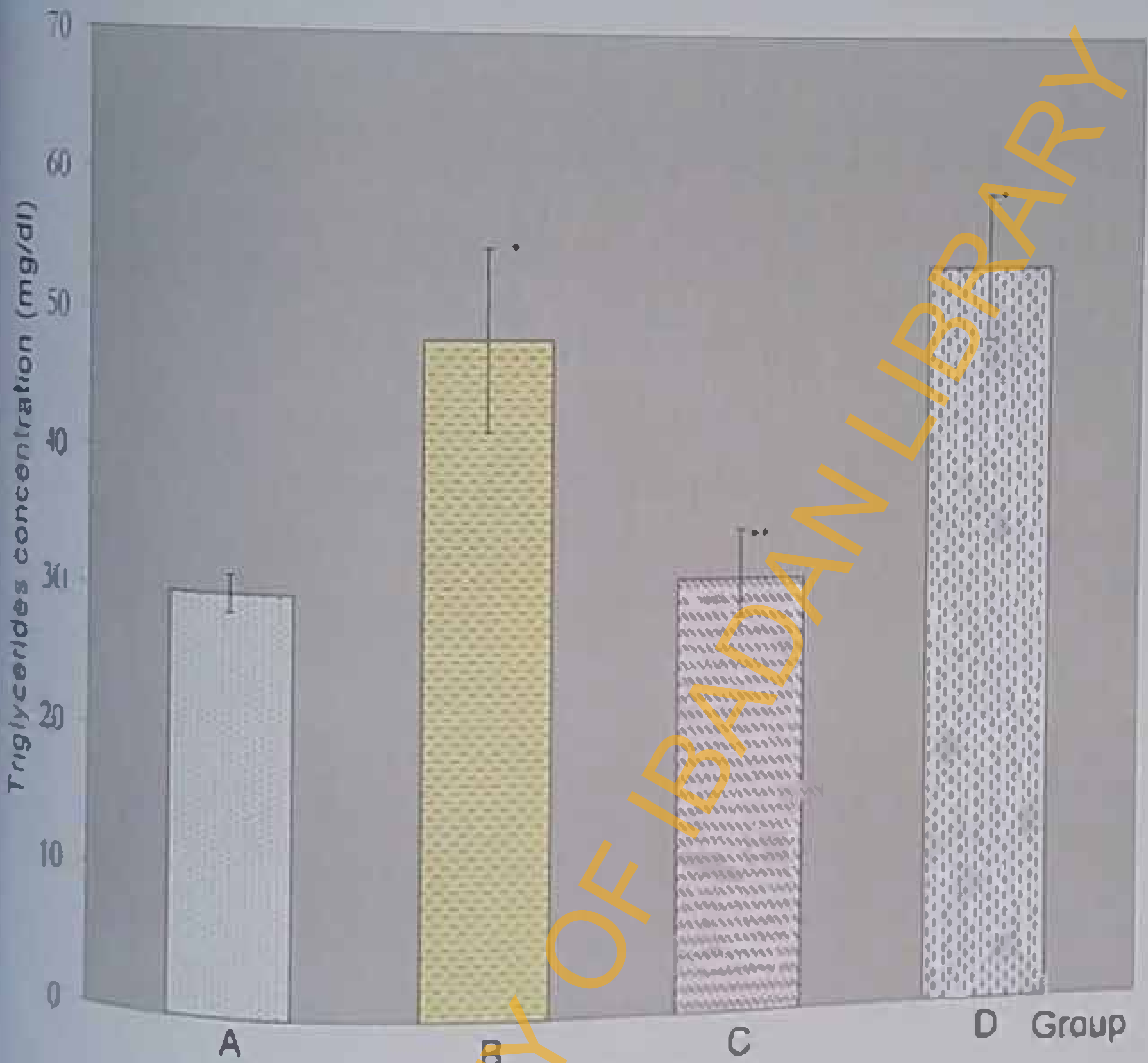

Fig.4. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma triglycerides in rats fed high lipid diet.

Values are means ± SEM (n = 6)
** Significantly different from hyperlipidaemic control rats ($p<0.05$)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b. wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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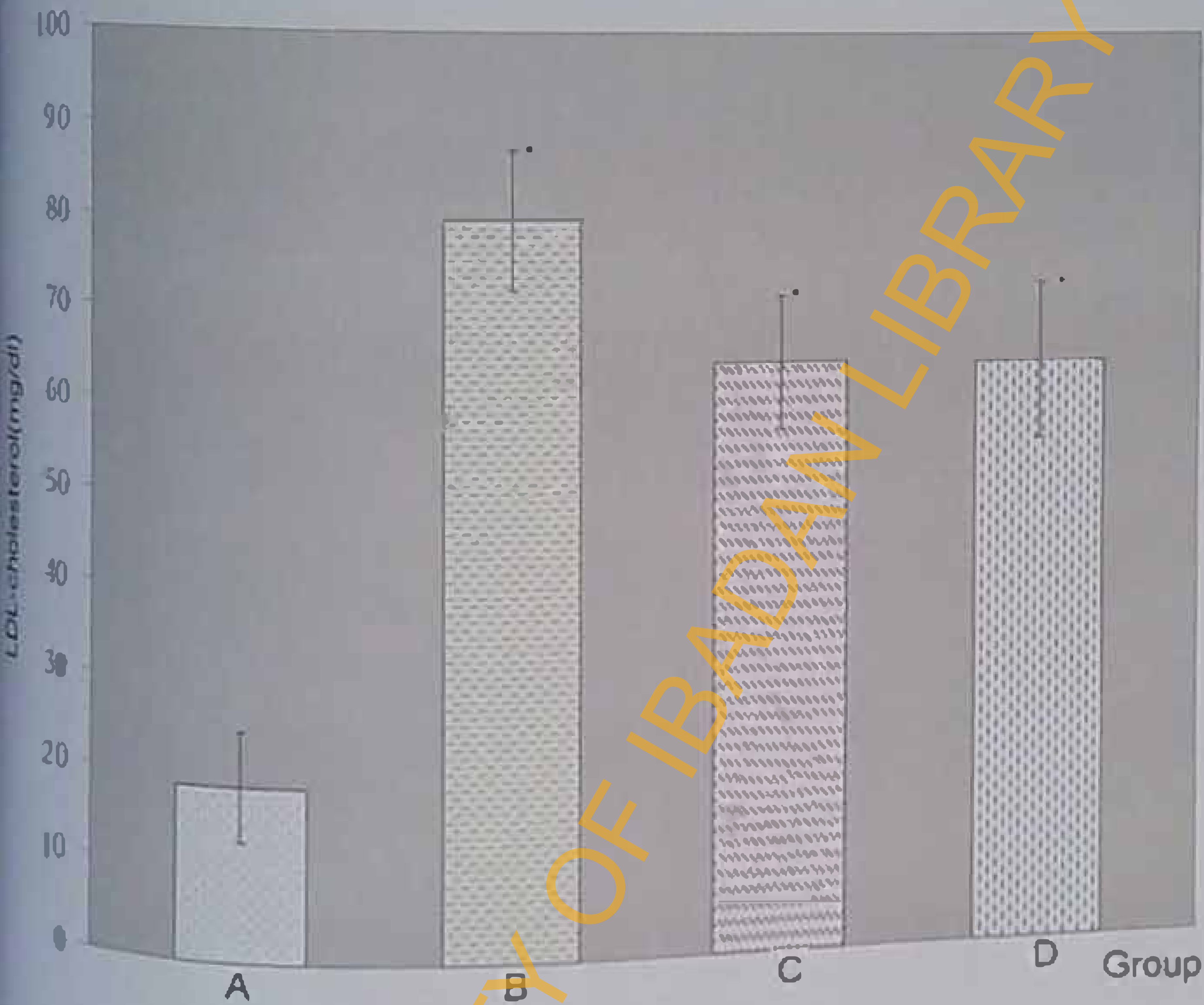

Fig.5. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma low density lipoprotein cholesterol in rats fed high lipid diet.

Values are means ± SEM (n = 6)
* Significantly different from normal control ($p<0.05$)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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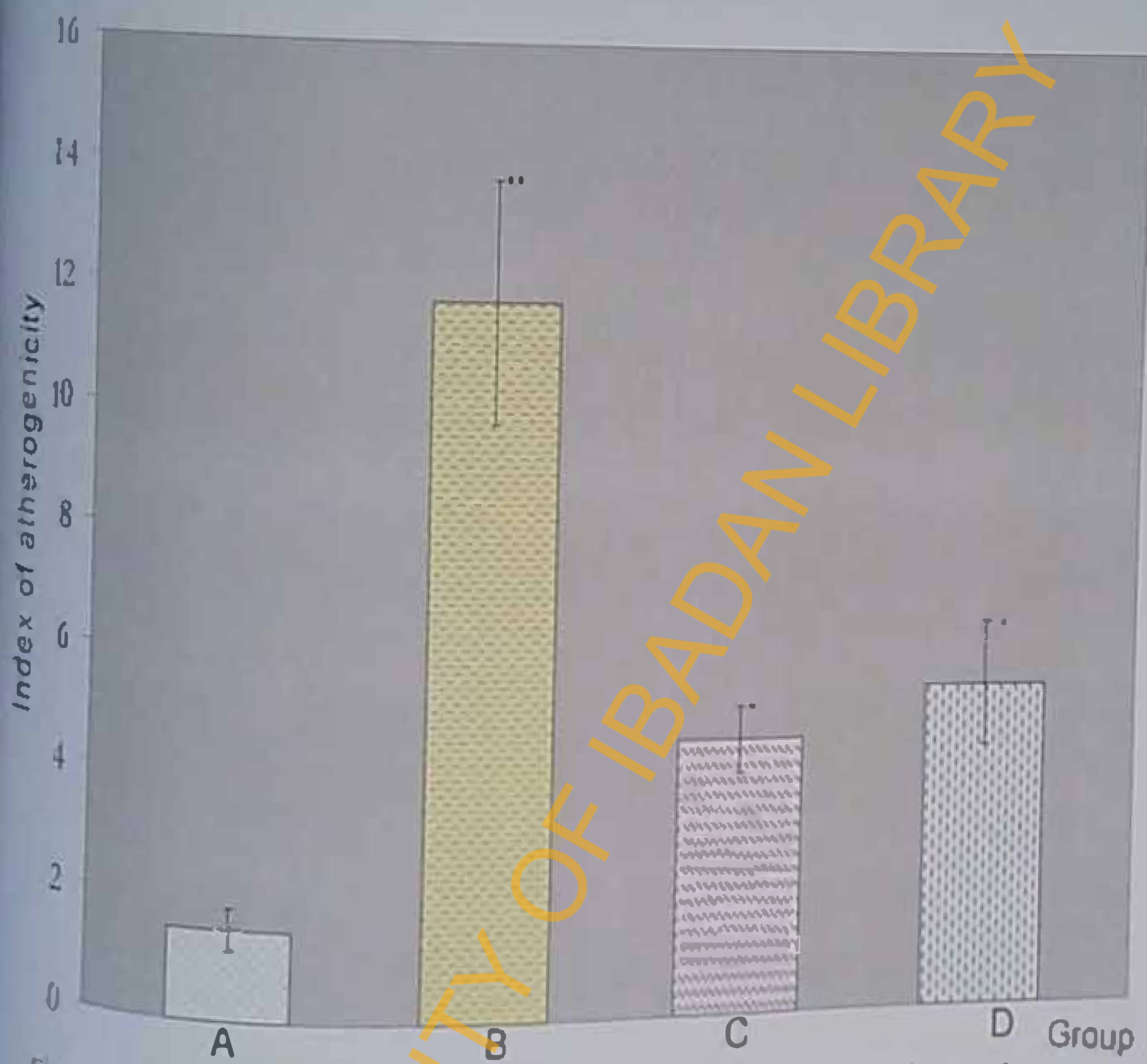

Fig.6. Effect of aqueous and methanolic leaf extracts of *P. americana* on index of atherogenicity (LDL/HDL ratio) in rats fed high lipid diet.

Values are means ± SEM (n = 6)
** Significantly different ($p<0.05$)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b. wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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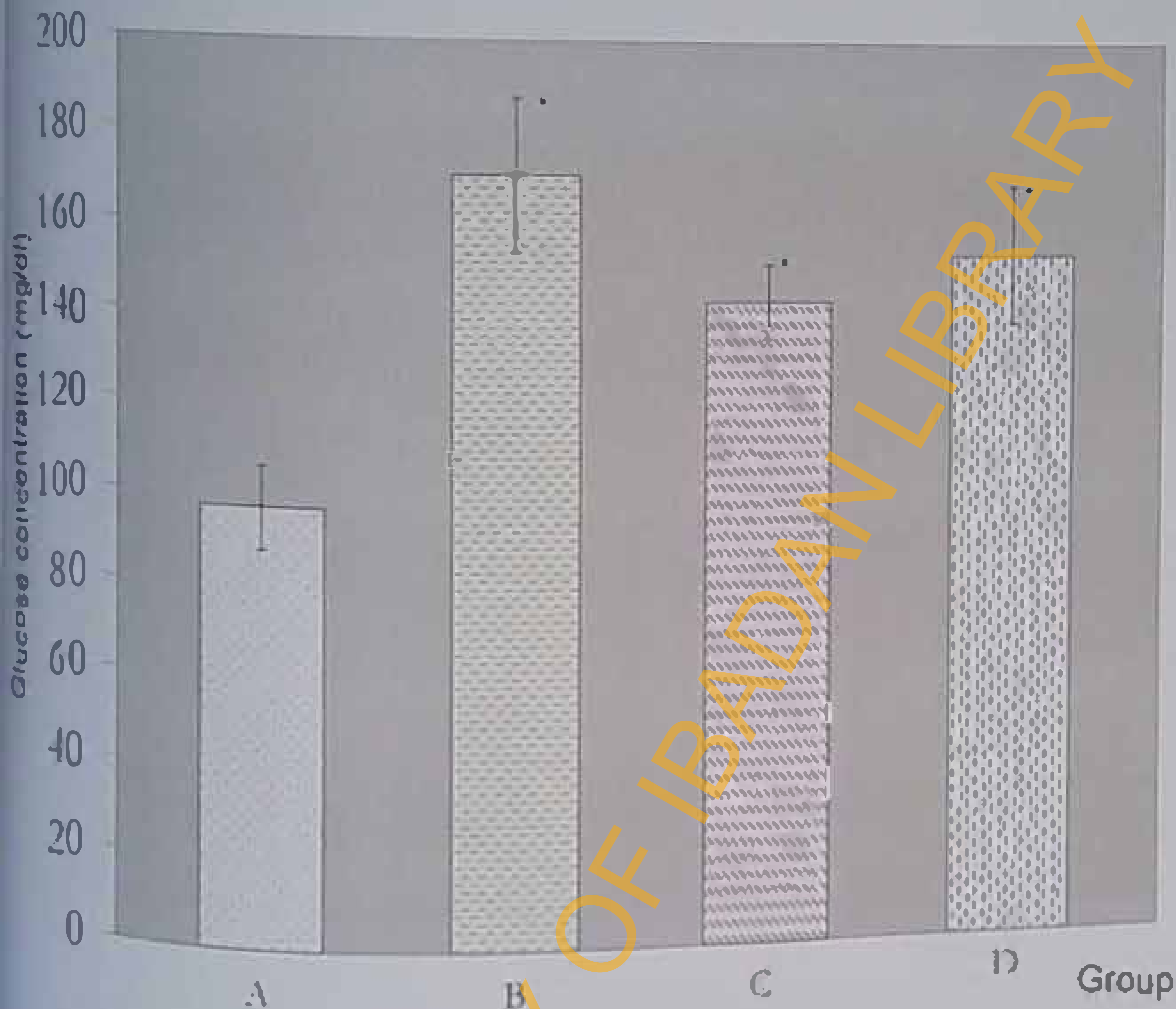

Fig.7. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma glucose in rats fed high lipid diet.

Values are means ± SEM (n = 6)
* Significantly different from normal control ($p<0.05$)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b. wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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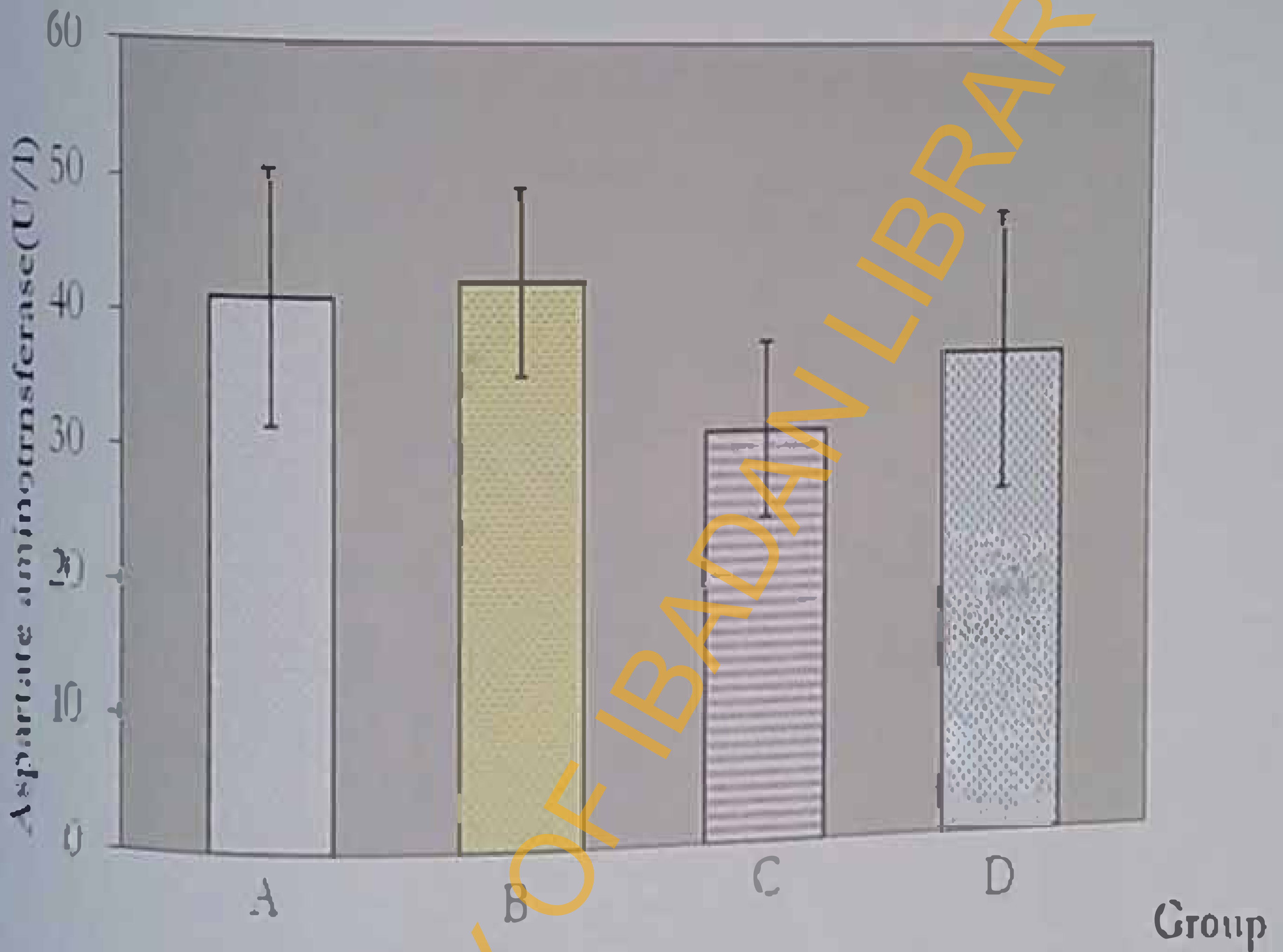

Fig.8. Effect of aqueous and methanolic leaf extracts of *P. americana* on aspartate aminotransferase activity in rats fed high lipid diet.

Values are means ± SEM (n = 6)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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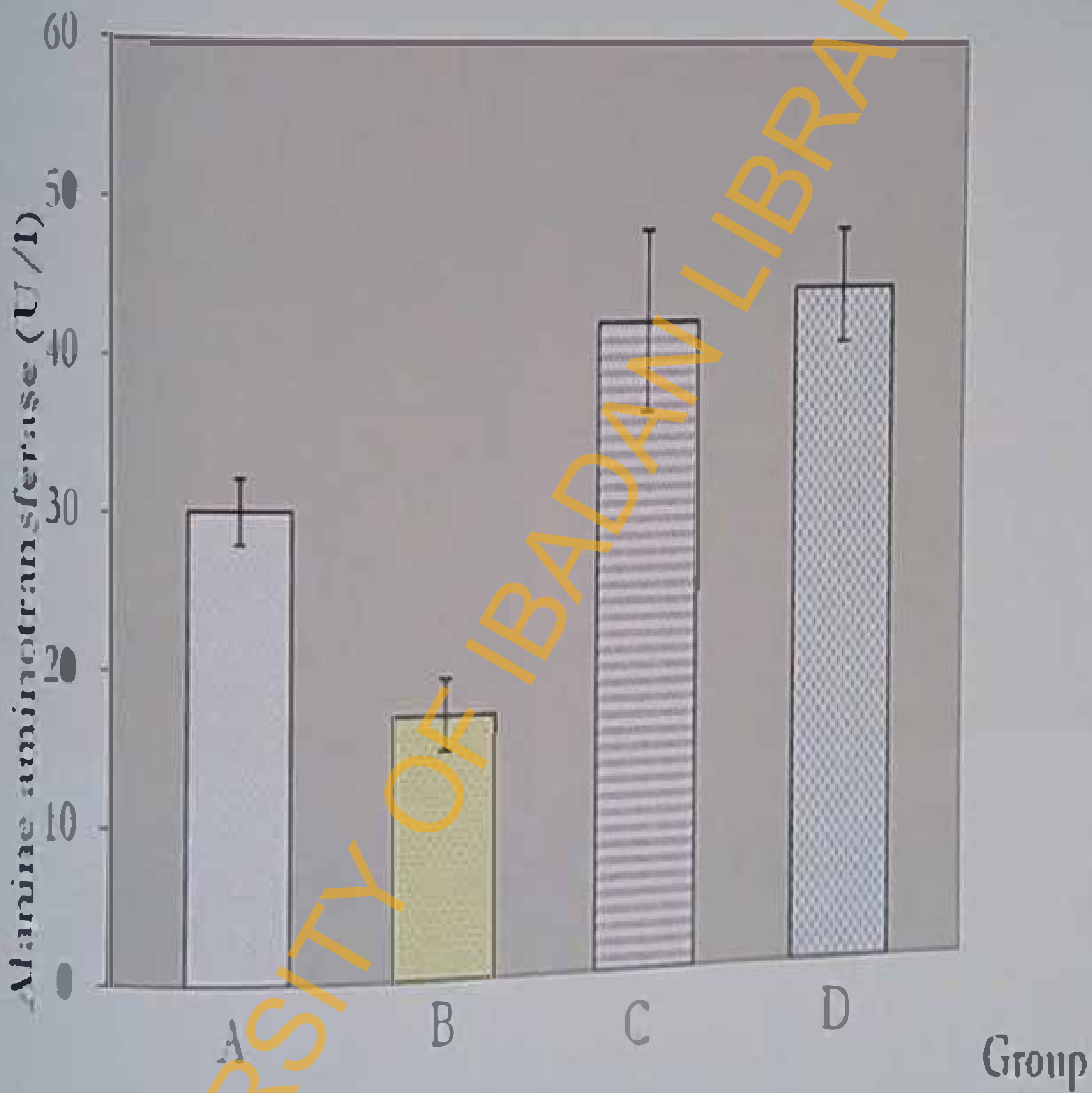

Fig.9. Effect of aqueous and methanolic leaf extracts of *P. americana* on alanine aminotransferase activity in rats fed high lipid diet.

Values are means ± SEM (n = 6)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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Plate 1: Liver section from normal rat showing preserved lobular (↓) architecture with normal hepatocytes [H&E, x 100].

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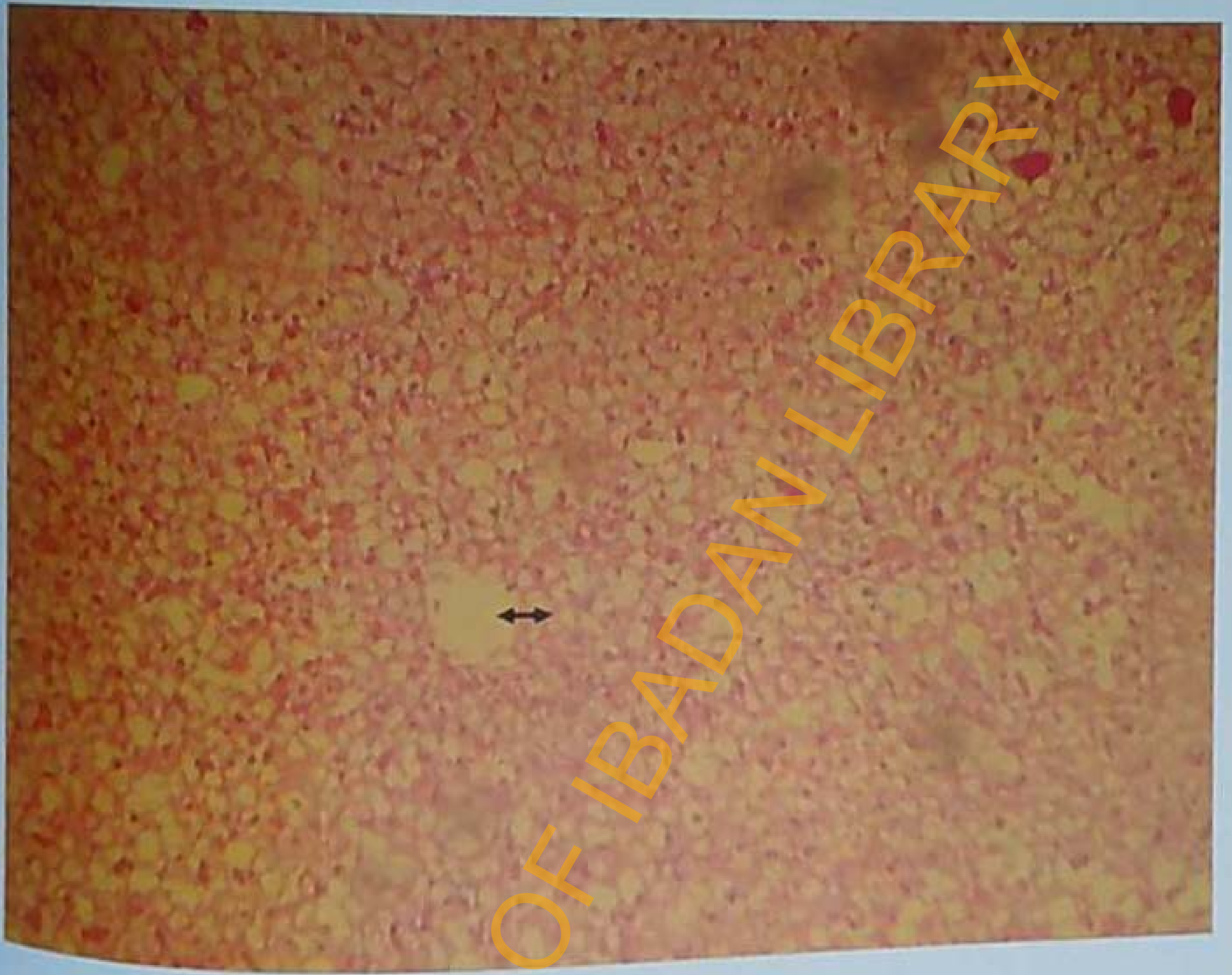

Plate 2: Liver section from hyperlipidaemic rat showing severe fatty change (↔) [H&E, x 100].

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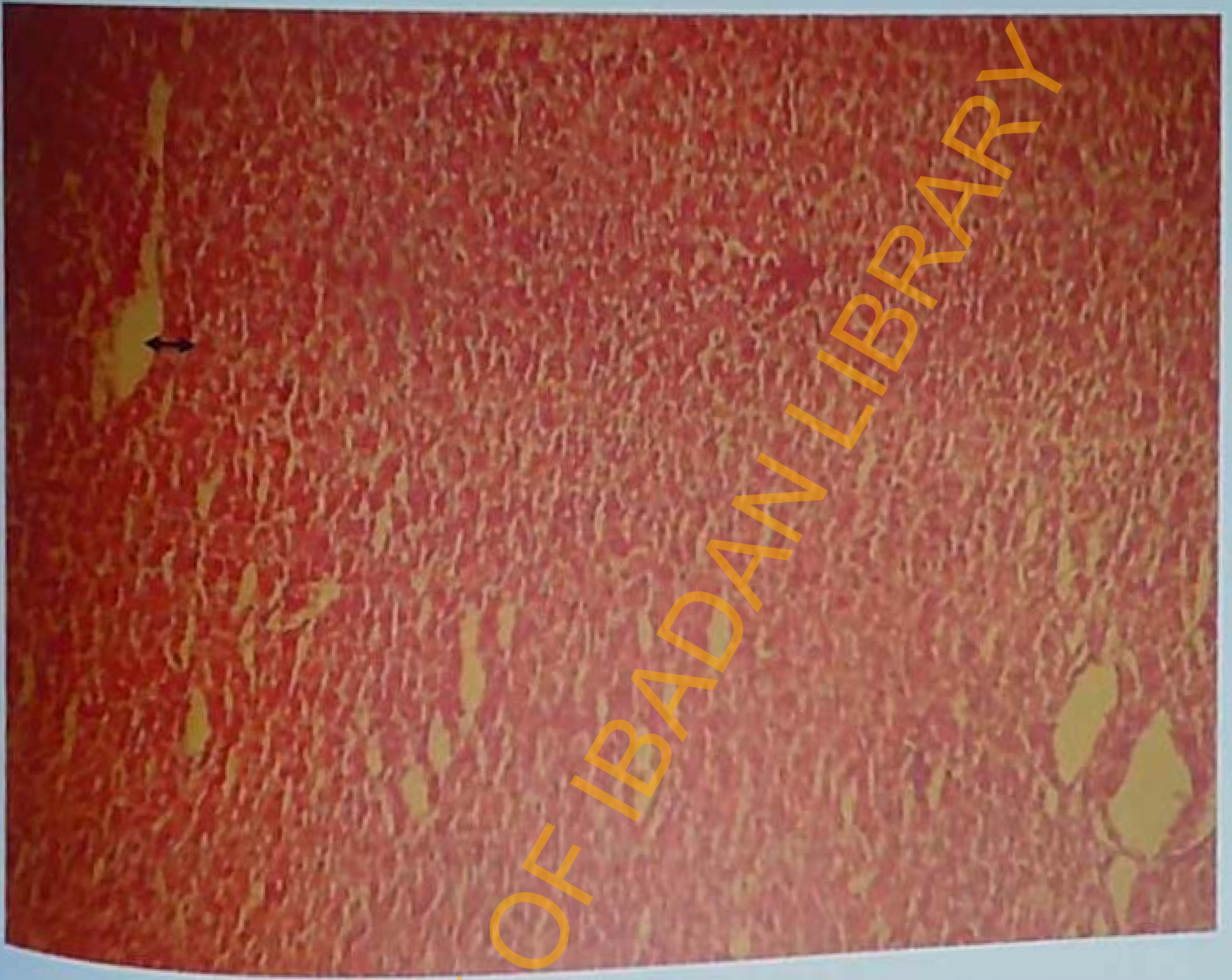

Plate 3: Liver section from hyperlipidaemic rat treated with 10 mg kg^{-1} b. wt ALEPA with mild fatty change (⟷) [H&E, x 100].

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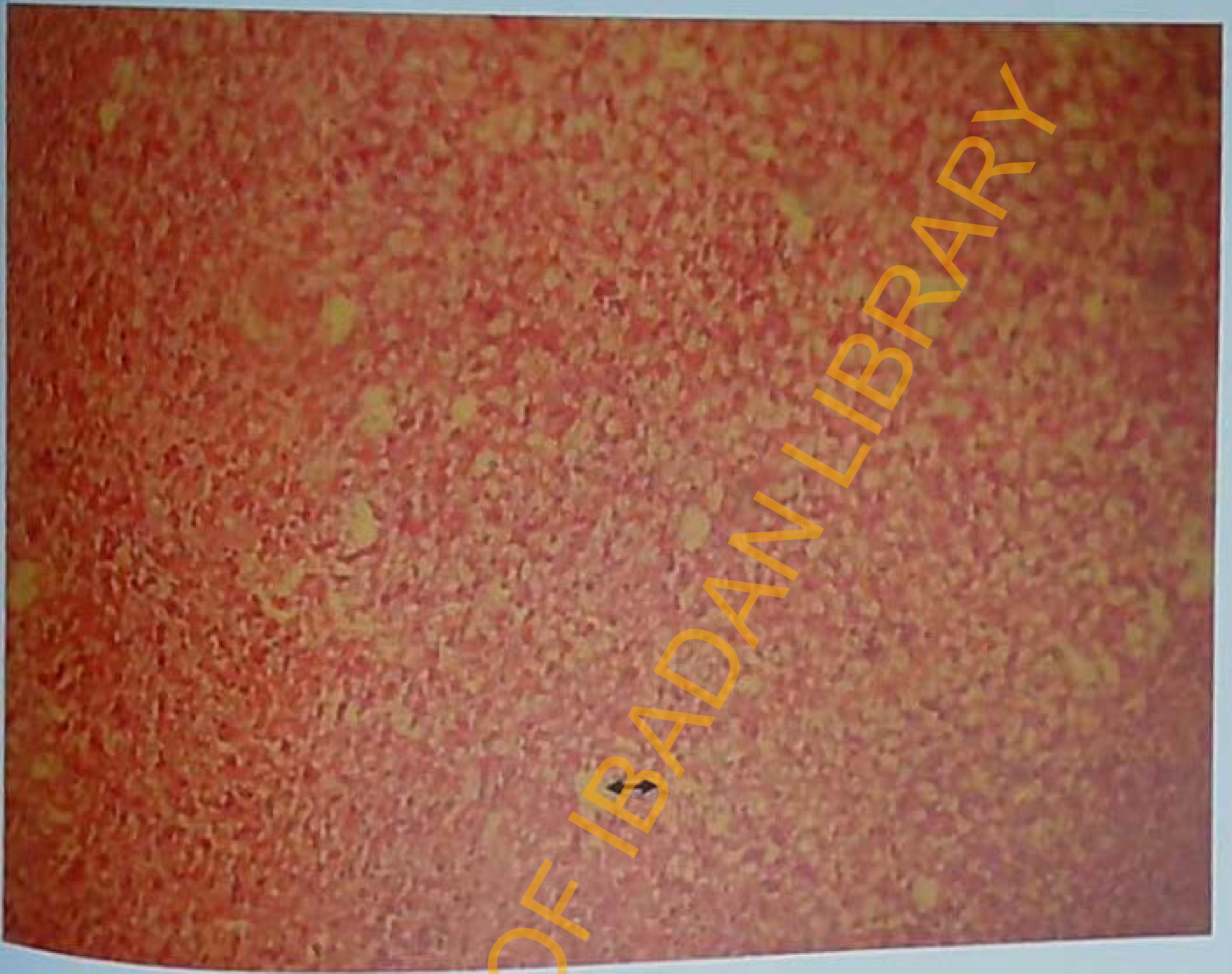

Plate 4: Liver section from hyperlipidaemic rat treated with 10 mg kg^{-1} b. wt MEPA with moderate fatty change (⟷) [H&E. x 100].

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4.2 Experiment 2: Antilipoperoxidative and antioxidant properties of aqueous and methanolic leaf extracts of *P. americana* in rats fed high lipid diet

Introduction

Biological membranes are characterized by the presence of large amounts of PUFAs which can undergo oxidation by a process known as lipid peroxidation.

Animals have several mechanisms for defence against free radicals and other reactive oxygen species. These include radical-scavengers and chain terminators such as vitamins C and E, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx), and non-enzymatic antioxidant glutathione (GSH).

The various defences are complementary to one another because they act on different oxidants or in different cellular compartments (Langseth, 2000). Natural substances that can inhibit lipid oxidation are obtained from many different sources, including plants (Marcia *et al.*, 2001).

Quantitative estimation of lipid peroxidation can be done by determining the concentration of peroxidation products in the form of thiobarburic acid reactive substances (TBARS), conjugated dienes and protein carbonyls.

This experiment was carried out to determine the effects of AEPA and MEPA on lipid peroxidation and antioxidant status in rats fed high lipid diet.

Procedure

Rats were fed high lipid diet to induce hyperlipidaemia as previously described in section 3.4.2.

Blood was withdrawn via cardiac puncture when animals were rendered unconscious under pentobarbital anaesthesia. The blood was collected in heparinised tubes followed

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by centrifugation at 3,000 rpm for 5 minutes at 4°C to separate the plasma. The plasma was stored in clean tubes at -20°C pending analysis.

10 % (w/v) homogenate was prepared from liver, kidney, heart and lung. Briefly, 1 g of tissue was homogenized in 10 ml of ice-cold homogenizing buffer (8 mM Na_2HPO_4, 12 mM NaH_2PO_4, 1.15 % KCl, pH 7.4) and centrifuged at 12,000 rpm for 20 min at 4°C.

Malondialdehyde (MDA), conjugated dienes (CD) and protein carbonyls were quantified as described in sections 3.4.16 to 3.4.18.

GSH, CAT, GSHPx and SOD were determined as outlined in sections 3.4.19 to 3.4.22.

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Results

Figure 10 shows the level of plasma MDA in the rats. Plasma MDA concentration was elevated ($p<0.05$) in the hyperlipidaemic control rats (Group B) compared with the normal control (Group A) and treated rats (Groups C and D). This indicates that administration of both aqueous and methanolic leaf extracts of *P. americana* inhibited plasma lipid peroxidation.

Table 4 shows the MDA concentration in rat tissues. Liver MDA concentration was increased ($p<0.05$) in rats fed high lipid diet (groups B, C and D: 0.32 ± 0.08, 0.42 ± 0.06 and 0.50 ± 0.04 µM/mg protein respectively) compared to the normal control (Group A, 0.22 ± 0.02 µM/mg protein). Similarly, MDA concentration in the lungs of treated rats was slightly higher compared with the hyperlipidaemic control rats (Group B, 0.07 ± 0.04 µM/mg protein) but significantly ($p<0.05$) higher compared with the lungs MDA concentration in normal control rats (group A, 0.05 ± 0.01 µM/mg protein). Brain MDA concentration was not statistically different ($p>0.05$) in rats treated with the extracts compared with the hyperlipidaemic control rats. Also, there were no significant differences ($p>0.05$) in heart and kidney MDA concentrations among the four experimental groups.

The concentrations of conjugated dienes (CD) in the various tissues are as shown in Figure 11. Tissue CD concentrations were generally higher in rats fed high lipid diet (Groups B – D). However, CD concentration was lower in rats treated with MEPA (Group D, 0.42 ± 0.14) and declined ($p<0.05$) in the liver of rats treated with AEPA (Group C, 0.28 ± 0.15 µM/mg protein) compared with the hyperlipidaemic control rats (Group B, 0.66 ± 0.15 µM/mg protein). Also kidney CD concentration in rats treated with

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AEPA (Group C, 0.70 ± 0.06 µM/mg protein) was higher than the hyperlipidaemic control (Group B, 0.58 ± 0.08) and MEPA treated rats (Group D, 0.50 ± 0.09).

Heart CD concentration in hyperlipidaemic control rats (Group B, 0.58 ± 0.07 µM/mg protein) was higher than in rats treated with AEPA and MEPA (Groups C and D, 0.31 ± 0.02 and 0.47 ± 0.04µM/mg protein respectively).

Protein carbonyls concentrations were lower in the plasma and liver of treated rats compared with the hyperlipidaemic control (Fig. 12). Also, protein carbonyls were increased ($p < 0.05$) in hyperlipidaemic control compared to the normal control rats. However, rats treated with MEPA showed higher protein carbonyl concentration in the kidney and heart compared to the hyperlipidaemic control rats.

Reduced glutathione (GSH) levels in the four groups are shown in Figure 13. There was significant ($p<0.05$) depletion in the plasma GSH level in the hyperlipidaemic control rats (Group B: 0.67 ± 0.22 µM/mg protein) compared with normal control rats (Group A, 6.98 ± 1.0 µM/mg protein). However, treatment with AEPA and MEPA elicited a restoration ($p<0.05$) of GSH level (Group C and D: 5.58 ± 0.82 and 5.83 ± 1.34 µM/mg protein respectively).

Table 5 shows the concentration of GSH in the various tissues of rats in the four groups. Liver GSH concentration in rats treated with AEPA (Group C, 16.04 ± 1.63 µM/mg protein) was lower ($p<0.05$) compared to GSH levels in the hyperlipidaemic control, normal control and MEPA treated rats (Groups B, A and D: 23.37 ± 2.83, 27.28 ± 2.44 and 24.98 ± 3.14 µM/mg protein respectively). Also, kidney GSH levels were significantly ($p<0.05$) lower in rats treated with AEPA (Group C, 17.43 ± 1.62 µM/mg protein) compared to normal control rats (Group A, 24.40 ± 2.04 µM/mg protein). Heart

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GSH level was decreased ($p<0.05$) in hyperlipidaemic control rats (Groups B. 13.07 ± 1.18) compared to normal control rats (Group A: 27.12 ± 1.89 μM/mg protein). Treatment with AEPA and MEPA increased heart GSH level (Groups C and D. 17.85 ± 2.02, 19.31 ± 1.68 and 24.87 ± 2.44 μM/mg protein respectively) compared to the untreated rats. Brain GSH concentration was lowest in rats treated with MEPA (Group D: 0.89 ± 0.21 μM/mg protein).

Lungs GSH concentration showed a decline in hyperlipidaemic control rats (Groups B. 17.85 ± 2.02 μM/mg protein) and rats treated with AEPA (Group C. 19.31 ± 1.68 μM/mg protein) compared with normal control and MEPA treated rats (29.58 ± 3.42 and 24.87 ± 2.44 μM/mg protein respectively).

Plasma and tissue catalase (CAT) activities are shown in Table 6. Plasma CAT activity was not significantly different ($p>0.05$) across the groups. Liver CAT activity was higher ($p<0.05$) in rats treated with MEPA (Group D. 4.78 ± 1.04 μM/mg protein) than the hyperlipidaemic control rats (Group B. 1.34 ± 0.41 μM/mg protein), normal control (Group A. 1.82 ± 0.26 μM/mg protein) and rats treated with AEPA (Group C. 0.85 ± 0.24 μM/mg protein). Also, liver CAT activity was significantly lower ($p<0.05$) in rats treated with AEPA than in normal control rats.

There were no significant differences ($p>0.05$) in kidney, heart and brain CAT activity among the groups. Lungs CAT activity showed a significant ($p<0.05$) decrease in the hyperlipidaemic control rats (1.19 ± 0.19 μM/mg protein) and in rats treated with MEPA (1.57 ± 0.38 μM/mg protein) compared to normal control (3.89 ± 0.88 μM/mg protein).

RBC and liver Glutathione Peroxidase (GSHPx) activities are shown in Figures 14 and 15 respectively. There was a decrease in RBC GSHPx activity in rats fed high lipid diet

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(Groups B, C and D: 60.32 ± 4.79, 35.00 ± 3.99 and 37.62 ± 9.58 U/mg protein respectively) compared to normal control rats (Group A, 121.02 ± 26.67 U/mg protein). RBC GSHPx activity was significantly ($p<0.05$) lower in rats treated with AEPA compared to the hyperlipidaemic and normal control rats.

Similarly, liver GSHPx activity declined ($p<0.05$) in rats fed high lipid diets (Groups B, C and D: 107.82 ± 33.71, 31.75 ± 6.36 and 38.01 ± 0.90 U/mg protein respectively) compared to normal control rats (Group A, 215. 13 ± 36.26 U/mg protein).

Table 7 shows plasma and tissue SOD activity. There were no significant ($p>0.05$) differences in the plasma SOD activity across the four experimental groups. However, plasma SOD activity was lowest in the hyperlipidaemic control rats (Group B, 2.30 ± 0.49 U/mg protein) and highest in the normal control rats (Group A, 3.29 ± 0.64 U/mg protein). Liver SOD activity was elevated in the hyperlipidaemic control rats (Group B, 5.48 ± 0.55 U/mg protein) and in rats treated with MEPA (Group D, 6.51 ± 1.32 U/mg protein) when compared with normal control (Group A, 4.80 ± 0.66 U/mg protein). Kidney SOD activity was lower in the treated rats (Groups C and D, 3.65 ± 0.47 and 3.44 ± 0.63 U/mg protein respectively) than in the hyperlipidaemic control rats (Group B, 4.29 ± 0.59 U/mg protein) and normal control rats (Group A, 4.43 ± 0.38 U/mg protein). Also, heart SOD activity was reduced in rats fed high lipid diet (Groups B, C and D, 3.88 ± 0.54, 4.50 ± 0.14 and 4.13 ± 0.22 U/mg protein respectively) compared with normal control rats (Group A, 6.49 ± 0.98 U/mg protein), with the heart SOD activity in the hyperlipidaemic control rats being lower than the treated rats and significantly lower ($p<0.05$) than in normal control rats. There were no significant ($p<0.05$) differences in the brain and lungs SOD activities across the four experimental groups although lungs

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SOD activity was slightly lower in the hyperlipidaemic control rats than in the other groups.

Conclusion

Treatment with AEPA and MEPA lowered plasma MDA concentration in the rats suggesting that the extracts have antiperoxidative effect. The leaf extract of *P. americana* was able to reduce conjugated diene concentration in the liver and heart of the rats suggesting that it protects against oxidative damage in these tissues.

AEPA reduced protein carbonyls in all the tissues while MEPA reduced protein carbonyls in the plasma and liver of rats. It could be concluded that the leaf extract of *P. americana* would inhibit oxidation of proteins during oxidative stress.

Treatment with AEPA and MEPA caused repletion of GSH in the hyperlipidaemic rats thus increasing the antioxidant status in circulation. However, MEPA was more effective than AEPA in the liver, lungs and kidney.

Treatment with AEPA and MEPA did not produce substantial changes in the activity of CAT in the tissues of hyperlipidaemic rats. However, CAT activity was elevated in the lungs and heart of rats treated with AEPA while treatment with MEPA raised CAT activity in the liver and kidney. The increase in CAT activity indicates enhancement of the antioxidant enzyme.

The decline in GSHPx in rats treated with AEPA and MEPA and this could be attributed to the involvement of GSHPx in free radical scavenging in the rats.

The decrease in plasma SOD activity suggests its involvement in scavenging the free radicals generated in the animals.

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TABLE [illegible] Effect of aqueous and methanolic leaf extracts of P. americana on tissue malondialdehyde concentration (µM/mg protein) in rats fed high lipid diet

	Group			
	A	B	C	D
Liver	0.22 ± 0.02	0.32 ± 0.08[b]	0.41 ± 0.06[b]	0.50 ± 0.04[b]
Kidney	0.07 ± 0.01	0.07± 0.01	0.08 ± 0.01	0.07 ± 0.01
Lung	0.05 ± 0.01	0.07 ± 0.04[b]	0.08 ± 0.02[a]	0.09 ± 0.02[a]
Heart	0.29 ± 0.11	0.30 ± 0.04	0.24 ± 0.10	0.32 ± 0.07
Brain	0.40 ± 0.02	0.41 ± 0.03[a]	0.37 ± 0.01[a]	0.32 ± 0.02[b]

Values are expressed as means ± SEM for six rats.
Values not sharing a common superscript differ significantly at $p < 0.05$.
A. fed standard chow; **B.** fed high lipid diet; **C.** fed high lipid diet + 10 mg kg^{-1} b.wt AEPA; **D.** fed high lipid diet + 10 mg kg^{-1} b.wt MEPA daily.

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TABLE 5.[illegible] Effect of aqueous and methanolic leaf extracts of *P. americana* on reduced glutathione levels (µM/mg protein) in rats fed high lipid diet

	Group			
	A	B	C	D
Liver	27.28 ± 2.44	23.37± 2.83[a]	16.04 ± 1.63[b]	24.98 ± 3.14[a]
Kidney	24.40 ± 2.04	21.48 ± 1.37	17.43 ± 1.62[a]	20.94 ± 1.11
Lung	29.58 ± 3.42	17.85 ± 2.02[b]	19.31 ± 1.68[b]	24.87± 2.44
Heart	27.12 ± 1.89	13.07 ± 1.18[a]	19.60 ± 0.36	18.85 ± 0.60
Brain	1.39 ± 0.10	1.96 ± 0.45	1.54 ± 0.26	0.89 ± 0.21[b]

Values are expressed as means ± SEM for six rats.
Values not sharing a common superscript differ significantly at $p < 0.05$.
A. fed standard chow; B. fed high lipid diet; C. fed high lipid diet + 10 mg kg^{-1} b.wt AEPA; D. fed high lipid diet + 10 mg kg^{-1} b.wt MEPA daily.

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TABLE 6. Plasma and tissue catalase concentrations (µM /mg protein) in rats fed high lipid diet

	Group			
	A	B	C	D
Plasma	0.90 ± 0.25	0.71 ± 0.19	0.76 ± 0.17	0.79 ± 0.38
Liver	1.82 ± 0.26	1.34 ± 0.41^{a}	0.85 ± 0.24^{a}	4.78 ± 1.04^{b}
Kidney	1.18 ± 0.24	0.87 ± 0.34	0.65 ± 0.13	0.97 ± 0.20
Lung	3.89 ± 0.88	1.19 ± 0.19^{b}	2.43 ± 0.63	1.57 ± 0.38
Heart	1.77 ± 0.28	1.50 ± 0.46	1.86 ± 0.79	1.26 ± 0.40
Brain	1.26 ± 0.53	0.87 ± 0.19	0.94 ± 0.25	1.56 ± 0.33

Values are expressed as means ± SEM for six rats.
Values not sharing a common superscript differ significantly at $p < 0.05$.
A, fed standard chow; B, fed high lipid diet; C, fed high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, fed high lipid diet + 10 mg kg^{-1} b.wt MEPA daily

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TABLE 7. Effect of aqueous and methanolic leaf extracts of P. americana on plasma and tissue superoxide dismutase concentrations in rats fed high lipid diet

	Group			
	A	B	C	D
Plasma	3.29 ± 0.64	2.30 ± 0.49	2.69 ± 0.34	2.41 ± 0.12
Liver	4.80 ± 0.66	5.48 ± 0.55	4.62 ± 0.84	6.51 ± 1.32
Kidney	4.43 ± 0.38	4.29 ± 0.59	3.65 ± 0.47	3.44 ± 0.63
Lung	4.43 ± 0.89	3.05 ± 0.40	4.12 ± 0.89	4.16 ± 0.59
Heart	6.49 ± 0.98	3.88 ± 0.54[a]	4.50 ± 0.14	4.13 ± 0.22
Brain	2.29 ± 0.40	2.12 ± 0.36	2.58 ± 0.37	2.30 ± 0.31

Values are expressed as means ± SEM for six rats.
[a]Significantly different from normal control $p \leq 0.05$.
A, fed standard chow; B, fed high lipid diet; C, fed high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, fed high lipid diet + 10 mg kg^{-1} b.wt MEPA daily.

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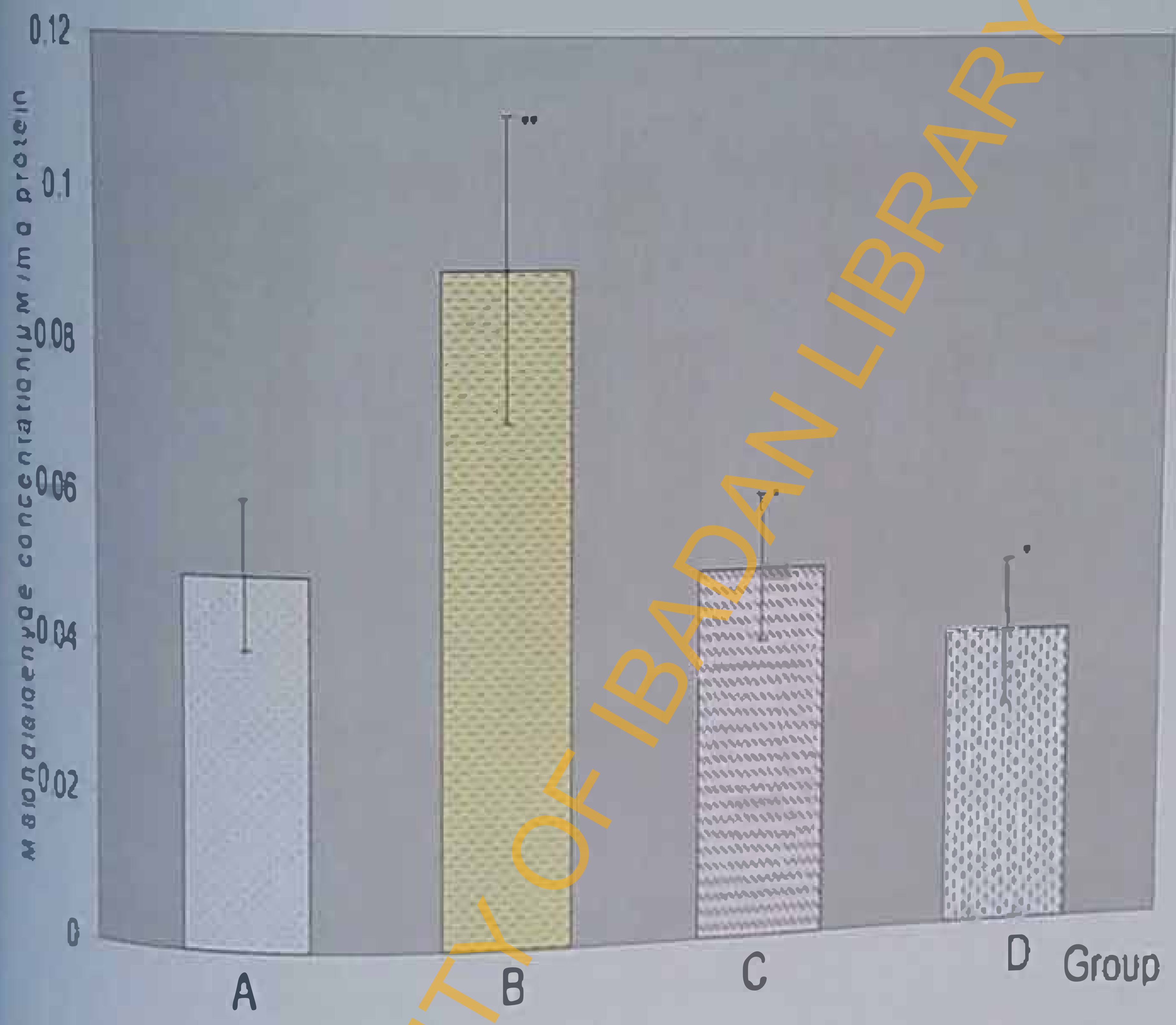

Fig.10. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma malondialdehyde in rats fed high lipid diet.

Values are means ± SEM (n = 6)
** Significantly different from normal control and treated rats ($p<0.05$)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b.wt MEPA.

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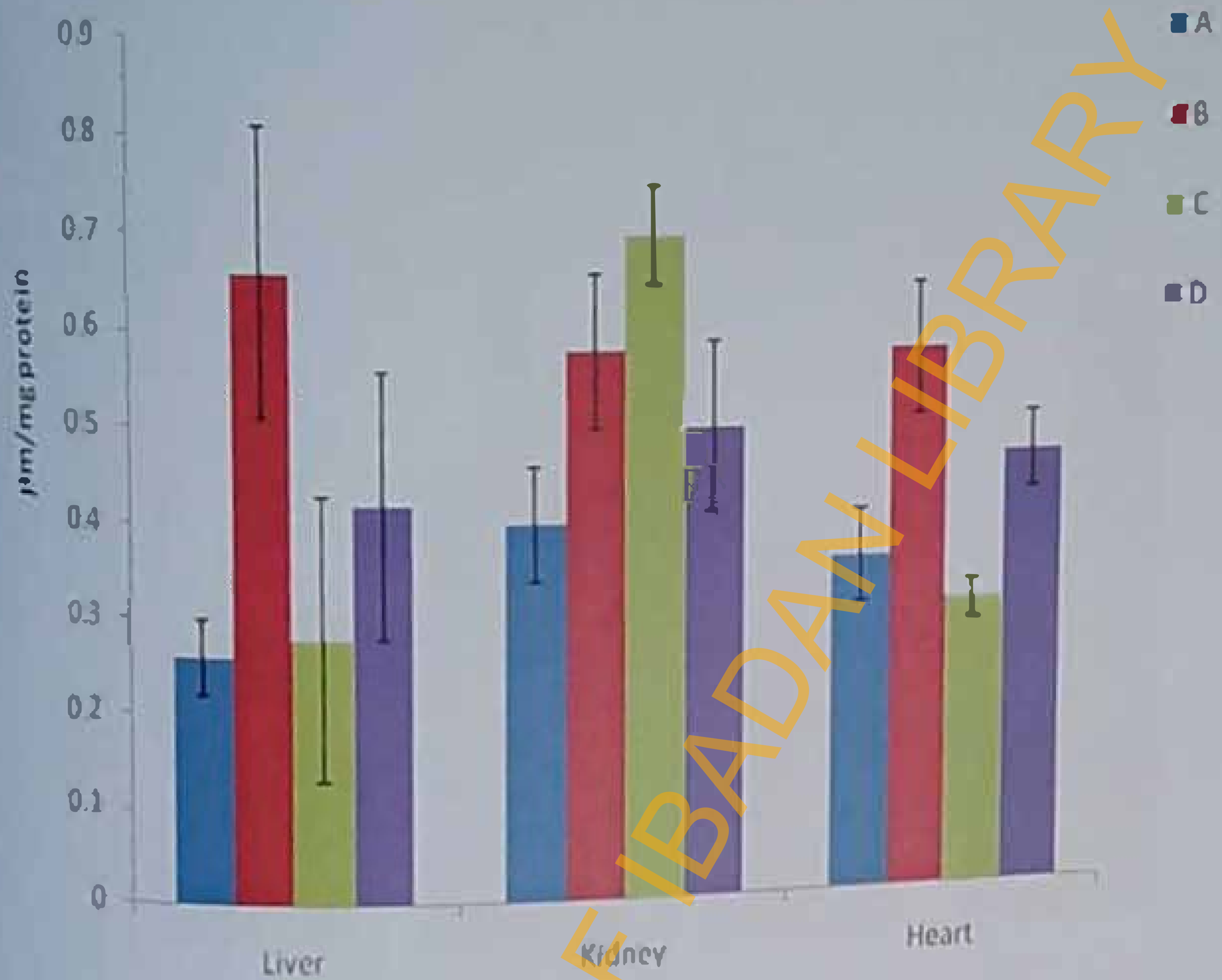

Fig.11. Effect of aqueous and methanolic leaf extracts of *P. americana* on liver, kidney and heart conjugated dienes in rats fed high lipid diet.

Values are means ± SEM (n = 6)
A, standard rat chow; **B**, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; **D**, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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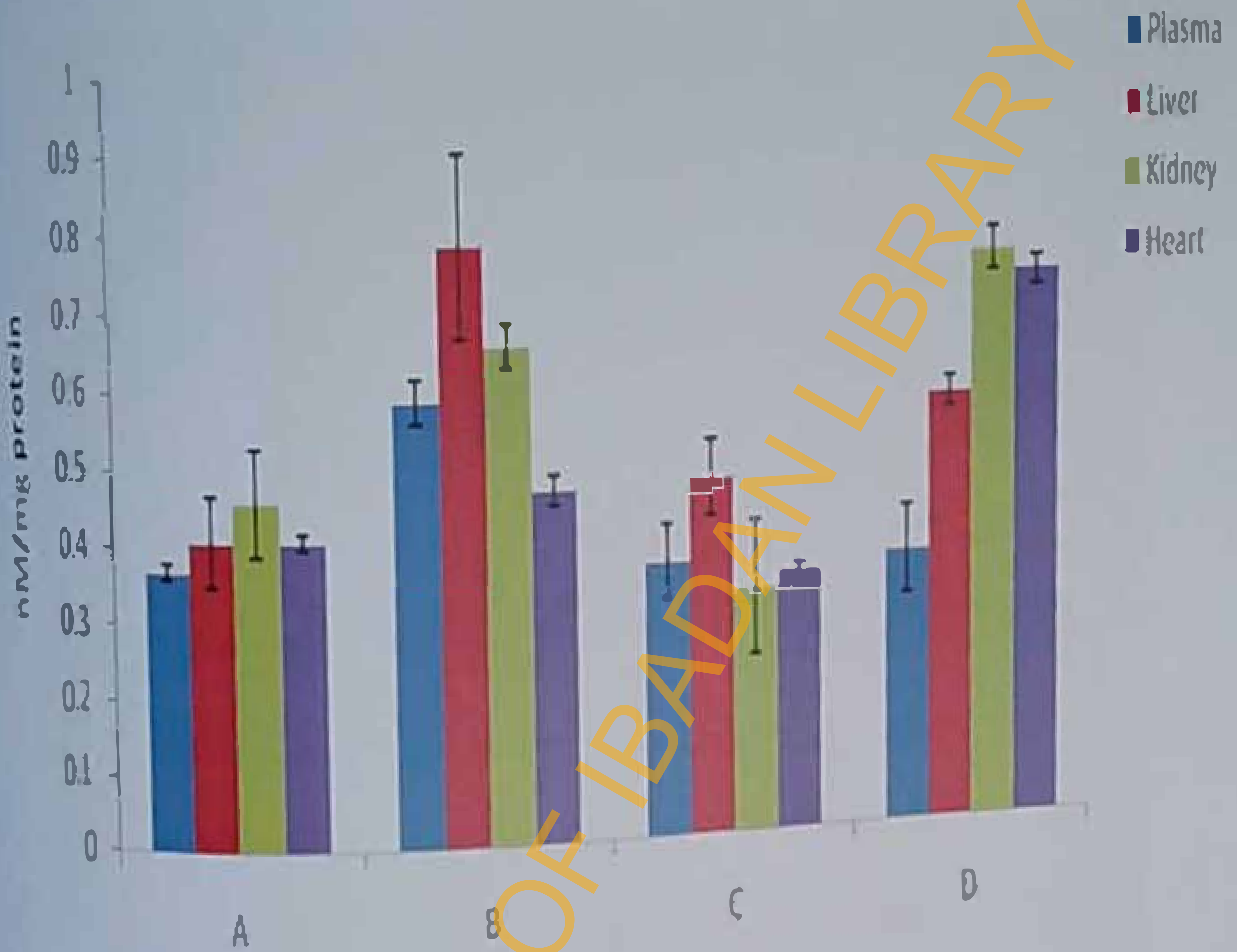

Fig.12: Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma, liver, kidney and heart protein carbonyl content in rats fed high lipid diet.

Values are means ± SEM (n = 6)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b.wt MEPA.

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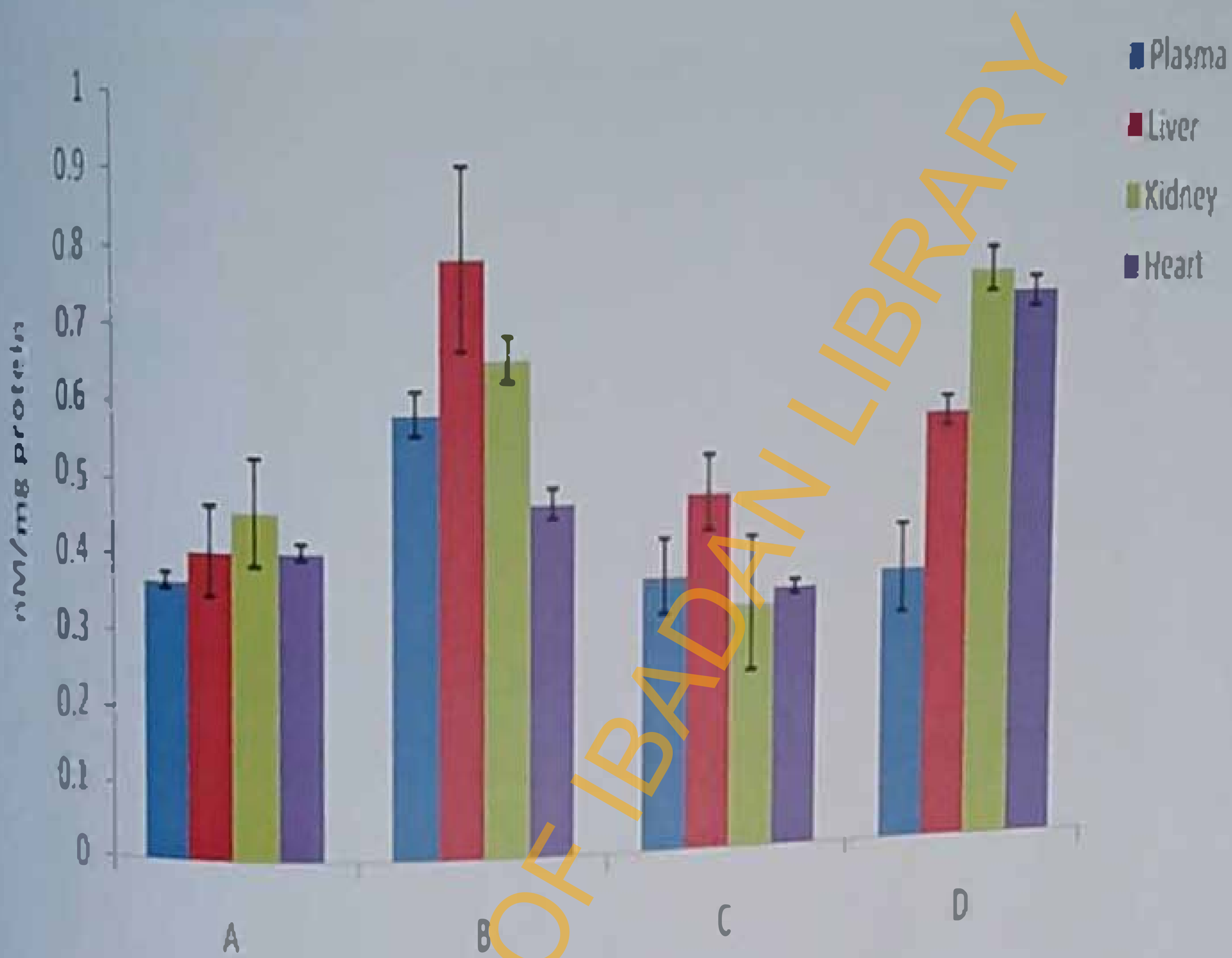

Fig 12. Effect of aqueous and methanolic leaf extracts of *P. americana* on plasma, liver, kidney and heart protein carbonyl content in rats fed high lipid diet.

Values are means ± SEM (n = 6)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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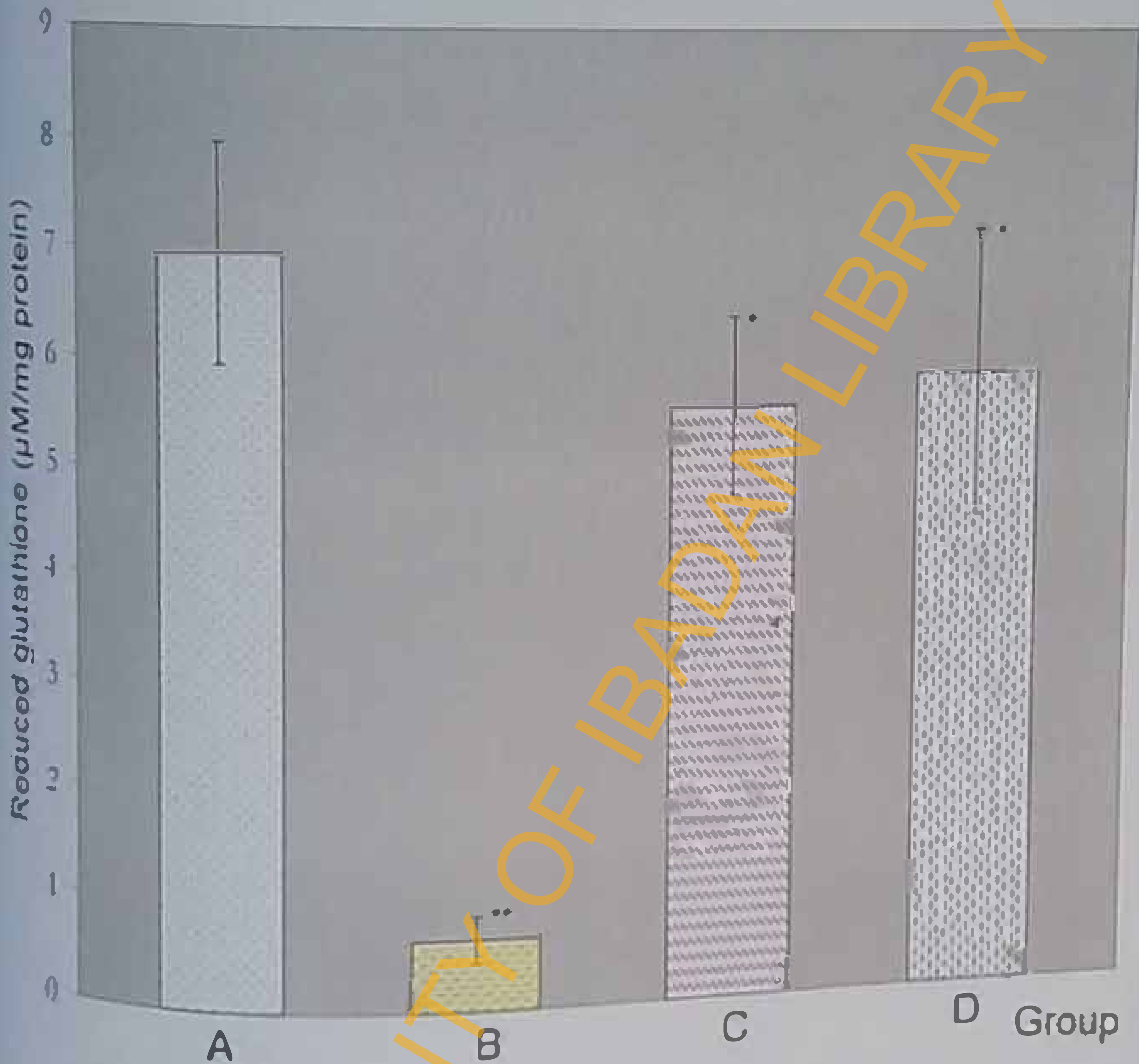

Fig.13. Effect of aqueous and methanolic leaf extracts of *P. americana* on red blood cell reduced glutathione in rats fed high lipid diet.

Values are means ± SEM (n = 6)
** Significantly different ($p<0.05$)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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Glutathione peroxidase activity (U/gmHb)

160
140
120
100
80
60
40
20
0

A B C D Group

Fig 14. Effect of aqueous and methanolic leaf extracts of *P. americana* on red blood cell glutathione peroxidase activity in rats fed high lipid diet

Values are means ± SEM (n = 6)

** Significantly higher than treated rats ($p<0.05$)

A, standard rat chow; B, high lipid diet; C, high libid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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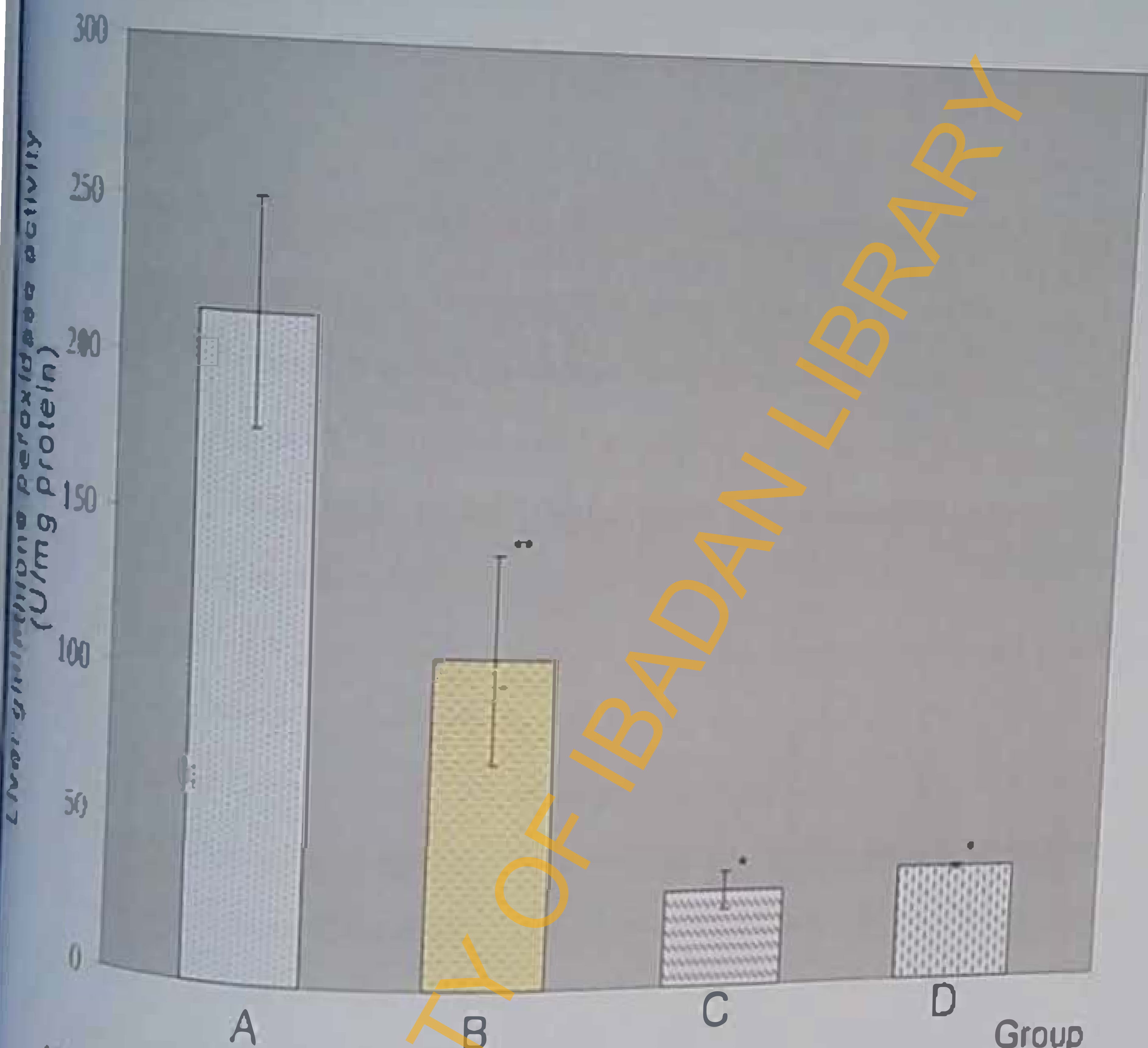

Fig.15. Effect of aqueous and methanolic leaf extracts of *P. americana* on liver glutathione peroxidase activity in rat fed high lipid diet

Values are means ± SEM (n = 6)
** Significantly higher than treated rats ($p<0.05$)
A, standard rat chow; B, high lipid diet; C, high lipid diet + 10 mg kg^{-1} b.wt AEPA; D, high lipid diet + 10 mg kg^{-1} b. wt MEPA.

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4.3 Experiment 3: Hepatoprotective activity of aqueous leaf extract of *P. americana* on CCl_4-induced hepatotoxicity in rats.

Introduction

The experimental intoxication induced by CCl_4 is widely used for modeling liver injury in rats. Hepatotoxicity is connected with severe impairment of cell protection mechanisms. The liver is the principal site for CCl_4-induced effects to manifest themselves. It is generally accepted that the hepatotoxicity of CCl_4 is the result of cytochrome P-450-dependent reductive dehalogenation to form a highly reactive trichloromethyl free radical, $CCl_3^{\cdot}$ (McCay *et al.*, 1984).

In the presence of oxygen, the $CCl_3^{\cdot}$ radical is converted to the trichloromethyl peroxy radical, $CCl_3OO^{\cdot}$ which is more reactive and thus more short-lived than the $CCl_3^{\cdot}$ radical (Mico and Pohl, 1983). $CCl_3OO^{\cdot}$ is far more likely than $CCl_3^{\cdot}$ to abstract a hydrogen from PUFA thereby initiating the process of lipid peroxidation, a complex series of reactions that terminate in the complete disintegration of the PUFA molecule with the formation of aldehydes, other carbonyls and alkanes (Forni *et al.*, 1983; Cheeseman *et al.*, 1985; Comporti, 1985; Tribble *et al.*, 1987).

CCl_4-induced damage is characterized by hepatocyte membrane damage caused by lipid peroxidation, increased plasma levels of hepatic enzymes such as AST, ALP and ALT, fatty degeneration (steatosis i.e. accumulation of triglycerides in the liver), reduced β-oxidation of fatty acids and necrosis. Thus, quantitative measurements of plasma levels of liver enzymes, total cholesterol and hepatic triglyceride level, together with histopathological examination of hepatocytes provide a good assessment of the extent of liver damage or regeneration when challenged with CCl_4.

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Plant derived natural products have received considerable attention in recent years due to their diverse pharmacological properties including antioxidants and hepatoprotective activity (Banskota *et al.*, 2000; Takeoka and Dao, 2003).

This experiment was designed to evaluate the hepatoprotective and antioxidative properties of AEPA against CCl4-induced hepatotoxicity in rats.

Procedure

Hepatotoxicity was induced in rats by treatment with CCl_4 as described in section 3.4.3. Blood samples were collected by cardiac puncture into plain sterile tubes and allowed to coagulate. The serum was separated by centrifugation at 3,000 rpm for 10 min at 4°C. A portion of the blood was placed in heparinized tubes for determination of some haematological parameters.

After sacrificing the rats the livers were quickly excised and perfused with chilled 1.15 % (w/v) KCl solution in order to remove all traces of haemoglobin. The livers were blotted dry, weighed and stored at -80°C pending analysis. Some portions of the livers were preserved in 10 % Formol saline for histopathological analysis.

Hepatoprotective activity of *P. americana* was assessed by the estimation of the activities of serum alkaline phosphatase and transaminases and the levels of serum total bilirubin. The activities of the enzymes AST, ALT and ALP were determined as previously described in sections 3.4.12 to 3.4.14. Also total bilirubin was determined according to the procedure outlined in section 3.4.15. Finally, the levels of the enzymes CAT, SOD, GSHPx and GST were estimated as described in sections 3.4.20 to 3.4.23.

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Hepatoprotective activity of the extract was calculated according to the formula of Singh *et al.* (1998).

$$\text{Hepatoprotective activity (\%)} = 1 - \frac{[PC - W]}{[C - W]} \times 100$$

where,

PC, C, and W are the measurable variables in rats treated with *P. americana* leaf extract plus CCl_4, CCl_4 and distilled water treated animals respectively.

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Results

The effects of AEPA on serum AST, ALT, ALP activities and total bilirubin concentration in CCl_4-intoxicated rats are shown in Table 8. Intoxication with CCl_4 caused hepatocellular damage as shown by elevation ($p < 0.05$) in serum AST (83 %), ALT (586 %) and ALP (195 %) compared to normal control. However, pre-treatment of rats with AEPA (100 mg kg^{-1} b. wt) protected against CCl_4-induced hepatotoxicity as evidenced by reductions in serum AST (43 %), ALT (66 %) and ALP (28 %) compared to CCl_4 control. Similarly, pre-treatment with 200 mg kg^{-1} b. wt AEPA caused significant reductions ($p<0.05$) in serum AST (57 %), ALT (63 %) and ALP (20 %) compared to CCl_4 control. Pre-treatment with the standard drug Reducdyn® also resulted in significant decreases ($p<0.05$) in the activities of these enzymes (AST, 51 %; ALT, 69 % and ALP, 37%) compared to CCl_4 control. Total bilirubin was significantly elevated ($p<0.05$) following intoxication of rats with CCl_4. Pre-treatment of rats with 100 and 200 mg kg^{-1} b. wt AEPA resulted in substantial decrease in total bilirubin (36 % and 78 % respectively) in the treated compared to the CCl_4 control rats.

Also pre-treatment with the standard drug Reducdyn® decreased serum total bilirubin by 57 % compared to CCl_4 control.

The results of the calculation of hepatoprotection as provided by Reducdyn® and AEPA are presented in Table 9. The calculated percentage protection shows that both Reducdyn® and the extract were hepatoprotective. The calculated hepatoprotective activity of AEPA at a concentration of 100 mg kg^{-1} b. wt was 94 % for AST, 77 % for ALT, 43 % for ALP and 49 % for total bilirubin while the hepatoprotective activity at a concentration of 200 mg kg^{-1} b. wt was 127 % for AST, 74 % for ALT, 30 % for ALP

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and 106 % for total bilirubin. These results are comparable to the hepatoprotective activity obtained by pre-treatment of rats with the standard drug Reducdyn®.

Table 10 shows the effect of pre-treatment with AEPA on liver antioxidant enzymes in CCl_4-intoxicated rats. CCl_4 administration resulted in significant elevation ($p < 0.05$) in the activities of CAT and SOD (294 % and 155 % respectively) compared to control rats. Pre-treatment with AEPA at 100 mg and 200 mg kg^{-1} b. wt produced significant reduction ($p<0.05$; 55 % and 63 % respectively) in liver CAT activity.

Similarly, SOD activity was significantly decreased ($p < 0.05$) by pre-treatment with 100 mg and 200 mg kg^{-1} b. wt AEPA (58 % and 56 % respectively).

Liver GSHPx activity was slightly increased in CCl_4-intoxicated rats. However, pre-treatment of rats with AEPA and Reducdyn® decreased GSHPx activity to lower levels than normal control rats.

Liver GST was markedly decreased ($p < 0.05$, 46 %) in CCl_4-intoxicated rats compared to normal control. Pre-treatment with 100 mg and 200 mg kg^{-1} b. wt AEPA caused an increase (37 % and 13 % respectively) in GST activity compared to CCl_4 control animals while pre-treatment with Reducdyn® increased GST activity by 36 %.

Serum GSH concentration was elevated ($p < 0.05$, 109 %) by CCl_4 intoxication but pre-treatment with 100 mg and 200 mg kg^{-1} b. wt AEPA reduced GSH concentration by 25 % and 42 % respectively compared with CCl_4 control (Fig. 16). Pre-treatment with the standard drug Reducdyn® decreased GSH concentration by 45 %.

There was no significant difference ($p>0.05$) in liver GSH concentration across the groups (Fig. 17).

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MDA levels and protein carbonyl content are shown in Tablel3. Liver MDA and protein carbonyl concentrations in CCl_4-intoxicated rats increased by 128% and 61% respectively compared to normal control rats. Pre-treatment with AEPA and Reducdyn® provoked significant ($p<0.05$) reductions in tissue MDA and protein carbonyls compared to CCl_4 control.

Table 14 shows some haematological parameters observed in the experimental animals. There was a decrease ($p > 0.05$) in the packed cell volume and haemoglobin concentration of CCl_4-treated rats compared to normal control. Also, total white blood cells (WBC) counts and neutrophils were significantly reduced ($p < 0.05$) while lymphocytes were increased by CCl_4 administration compared to normal control. Pre-treatment with 100 mg and 200 mg kg^{-1} b. wt AEPA restored WBC counts while pre-treatment with 100 mg kg^{-1} b. wt AEPA only increased neutrophils and lowered lymphocytes counts.

Results of the histopathological examination are depicted in Plates 1 – 5. Liver sections from normal control rats revealed hepatocytes with numerous portal tracts dividing them into lobules. Livers of CCl_4-treated rats showed marked widespread necrosis of hepatocytes with areas of fatty change, ballooning degeneration and diffuse mononuclear infiltration. However, pre-treatment with 100 mg and 200 mg kg^{-1} b. wt AEPA reduced the severity of hepatic damage as shown by the mild, diffuse fatty change and less periportal necrosis.

Conclusion

CCl_4 intoxication caused marked increases ($p<0.05$) in the activities of AST, ALT and ALP in the rats. Also the concentration of total bilirubin was significantly increased after

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CCl_4 administration. These increases indicate cellular leakage and loss of functional integrity of the membrane resulting from liver damage.

The significant reduction in liver enzymes and bilirubin after pre-treatment with AEPA suggests that the extract is hepatoprotective. Also, the reduction in the severity of necrosis and fatty infiltration shows that pre-treatment with AEPA has hepatoprotective activity against CCl_4-induced liver damage in the rat.

Pre-treatment with AEPA decreased the activities of CAT, SOD and GSHPx enzymes that were raised by CCl_4-intoxication. The extract may have scavenged the free radicals generated thereby decreasing lipid peroxidation and oxidative stress in the animals.

The elevation of serum GSH in this study may be due to free radical generated by CCl_4-intoxication. Pretreatment with AEPA decreased GSH concentration that was elevated in response to the toxicant.

The increase in lipid peroxidative products resulting from CCl_4-intoxication was substantially reversed by pre-treatment with AEPA showing that the extract possesses antiperoxidative properties.

CCl_4-intoxication caused leucopenia and lymphocytosis in the animals. Pre-treatment with AEPA ameliorated these conditions. It could therefore be suggested that AEPA has the potential to restore CCl_4-induced alterations of hematological parameters in rat.

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TABLE 8. Effect of pre-treatment with aqueous leaf extract of *P. americana* on CCl_4-induced liver damage in rats

Treatment (Dose, mg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	TBL (µmol/L)
Control	64.18 ± 11.63	11.74 ± 3.25	27.63 ± 9.32	12.75 ± 6.19
CCl_4 + AEPA (100)	67.36 ± 15.04[a]	27.74 ± 9.34[a]	58.52 ± 8.0	31.12 ± 8.07[a]
CCl_4 + AEPA (200)	50.05 ± 5.36[a]	29.68 ± 5.18[a]	65.57 ± 3.75	10.78 ± 1.50[b]
CCl_4 + Reducdyn (100)	57.05 ± 1.53[a]	24.87 ± 4.70[a]	51.46 ± 8.62	21 .08 ± 2.73[b]
CCl_4 only	117.44 ± 20.74[b]	80.52 ± 23.80[b]	81.51 ± 21.17	48.46 ± 18.87[a]

Values are expressed as means ± SEM (n = 6).
Values not sharing a common superscript differ significantly at $p<0.05$.

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TABLE 9. Hepatoprotective activity of aqueous leaf extract of *P. americana* against CCl_4-induced hepatotoxicity in rats

Liver Function Indicator	Pre-treatment		
	AEPA		Reducdyn
	(100 mg kg^{-1}) [% protection]	(200 mg kg^{-1}) [% protection]	(100 mg kg^{-1}) [% protection]
AST	94.03	126.53	113.39
ALT	76.70	73.92	80.91
ALP	42.67	29.58	55.77
Bilirubin	48.56	105.52	76.67

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TABLE 10. Effect of pre-treatment with aqueous leaf extract of *P. americana* on liver catalase, glutathione peroxidase, superoxide dismutase and glutathione S-transferase activities in CCl_4-induced hepatotoxicity in rats.

Treatment (Dose, $mg\ kg^{-1}$)	CAT ($U\ mg^{-1}$ protein)	GSHPx ($U\ mg^{-1}$ protein)	SOD ($\mu M\ mg^{-1}$ protein)	GST ($\mu M\ mg^{-1}$ protein)
Control	0.17 ± 0.01	147.63 ± 50.98	42.80 ± 1.28	26.18 ± 1.75
CCl_4 + AEPA (100)	0.30 ± 0.01^a	135.78 ± 14.41^a	46.40 ± 0.85^a	19.46 ± 1.05
CCl_4 + AEPA (200)	0.25 ± 0.06^a	136.44 ± 13.82^a	47.46 ± 1.40^a	15.99 ± 1.20
CCl_4 + Reducdyn (100)	0.18 ± 0.01^a	122.95 ± 34.38^b	43.30 ± 1.50^a	19.31 ± 0.84
CCl_4	0.67 ± 0.20^b	157.03 ± 6.48^a	108.93 ± 18.29^b	14.21 ± 1.07

Values are expressed as means ± SEM (n = 6).
Values not sharing a common superscript differ significantly at $p<0.05$.

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TABLE [illegible]: Effect of pre-treatment with aqueous leaf extract of P. americana on serum total protein, total cholesterol and triglycerides in CCl_4-induced hepatotoxicity in rats

Treatment (Dose, mg/kg)	T-CHOL (mg/dl)	TAG (mg/dl)	Total Protein (mg/g tissue)
Control	63.05 ± 4.76	59.65 ± 3.96	84.88 ± 2.33
CCl_4 + AEPA (100)	57.25 ± 2.66^b	44.68 ± 3.95^a	85.07 ± 1.39^a
CCl_4 + AEPA (200)	46.57 ± 7.10^b	46.28 ± 6.67^a	89.11 ± 1.85^a
CCl_4 + Reducdyn (100)	33.89 ± 0.33^a	58.42 ± 3.96^a	82.62 ± 3.60^a
CCl_4	70.79 ± 8.95^b	126.94 ± 11.63^b	38.79 ± 4.91^b

Values are expressed as means ± SEM (n = 6).
Values not sharing a common superscript differ significantly at $p<0.05$.

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TABLE 12: Effect of pre-treatment with aqueous leaf extract of P. americana on liver total protein, total cholesterol and triglycerides in CCl_4-induced hepatotoxicity in rats

Treatment (Dose, mg/kg)	T-CHOL (mg/dl)	TAG (mg/dl)	Total Protein (mg/g tissue)
Control	36.95± 8.93	176.42 ± 13.93	44.96 ± 0.73
CCl_4 + AEPA (100)	49.04 ± 3.8[b]	395.56 ± 53.55[a]	42.02 ± 0.44
CCl_4 + AEPA (200)	47.73 ± 7.98[b]	388.11 ± 31.05[a]	40.80 ± 0.72
CCl_4 + Reducdyn (100)	53.87 ± 9.07[b]	371.53 ± 20.51[a]	43.49 ± 0.35
CCl_4	113.13 ± 8.50[a]	795.50 ± 41.2[b]	36.77 ± 0.54

Values are expressed as means ± SEM (n = 6).
Values not sharing a common superscript differ significantly at $p<0.05$.

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TABLE 13. Effect of pre-treatment with aqueous leaf extract of *P. americana* on lipid peroxidation in CCl_4 -in toxicated rats

Treatment (Dose, mg/kg)	Liver MDA (μM/mg protein)	Liver Carbonyls (μM/mg protein)
Control	0.25 ± 0.06	4.96 ± 0.17
CCl_4 + AEPA (100)	0.27 ± 0.06[a]	3.23 ± 0.85[b]
CCl_4 + AEPA (200)	0.20 ± 0.01[a]	2.42 ± 0.14[b]
CCl_4 + Reducdyn (100)	0.28 ± 0.04[a]	1.99 ± 0.81[b]
CCl_4	0.57 ± 0.03[b]	7.99 ± 1.26[a]

Values are expressed as means ± SEM (n = 6).
Values not sharing a common superscript differ significantly at $p<0.05$.

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TABLE 14. Effect of pretreatment with aqueous leaf extract of *P. americana* on peripheral blood smears in CCl_4- intoxicated rats.

Treatment (Dose, mg kg^{-1})	WBC	PCV	Hb	N	L	M	E	B
Control	8666.67 ± 785.99	36.08 ± 1.54	12.03 ± 0.51	45.60 ± 8.15	55.80 ± 8.50	0.20 ± 0.20	0 ± 0	0 ± 0
CCl_4 + AEPA (100)	8700.00 ± 687.75	38.67 ± 3.25	12.89 ± 1.08	33.50 ± 6.48	66.17 ± 6.33	0.33 ± 0.21	0 ± 0	0 ± 0
CCl_4 + AEPA (200)	9683.33 ± 656.97	27.08 ± 3.10	9.77 ± 0.88	20.00 ± 2.97	77.60 ± 1.12	0.20 ± 0.20	0.40 ± 0.40	0 ± 0
CCl_4 + Reducdyn (100)	10066.67± 635.96	34.33 ± 1.45	11.61 ± 0.61	39.60 ± 6.42	60.20 ± 6.37	0.20 ± 0.20	0 ± 0	0 ± 0
CCl_4	4833.33 ± 811.04	33.50 ± 1.70	11.17 ± 0.57	22.60 ± 2.66	76.00 ± 2.68	0.60 ± 0.40	0.20 ± 0.20	0 ± 0

WBC, white blood cells (10^3/μL; PCV, packed cell volume (%); Hb, haemoglobin (g/dl); N, neutrophils (%); L, lymphocytes (%); M, monocytes (%); E, eosinophils (%); B, basophils (%).
All the values are presented as mean ± S.E.M.

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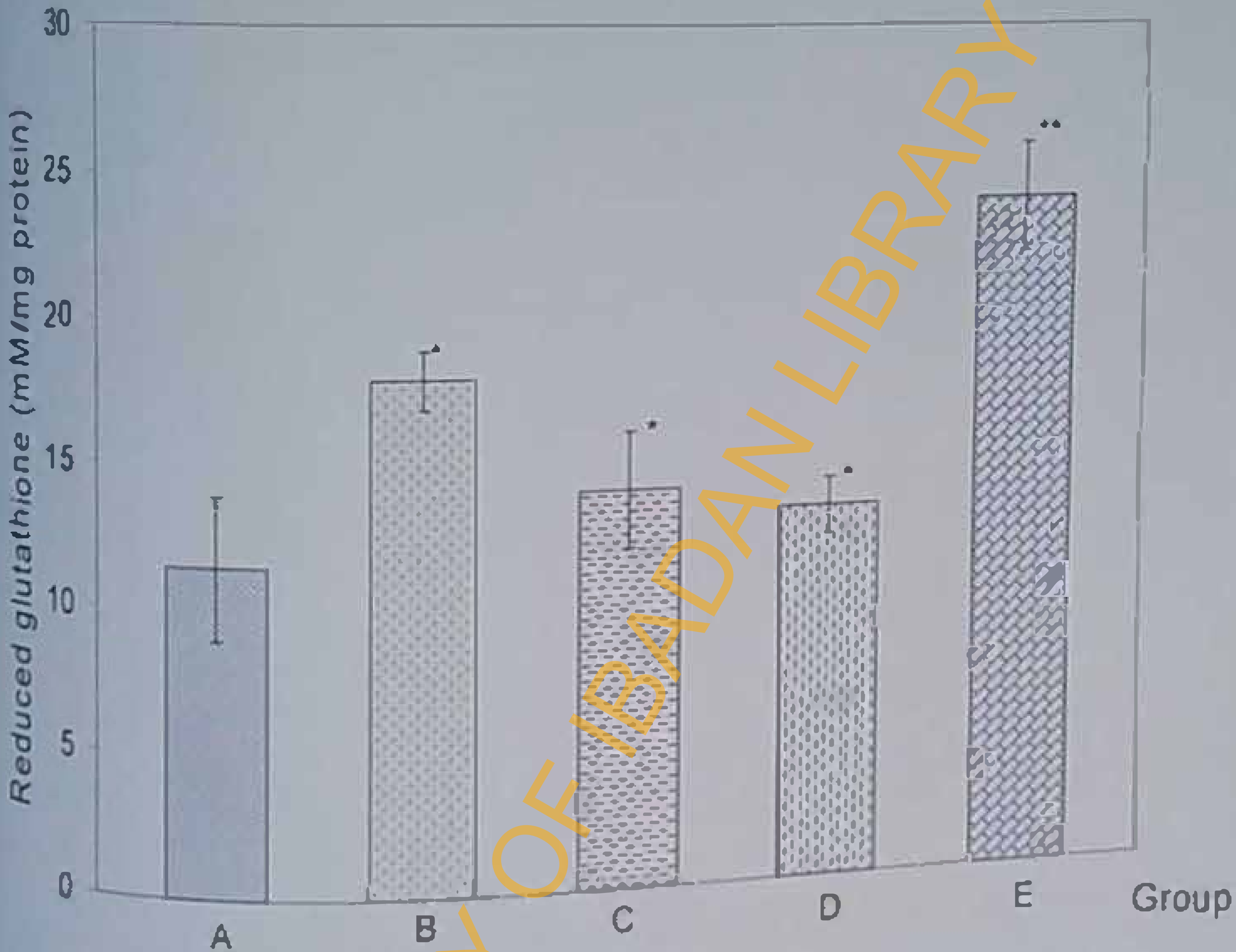

Fig. 16. Effect of aqueous extract of *P. americana* on reduced glutathione content in the serum of CCl_4-treated rats.

Values are expressed as means ± SEM (n = 6).
** Significantly different at $p<0.05$.
A, standard rat chow; B, CCl_4 + 100 mg kg⁻¹ b.wt AEPA ; C, CCl_4 + 200 mg kg⁻¹ b.wt AEPA; D, CCl_4 + 100 mg kg⁻¹ b.wt Reducdyn®; E, CCl_4 only.

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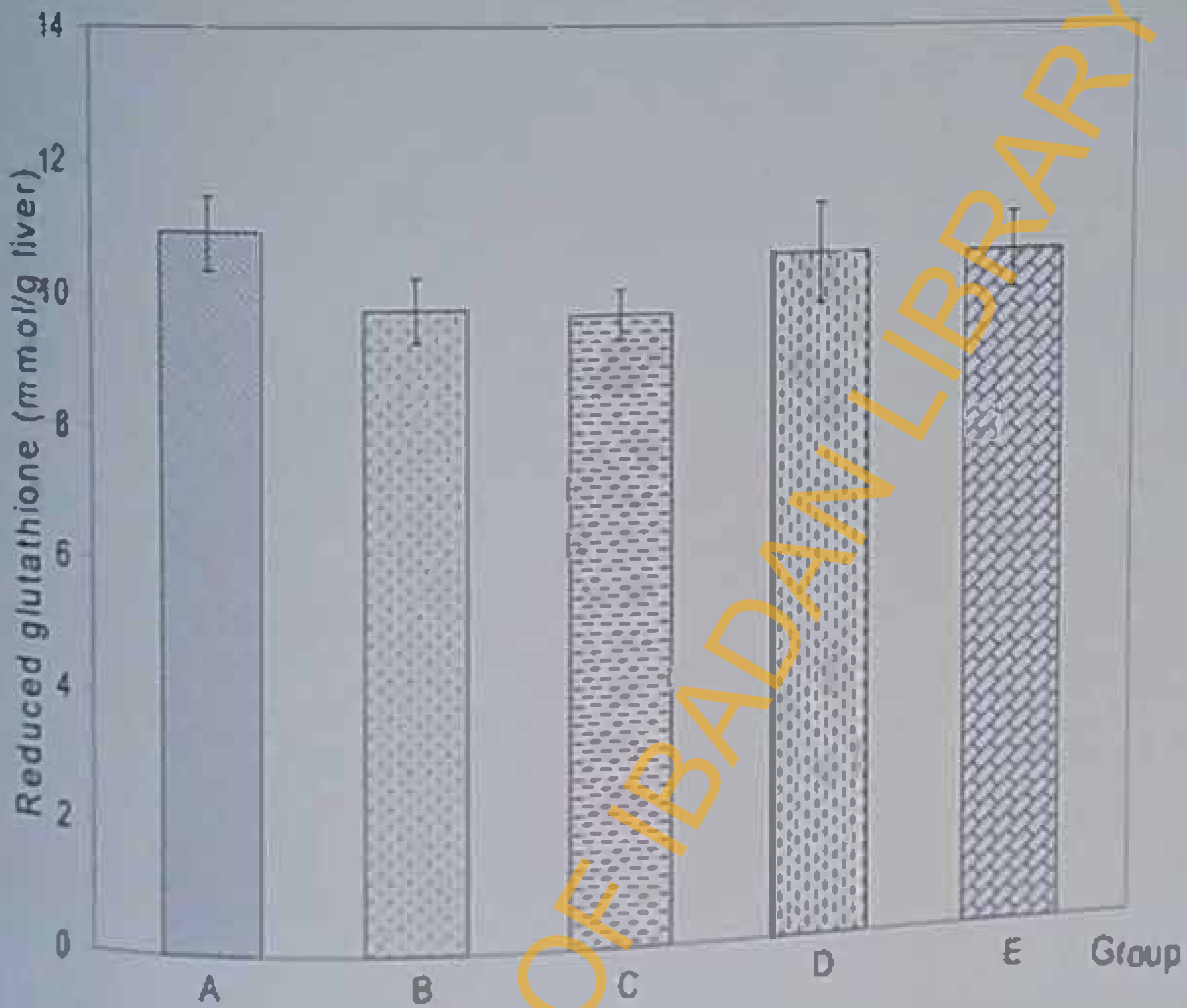

Fig. 17. Effect of aqueous extract of *P. americana* on reduced glutathione content in the liver of CCl_4-treated rats.

Values are expressed as means ± SEM (n = 6).
A, standard rat chow; **B**, CCl_4 + 100 mg kg^{-1} b.wt AEPA ; **C**, CCl_4 + 200 mg kg^{-1} b.wt AEPA; **D**, CCl_4 + 100 mg kg^{-1} b.wt Reducdyn®; **E**, CCl_4 only.

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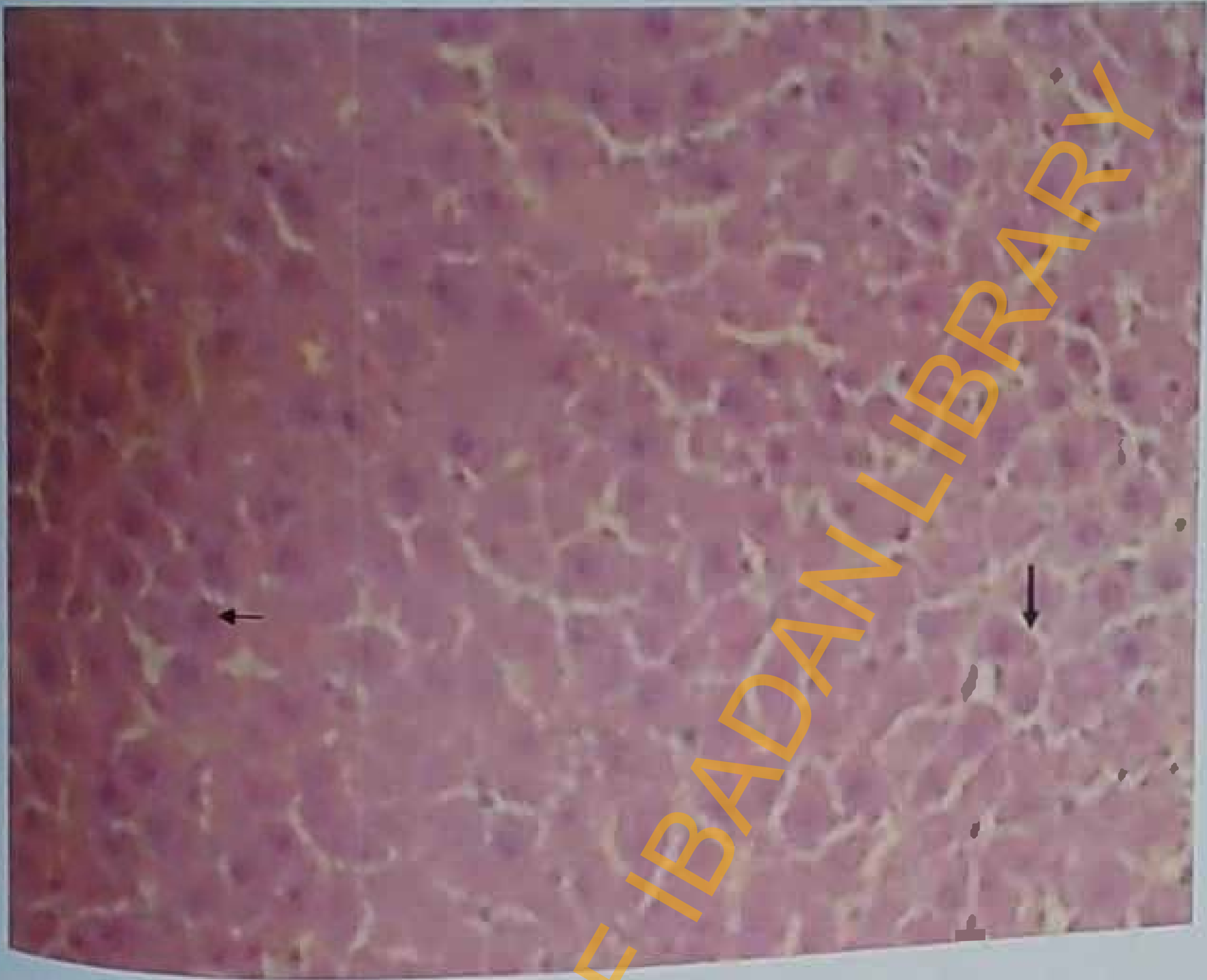

Plate 5. Liver section from normal rat showing normal liver architecture and hepatocytes (←) with numerous portal tracts (↓) dividing them into lobules (H&E, x 100).

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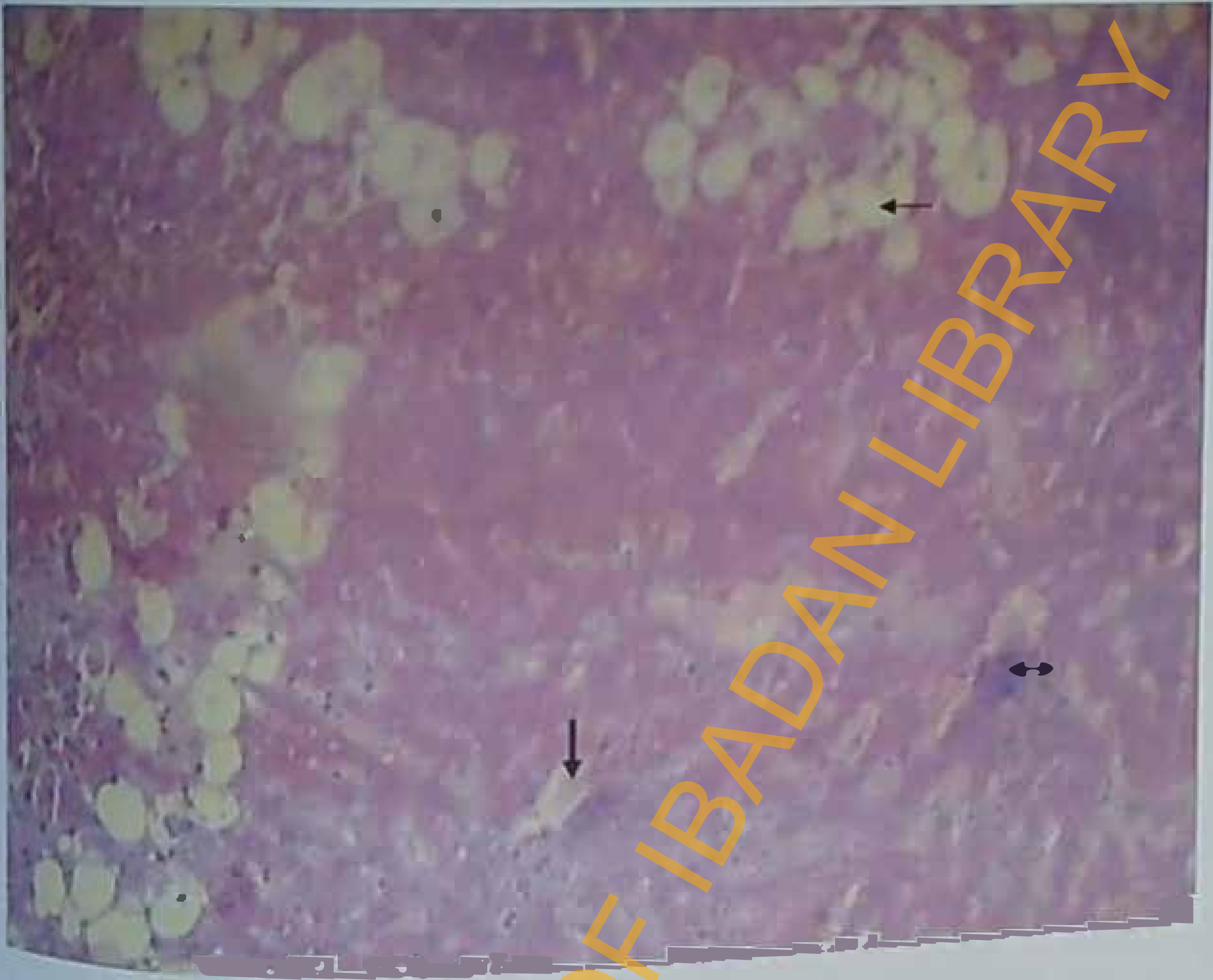

Plate 6. Liver section from CCl_4-treated rat pre-treated with 100 mg kg^{-1} b. wt AEPA showing mild, diffuse fatty change (←), periportal necrosis (↓) and mononuclear cell infiltration (↔) (H&E, x 100).

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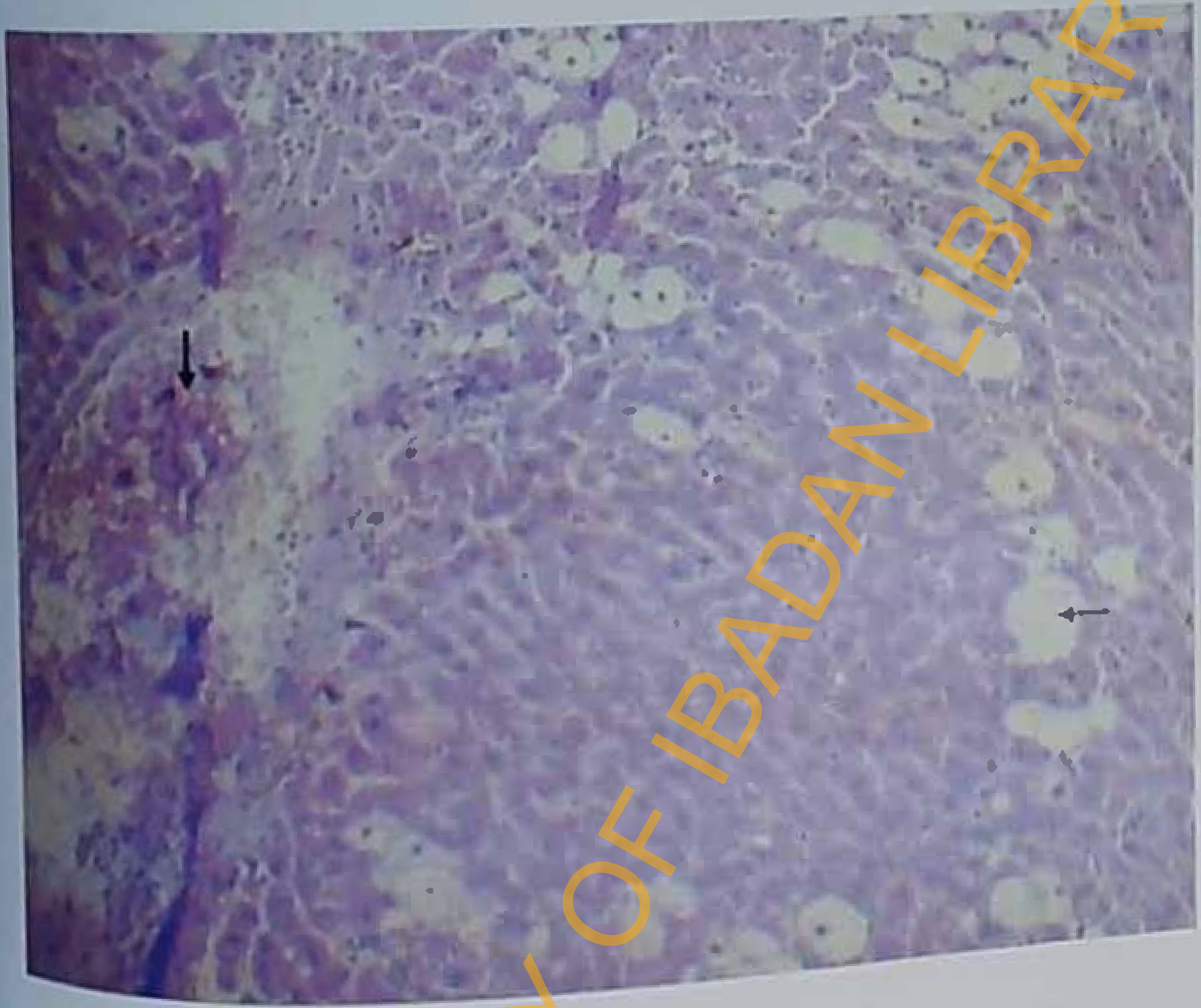

Plate 7. Liver section from CCl_4-treated rat pre-treated with 200 mg kg^{-1} b. wt AEPA showing mild, diffuse fatty change (◄—) with less periportal necrosis (↓) (H&E, x 100).

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Plate 8. Liver section from rat pre-treated with 100 mg kg^{-1} b. wt Reducdyn® showing diffuse fatty change (◄—) and mild mononuclear infiltration (◄►) (H&E, x 100).

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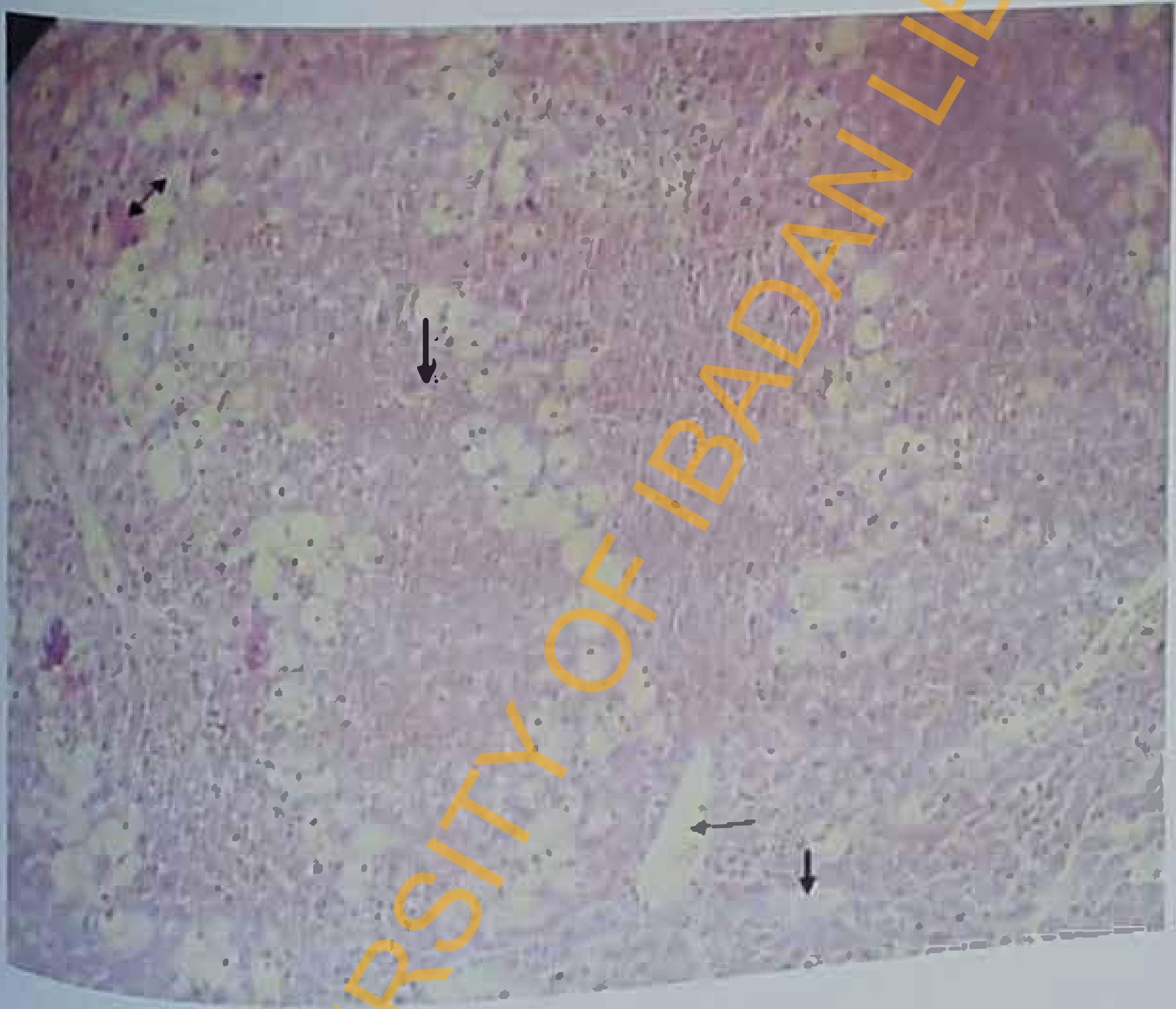

Plate 9. Liver section from CCl_4-control rat showing extensive necrosis (↓) of hepatocytes with areas of fatty change (←) and diffuse mononuclear infiltration (↔) (H&E, x 100).

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CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 Discussion

This present study examined whether the leaf extracts of *Persea americana* would lower hypercholesterolemia and lipid peroxidation in rats fed high lipid diet, and ameliorate liver injury caused by CCl_4-intoxication.

Based on the non-significant difference in the overall body weight gain among the four groups of rats it could be inferred that the test rats tolerated the administration of the high lipid diet and plant extracts when compared with rats fed on standard chow. Plasma glucose and triglycerides concentrations were markedly increased in rats fed high lipid diet compared to the normal control. This observation is similar to findings by other workers that feeding rats with high lipid diet increases plasma glucose and triglycerides (Srinivasan *et al*., 2004; Schaalan *et al*., 2009). It has been demonstrated that elevations of saturated fatty acids are associated with increased endogenous glucose production in non-diabetic subjects (Clore *et al*., 2004) thus suggesting that increased plasma saturated fatty acids, derived either from diet or endogenous synthesis may play an important role in the regulation of hepatic glucose disposal. Elevated free fatty acids have been shown to inhibit glucose oxidation and glycogen synthesis (Boden, 1997).

The increased glucose concentration in rats fed high lipid diet in this study could be due to increase of free glucose derived from either gluconeogenesis or glycogenolysis. Glucose-oxidised LDL stimulates macrophage proliferation, indicating that hyperlipidaemia in combination with hyperglycemia may induce macrophage

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proliferation *in vivo* (Lamharzi *et al.*, 2004). Studies have implicated the proliferation of macrophage-derived foam cells as a critical event in the evolution of atherosclerotic lesions (Gordon *et al.*, 1990; Rosenfeld and Ross, 1990). It is therefore possible that untreated hyperglycemia and hypertriglyceridemia could contribute to the development of atherosclerotic lesions. Treatment of hyperlipidaemic rats with AEPA and MEPA had a lowering effect on the plasma glucose concentrations compared with the untreated rats. The bark extract of *P. americana* has been shown to have anti-hyperglycemic and anti-diabetic properties (Alarcon-Aguilara *et al.*, 1998). The result of this study agrees with earlier report that the aqueous leaf extract of *P. americana* possesses hypoglycemic activity (Antia *et al.*, 2005) and this could justify the use of the extract in the treatment of diabetes.

Rats fed with the high lipid diet showed significant increase in cholesterol levels. Rats are known to have high rate of hepatic cholesterol synthesis and can also markedly increase their rate of bile acid synthesis. Consequently, rats do not have elevated plasma lipoprotein cholesterol and fatty acids when fed high cholesterol diet. They respond primarily to dietary cholesterol challenge by down-regulating hepatic synthesis and up-regulating hepatic bile production so that the plasma lipoprotein cholesterol concentration remains relatively unchanged (Kris-Etherton and Dietschy, 1997). However, results from this study show a 2-fold and a 14-fold increase in plasma and hepatic cholesterol concentrations respectively in the rats fed high lipid diet compared to the normal control. This is in line with the report of Hwa *et al.* (1992) which found a 16-fold increase in hepatic cholesterol in C57BL/6 mice. Shefer *et al.*, (1992) obtained a 2-

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fold increase in hepatic cholesterol in rats relative to controls when both were fed a high-fat atherogenic diet containing cholic acid. The increase in cholesterol levels in this study can be attributed to the inclusion of cholic acid in the diet since cholate is known to facilitate micelle formation in the intestines thus enhancing cholesterol absorption leading to cholesterolemia and changes in the lipoprotein concentrations (Shefer *et al.*, 1992, Johnston *et al.*, 1999, Wang *et al.*, 1999, Bobkova *et al.*, 2004). Treatment of hyperlipidaemic rats with AEPA and MEPA lowered both plasma and liver cholesterol. The cholesterol-lowering activity observed in this study could be attributed to the presence of flavonoids in the extracts. Flavonoids are known to possess hypocholesterolemic activity and the mechanism of action is thought to be by inhibition of HMG-CoA reductase, which catalyzes the rate limiting step in the biosynthesis of cholesterol, and suppression of cholesterol esterification (Theriault *et al.*, 2000, Koshy *et al.*, 2001, Anila and Vijayalakshmi, 2002). The lowered levels of cholesterol in the plasma and liver of AEPA and MEPA treated rats could be due to inhibition of cholesterogenesis.

Dietary cholesterol appears to contribute to the accumulation of liver triglycerides by stimulation of hepatic TG biosynthesis and a decrease in oxidation of fatty acids in the rat (Fungwe *et al.*, 1993).

In this study, there was a 28-fold increase in the hepatic concentration of TG in the hyperlipidaemic rats compared to the normal control. It is not well known how plasma TG concentrations influence the development of atherosclerosis. Minnich and Zilversmit (1989) demonstrated that severe hypertriglyceridemia in the alloxan-treated, cholesterol-

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fed diabetic rabbit model of atherosclerosis can only partially be attributed to VLDL hypersecretion, whereas a removal defect, resulting in saturation of the TG-removal mechanism was shown to be largely responsible. The impaired removal of plasma TG was also related to the presence of cholesterol predominantly in lipoproteins of increased size, which resulted in protection of atherosclerosis due to the exclusion of very large cholesterol-containing lipoproteins from the arterial wall (Minnich and Zilversmit, 1989). Treatment with AEPA significantly lowered plasma TG level as well as decreased liver TG level in hyperlipidaemic rats while MEPA decreased TG level in the liver only. It seems that both AEPA and MEPA exert their antihypertriglyceridemic action by suppression of TG synthesis.

The HDL-CHOL concentration and the HDL-CHOL: LDL-CHOL ratio (which is a more useful index of atherogenicity) were lowered in the hyperlipidaemic rats. However, treatment with AEPA and MEPA caused significant increases in HDL-CHOL concentrations when compared with hyperlipidaemic control rats. Epidemiological studies show that high levels of HDL-CHOL protect against the development of atherosclerosis (Gordon *et al.*, 1977; Castelli *et al.*, 1986). HDL-CHOL has the ability to promote the efflux of cholesterol from cells. This process may minimize the accumulation of foam cells in the artery wall (Barter *et al.*, 2004). Also, the major proteins of HDL-CHOL, apo A-I and apoA-II, as well as other proteins such as paraoxonase that cotransport with HDL in plasma are well known to have antioxidant properties. As a consequence, HDL-CHOL has the capacity to inhibit the oxidative modification of LDL-CHOL in a process that reduces the atherogenicity of these

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lipoproteins (Barter *et al.*, 2004). The restoration of HDL-CHOL levels in rats after treatment with AEPA and MEPA in this study may therefore serve to protect against lipoprotein peroxidation and the development of atherosclerosis.

Raised levels of LDL-CHOL as well as reduced HDL-CHOL: LDL-CHOL ratios are risk factors in atherosclerosis. The leaf extracts of *P. americana* have been shown to possess anti-inflammatory (Guevarra *et al.*, 1998; Adeyemi *et al.*, 2002) and anti-hypertensive/hypotensive properties (Girow *et al.*, 1991; Adeboye *et al.*, 1999). The administration of AEPA and MEPA in this study resulted in lowering of plasma T-CHOL and LDL-CHOL levels as well as restoration of HDL-CHOL level and improvement of HDL-CHOL: LDL-CHOL ratio in the treated rats. This could serve as a protective mechanism against the formation of foam cells and the development of atheriosclerosis and possibly account for the anti-inflammatory and hypotensive properties earlier reported.

There was no significant change in the activities of the hepatic enzymes AST and ALT in the plasma of rats treated with AEPA and MEPA. This suggests that the administration of *P. americana* leaf extracts was well tolerated and did not adversely affect liver function, based on the activity of these two enzymes.

It was observed that excised livers of rats that ingested the high lipid diet were golden yellow in colour. This is similar to the observation of Palmer *et al.*, (1997) after feeding rats with high fats diet.

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As observed, mean plasma concentrations of glucose in rats fed high lipid diet were significantly increased compared to the normal control rats. Hyperglycemia leads to protein glycation, glucose auto-oxidation and fatty acid oxidation which may contribute to increased ROS generation (Latha and Pari, 2004). There is some evidence that glycation itself may induce the formation of oxygen-derived free radicals (Inouye *et al.*, 1998). Also, glucose is known to induce lipid peroxidation through activation of lipoxygenase enzymes (Rajeswari *et al.*, 1991). It has been confirmed that hyperglycemia is related to the activation of the polyol pathway leading to increased oxidative stress (Cameron and Cotter, 1997). Probably, the increase in plasma glucose levels in rats fed high lipid diet in this study may have contributed to the observed higher concentrations of lipid peroxidation products in the plasma of these rats. Increased incorporation of PUFA from vegetable oil dietary sources into plasma lipoprotein has been shown to increase both lipoprotein (Nardini *et al.*, 1995) and tissue susceptibility to lipid peroxidation (L'Abbé *et al.*, 1991; De Schrijver *et al.*, 1992; Skúladóttir *et al.*, 1994). Lipid peroxidation products have been shown to rise with increased amount of fatty acids susceptible to peroxidation in the high fat diets (Ima-Nirwana *et al.*, 1996). In this study, feeding rats a high lipid diet was found to induce pro-oxidant changes in markers of oxidative stress in the plasma. These changes were manifested as depletion of plasma concentration of GSH and non-significant increase in plasma MDA. This finding is similar to an earlier report which showed that feeding rats a high cholesterol diet containing currant oil induced pro-oxidant changes in markers of oxidative stress in the blood (Večeřa *et al.*, 2003). Also, higher levels of MDA, CD and protein carbonyls were observed in the plasma and tissues of hyperlipidaemic rats compared to normal control. This agrees with an earlier observation

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that hypercholesterolaemia is associated with increased oxidant stress (Prasad and Kalra, 1993). As the precursor of a large number of highly reactive oxidizing agents, superoxide has the potential to inflict considerable damage to biological systems (Babior, 1997). Damage to DNA, proteins and lipids have all been documented as consequences of exposure to $O_2^{\cdot -}$ and its descendants (Thomas *et al*., 1985; Imlay and Linn, 1988; Aikens and Dix, 1991; Stadtman, 1992). MDA is known to cause cross-linkage of membrane components containing amino groups which makes the membrane fragile (Cameron and Cotter, 1994).

The increase in protein carbonyls content in the plasma, liver, heart and kidney of hyperlipidaemic rats is indicative of oxidative damage as well as chemical modification of proteins in these tissues. Oxidative modification alters the function of proteins and is thought to play an important role in the decline of cellular functions during ageing (Leeuwenburgh *et al*., 1998). Because proteins have many different and unique biological functions, oxidative modifications to proteins can lead to diverse functional consequences such as inhibition of enzyme activities and loss of protein function (Fucci *et al*., 1983; Stadtman, 1990; Shacter *et al*., 1995). The administration of AEPA and MEPA helped to lower oxidative stress in the treated rats as shown in the decline of indices of oxidative stress in the treated rats compared to the hyperlipidaemic control rats. Qualitative screening of the leaf extracts of *P. americana* indicated the presence of flavonoids and this corroborates earlier reports that the leaves of *P. americana* are rich in flavonoids (King and Knight, 1992; Merici *et al*., 1992). Flavonoids are known to be antioxidants and free radical scavengers. They have the ability to alter peroxidation kinetics by modifying the lipid packing order and decreasing fluidity of the membrane (Arora *et al*.,

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2000). These changes could sterically hinder diffusion of free radicals and restrict peroxidative reactions. Hence it may be possible that flavonoids are responsible for the antioxidant effect of AEPA and MEPA.

Glutathione levels are maintained by the activities of glutathione reductase and GSH synthases. GSH plays a pivotal defensive role against oxidative insults as an endogenous scavenger of free radicals (Cooper and Kristal, 1997). Its level in the blood is a sensitive indicator of antioxidant status in circulation (Piemonte *et al.*, 2001). There was a 10-fold decline in plasma GSH concentrations in the untreated, hyperlipidaemic rats compared to normal control. However, treatment with AEPA and MEPA restored plasma GSH concentrations to almost normal compared with the control. This suggests that AEPA and MEPA could improve antioxidant status in circulation by causing an increase in the concentration of plasma GSH thus protecting against oxidative damage.

However, no significant decrease in hepatic GSH concentration was noticed in the untreated hyperlipidaemic rats compared to the normal control rats. Maintenance of liver GSH under conditions of increased lipoperoxidation has been suggested as a supportive and a compensatory mechanism (Cooper and Kristal, 1997; Spolarics and Meyenhofer, 2000) reflecting higher capacity of liver to maintain GSH concentration compared to erythrocytes (Valencia *et al.*, 2001). A decrease in liver GSH is often related to hepatic fatty infiltration in different experimental models (Soltys *et al*, 2001; Vendemiale *et al*, 2001). Results of this study indicate lower concentration of liver GSH after high lipid feeding which caused an accumulation of both hepatic cholesterol and TG. This is similar to an earlier observation of lower liver GSH concentration after feeding rats with high

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cholesterol diet containing lard fat (Večeřa *et al*., 2003). The pathophysiological consequences of GSH depletion have been extensively studied. GSH, GSHPx, and GST depletion promotes generation of reactive oxygen species and oxidative stress with the subsequent cascade of effects affecting the functional and structural integrity of cell and organelle membranes (Raza *et al*., 2000).

SOD functions as an antioxidant to convert O_2^- to the less toxic H_2O_2 and therefore has a protective action against the possible deleterious effects of O_2^- (Murray *et al*., 1993). This could account for the higher levels of plasma SOD activity in hyperlipidaemic rats. The elevation of SOD and CAT activities in the hyperlipidaemic rats is probably a response to increased production of lipid peroxides. Enzymes are known to fit into a genetic scheme of regulation in that their concentrations in the cell are rapidly elevated in response to transcriptional regulators that sense sudden changes in oxidant levels (Harris, 1992). Although H_2O_2 production was not quantified in this study, the increase in CAT activity, a specific H_2O_2 scavenger may be due to an increase in its formation in the tissues.

GSHPx activity in the red cells and liver of hyperlipidaemic rats was low compared to normal control. This might be due to the depletion of GSH in both plasma and liver observed in this study. The opposing responses of CAT and GSHPx, both of which breakdown H_2O_2, are in agreement with earlier reports (Kakkar *et al*., 1997; Bhor *et al*., 2004). Bhor *et al*. (2004) suggested the existence of compensatory mechanisms in response to increased oxidative stress such that tissues lacking one of the enzymes may be critically dependent upon another.

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In conclusion, feeding rats with high lipid diet containing cholic acid caused 2-fold and 13-fold increases in plasma and hepatic cholesterol concentrations respectively. The administration of AEPA and MEPA at a dose of 10mg kg^{-1} b. wt caused a reduction in body weight gain, a lowering of both plasma glucose and LDL-CHOL and maintenance of HDL-CHOL concentration in the rat. It could be hypothesized that the leaf extract of *P. americana* increases catabolism of lipids accumulated in adipose tissue thereby causing a decrease in body weight gain.

The data obtained in hyperlipidaemic rats treated with the leaf extracts of *P. americana* provide useful information by showing that the extracts have antihyperglycemic and antihypercholesterolemic effects.

The administration of extracts of *P. americana* helped to lower oxidative stress in the treated rats as shown in the decline of indices of oxidative stress in the rat. Also, *P. americana* leaf extracts could improve antioxidant status in circulation in the rat by causing an increase in the concentration of plasma GSH, an endogenous antioxidant that plays a pivotal role in the defence against oxidative insults.

These findings suggest that both AEPA and MEPA lower plasma glucose, T-CHOL, and LDL-CHOL levels and increase HDL-CHOL and GSH concentrations in hyperlipidaemic rat. However, these results also show that AEPA appears more beneficial and could further be exploited as a potential botanical in the management of the emerging diseases associated with hyperlipidaemia.

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In order to ascertain whether the aqueous leaf extract of *P. americana* (AEPA) would reduce hepatic lipid accumulation in fatty liver disease and ameliorate liver damage rats were pre-treated with AEPA and intoxicated with CCl_4

Administering CCl_4 to rats markedly increased serum AST, ALT and total bilirubin levels. Increase in the levels of serum aminotransferases is known to reflect the severity of liver injury (Lin *et al.*, 1996). The leakage of large quantities of enzymes into the blood stream is associated with massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver. The increase in the transaminases and alkaline phosphatase is a clear indication of cellular leakage and loss of functional integrity of the membrane (Saraswat *et al.*, 1993). However, the increased activities of enzymes and total bilirubin in this study were considerably reduced by pre-treatment with AEPA suggesting that the extract tended to prevent liver damage and suppress the leakage of enzymes through cellular membrane into the blood stream. Also the calculated percentage hepatoprotection shows that the administration of AEPA was substantially hepatoprotective and this was comparable to the standard drug Reducdyn® used in this study. This result is similar to the hepatoprotective activity against CCl_4 exhibited by *Garcinia kola* (Farombi, 2000), *Vernonia amygdalina* (Babalola *et al.*, 2001), *Bauhinia racemosa* (Gupta *et al.*, 2004), *Bupleurum kaoi* (Wang *et al.*, 2004), *Telfairia occidentalis*, *Amaranthus caudatus*, *Occimum gratissimum* (Salawu and Akindahunsi, 2007) and *Acalypha racemosa* (Iniaghe *et al.*, 2008).

Histopathological analysis and the decrease in the serum transaminases levels provided supportive evidence that pre-treatment with AEPA reduced the severity of

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histopathological injuries (such as necrosis, ballooning degeneration and cellular infiltration) by CCl_4.

Serum ALP and bilirubin levels are related to the function of the hepatic cell and increase in serum level of ALP is due to increased synthesis (Moss and Butterworth, 1974). Results from this study demonstrate that pre-treatment of rats with AEPA caused substantial decrease in ALP and bilirubin levels and this decline was significant for bilirubin at extract concentration of 200mg kg^{-1} b. wt. Effective control of bilirubin level and ALP activity points towards an early improvement in the secretory mechanism of the hepatic cell (Gupta *et al*., 2004).

Administration of CCl_4 caused inhibition of protein synthesis manifested as decrease in both serum and liver total proteins compared with normal control. This effect confirms earlier reports by other workers (Venukumar and Latha, 2002; Mankani *et al*., 2005; Abdel-Hamid, 2006; Manjunatha, 2006). Inhibition of protein synthesis in the liver is primarily considered to lead to depression of lipoprotein synthesis and accumulation of fat in the liver, leading to fatty liver (Pirlou *et al*., 1979). A decline in total protein content has been suggested as a useful index of the severity of cellular dysfunction in chronic liver diseases (Venukumar and Latha, 2002). Pre-treatment with AEPA restored serum and liver total protein to near normal levels. The restoration of total protein content in serum and liver of rats treated with AEPA further elucidates its hepatoprotective activity. Stimulation of protein synthesis accelerates the regeneration process and the production of liver cells.

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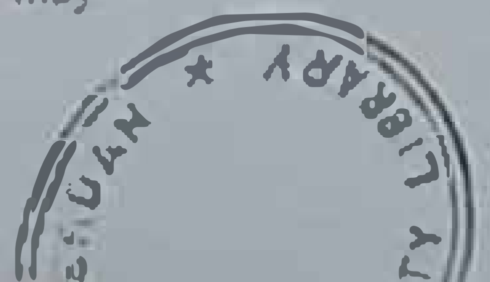
An increase in the levels of cholesterol and triglycerides were noted in serum and hepatic tissues. It has previously been reported that carbon tetrachloride treatment provokes increase in cholesterol and triglyceride levels in rat liver (Seakins and Robinson, 1963; Venukumar and Latha, 2002; Kamalakkannan *et al.*, 2005). CCl_4 increases the synthesis of fatty acids and triglycerides from acetate. This could be due to the transport of acetate into the liver cell, resulting in increased substrate availability. Also, the major metabolic defect induced by CCl_4 intoxication to rats appears to be inhibition of hepatic triglyceride release. This inhibition of outward transport would allow the accumulation of triglycerides within the liver and the occurrence of fatty liver associated with CCl_4 poisoning (Heimberg *et al.*, 1962). In CCl_4 toxicity, the synthesis of cholesterol is also increased (Boll et al., 2001).

On the other hand, CCl_4 lowers β-oxidation of fatty acids and hydrolysis of triglycerides. This increases the availability of fatty acids for esterification (Lieber, 2000). Severe impairment of mitochondrial fatty acid β-oxidation causes microvesicular steatosis, characterized by accumulation of tiny lipid vesicles in the cytoplasm of hepatocytes (Fromenty and Pessayre, 1995). Because of poor mitochondrial oxidation, nonesterified fatty acids (NEFAs) accumulate in the liver and become esterified into triglycerides. Reports have also shown that during CCl_4 toxicity, fat from the peripheral adipose tissue is translocated to the liver and kidney leading to its accumulation (Devarshi et al., 1986). It is suggested that an essential step in the outward transport of hepatic triglyceride is the synthesis of lipoproteins at the endoplasmic reticulum by the utilization of triglycerides previously synthesized at another site. Interference with lipoprotein synthesis by damage to the endoplasmic reticulum, as seen in CCl_4 intoxication may effectively depress



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outward triglyceride transport and result in the development of a fatty liver (Recknagel *et al.*, 1960; Heimberg *et al.*, 1962; Pencil *et al.*, 1984; Honma & Suda, 1997).

These factors could help to explain the significant increase in hepatic triglycerides observed in CCl_4- intoxicated rats in this study.

However, pre-treatment with AEPA produced a substantial reduction in the elevated hepatic cholesterol and triglycerides levels, suggesting that the extract prevented CCl_4-induced hyperlipidaemia probably due to its hepatoprotective activity.

The mechanism by which CCl_4 causes liver damage involves the biotransformation of CCl_4 by the cytochrome P-450 enzyme system to the toxic trichloromethyl free radical ($CCl_3^{\bullet}$), and then transforming this free radical into a more reactive trichloromethyl peroxyl radical ($CCl_3O_2^{\bullet}$), which causes lipid peroxidation, disrupts Ca^{2+} homeostasis, and eventually kills cells (McCay *et al.*, 1984; Recknagel *et al.*, 1989; Farombi, 2000).

Elevation in the levels of end products of lipid peroxidation in the liver of rat treated with CCl_4 was observed. The increase in MDA and protein carbonyls levels in the liver suggests enhanced lipid peroxidation. This observation is similar to earlier reports that there is an elevation of MDA in liver of rats treated with CCl_4 which is attributed to enhanced lipid peroxidation, leading to tissue damage and failure of antioxidant defence mechanisms to prevent the formation of excessive free radicals (Shenoy *et al.*, 2001; Wang *et al.*, 2004).

Pre-treatment with AEPA decreased MDA concentration and significantly reduced protein carbonyl levels. Hence, it may be that the mechanism of hepatoprotection of AEPA is due in part to its antioxidant effect. It is possible that trichloromethyl radical or

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lipid peroxides generated by CCl_4 treatment may be scavenged by the extract resulting in depression of lipid peroxidation in the liver.

It has been suggested that the protective effect of plant extracts against CCl_4-induced liver damage may be attributed to the presence of constituents including flavonoids, tannins, triterpenoids and alkaloids (Gilani and Janbaz, 1995; Tran *et al.*, 2001; Gupta *et al.*, 2004). Flavonoids are known to be antioxidants, free radical scavengers and antilipoperoxidants leading to hepatoprotection (Yuting et al., 1990; Cook and Samman 1996; Khalid et al., 2002; Al-Qarawi, et al., 2004; Mankani et al., 2005). Many compounds known to be beneficial against CCl_4-mediated liver injury exert their protective action by toxin-mediated lipid peroxidation either via a decreased production of CCl_4-derived free radicals or through the antioxidant activity of the protective agents themselves (Thabrew *et al.*, 1987; Jayatilaka *et al.*, 1990).

The hepatoprotective effect of *P. americana* against CCl_4-induced liver damage could also be attributed in part to its antioxidant effect and free radical scavenging activity, thus eliminating deleterious effects of toxic metabolites from CCl_4 and inducing liver cell regeneration. The antioxidant and free radical scavenging of AEPA could be due to the presence of flavonoids, saponins, terpenoids, tannins, and alkaloids.

The elevation of serum GSH in CCl_4-intoxicated rats agrees with the findings of Harisch and Meyer. (1985). Increased GSH level is known to represent increased GSH synthesis due to transcriptional activation of the gamma-glutamyl cysteinyl synthetase gene (Mari and Cederbaum, 2000). Up regulation of these antioxidant genes may reflect an adaptive mechanism to detoxify CYP2E1-derived oxidants.

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GSH plays a pivotal defensive role against oxidative insult as an endogenous scavenger of free radicals and maintenance of liver GSH under conditions of increased lipoperoxidation has been suggested as a supportive and compensatory mechanism (Cooper and Kristal, 1997; Spolarics and Meyenhofer, 2000). Also, CCl_4 is known to cause lipid peroxidation but do not deplete GSH (Jaeschke *et al.*, 2002). These observations and the free radical scavenging activity of the extract could explain the non-depletion of GSH in liver of rats in this study.

Liver cytosolic GST activity was slightly decreased in CCl_4-treated rats compared with control. Hepatic GST is known to be released into the serum after treatment with CCl_4 (Aniya and Anders, 1985; Recknagel *et al.*, 1989). The reduction in GST activity in this study could be due to the release of the enzyme into the serum following CCl_4-intoxication. Hepatic GST activity was however recovered by pre-treatment with AEPA.

CCl_4-intoxication also caused significant elevation in SOD and CAT activities and an increase in GSHPx activity in the liver of CCl_4-control rats. It is known that under oxidative stress some endogenous protective factors such as SOD and CAT are activated in the defence against oxidative injury (Kyle *et al.*, 1987; John *et al.*, 2001). The increase in the enzymes activities in the liver observed in this study was probably a response to increased reactive oxygen species generation and pre-treatment with AEPA elicited reductions in liver GSHPx, SOD and CAT activities.

Similarly, CCl_4 may cause oxidative stress and the consequent up-regulation of antioxidant enzymes to render cells more resistant to subsequent oxidative damage (Halliwell, 2000). In this study, pre-treatment with AEPA reduced SOD and CAT

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activities to near control levels, implying that *P. americana* may prevent CCl_4-induced lipid peroxidation.

Administration of CCl_4 alone caused leucopenia, neutropenia and lymphocytosis in the rats. This observation is similar to the findings of Mandal *et al.* (1998). The administration of AEPA at a concentration of 100 mg and 200 mg kg^{-1} b. wt restored WBC count by 99% and 85% respectively compared to CCl_4 control rats. It could therefore be suggested that AEPA has the potential to restore CCl_4-induced alterations of haematological parameters in the rat.

These results show that AEPA possesses significant protective effect against hepatotoxicity induced by carbon tetrachloride which may be attributed to the individual or combined action of phytoconstituents present in it. Further investigations are needed to determine the exact phytoconstituents that are responsible for its hepatoprotective effect.

However, a comparison of the effects of AEPA and MEPA shows that AEPA was more effective in reducing the levels of plasma glucose, total cholesterol and triglycerides as well as index of atherogenicity in the hyperlipidaemic rats. Also treatment with AEPA resulted in lower levels of markers of oxidative stress in the liver, lungs and heart of the rats.

Similarly, pre-treatment with 200 mg kg^{-1} b. wt provided better protection against CCl_4-induced hepatotoxicity as shown by the percentage protection against AST (127 %) and total bilirubin (106 %), reduction in serum total cholesterol (34 %), liver MDA (65 %), liver carbonyls (70 %) and increase in total protein (130 %).

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5.2 Conclusion

The results obtained from this study indicate that the extracts of the leaves of *P. americana* lower plasma glucose, total cholesterol and LDL cholesterol in the hypercholesterolemic rat. Also, the extracts caused a decline in the indices of oxidative stress and a restoration of HDL cholesterol and glutathione. Furthermore, the aqueous extract possesses significant protective effect against CCl_4-induced hepatotoxicity in the rat and the hepatoprotection appears to be dose dependent.

These beneficial effects may be attributed to the individual or combined action of the phytoconstituents.

Thus, this study shows for the first time that *P. americana leaf* extracts possess hypolipidaemic, antioxidative and hepatoprotective effects. This may account for its use in ethnomedicine and could be further exploited in the management of diseases associated with hyperlipidaemia.

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Contribution to knowledge

The results of this study show for the first time that:

1. Aqueous and methanolic leaf extracts of *P. americana* lower low density lipoprotein (LDL) cholesterol in hyperlipidaemic rats.
2. The extracts increase the level of high density lipoprotein (HDL) cholesterol.
3. The extracts help to reduce the index of atherogenicity which may represent a protective mechanism against the development of atherosclerosis.
4. *P. americana* could improve antioxidant status in circulation by increasing the concentration of reduced glutathione (GSH).
5. The extracts of *P. americana* are protective against lipid peroxidation.
6. The aqueous leaf extract of *P. americana* possesses significant protective effect against CCl_4-induced hepatotoxicity.

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