BIOCHEMICAL AND PHARMACOLOGICAL PROPERTIES OF METHANOLIC LEAF EXTRACT OF *SPONDIAS MOMBIN* LINN

BY

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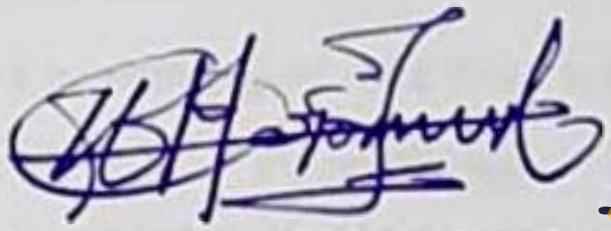
Certification

I certify that this work was carried out by Mr. A. C. Akinmoladun in the Department of Biochemistry, University of Ibadan.



5th Aug 2011

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Dedication

To

The all-wise and all-knowing God; the One who knows everything about everything.

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Afolahi C. Akinmoladun
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ABSTRACT

Spondias mombin is used traditionally in Nigeria for the management of malaria, diarrhoea and microbial infections but there is insufficient data from pharmacological investigations on its properties. The effects of the Methanolic Extract of *Spondias mombin* (MES) leaves on ischemia-induced cardiac and cerebral damages in rats were investigated. The antioxidative and antiproliferative properties of MES and its fractions were also investigated.

The MES was prepared by macerating *Spondias mombin* leaves in methanol and concentrating the filtrate obtained and then phytochemically screened according to standard procedures. Total phenolic content (TPC), total flavonoid content (TFC), radical scavenging activities, lipid peroxidation inhibitory activity (LPIA) and reductive potential (RP) were determined spectrophotometrically. Effect of MES on cardiac contractility was evaluated *ex vivo* in rat hearts subjected to ischemia using a GRASS polygraph and compared with that of a standard, ramipril. In a separate *in vivo* study, rats were treated with carboxymethyl cellulose (vehicle) or 85 mg/kg isoproterenol (ISP) with or without MES (100 and 250 mg/kg). Thirty days after treatment, sera, plasma and tissue homogenates were prepared. Levels of malondialdehyde (MDA), glutathione (GSH), cholesterol, phosphate and nitrite as well as the activities of superoxide dismutase (SOD), catalase and lactate dehydrogenase (LDH) were evaluated spectrophotometrically. In a neuropharmacological study, rats were subjected to Middle Cerebral Artery Occlusion (MCAO) and treated with vehicle or MES. Neurological deficit (ND) was estimated from observation of flexion, circling, hemiparesis and non-spontaneous movement. Infarct size, MDA and GSH levels were also evaluated. Protein expressions of $gp91^{phox}$ and $p22^{phox}$ (subunits of nicotinamide adenine dinucleotide phosphate oxidase), eNOS and nNOS (isoforms of nitric oxide synthase) and SOD were evaluated by Western blotting. The MES was fractioned into water, n-butanol, ethyl acetate, dichloromethane and hexane. Antioxidant and antiproliferative assays were carried out by spectroscopy on the fractions. Data were analyzed using ANOVA and Student's t-test at $P < 0.05$.

Phytochemical screening confirmed the presence of tannins, terpenoids and flavanoids. The TPC and TFC were 329.0 ± 11.4 mg/l gallic acid equivalent and 228.0 ± 3.5 µg/ml quercetin

equivalent respectively. Radical scavenging activities ranged from 43.0 ± 0.9% to 88.6 ± 3.0% while LPIA and RP were 54.0 ± 1.3% and 0.6 ± 0.0 respectively. The MES stimulated significant cardiac contractile activities similar to that of ramipril. The MES also reduced ISP-induced elevation of LDH activity and MDA, phosphate, cholesterol and nitrite levels and reversed the decreased GSH level and SOD and catalase activities in the ISP-challenged group. It reduced ND and infarct size by 43.0% and 75.0% respectively and significantly ameliorated MCAO-induced elevation of MDA and decrease in GSH levels. It suppressed the expressions of $gp91^{phox}$ and nNOS but enhanced those of $p22^{phox}$, eNOS and SOD. The ethyl acetate and n-butanol fractions showed the highest antioxidant activity while the dichloromethane fraction had the highest antiproliferative activity. Quercetin-3-O-ß-D-glucopyranoside and undec-1-ene were characterized from the ethyl acetate and n-butanol fractions.

Methanolic extract of *Spondias mombin* leaves exhibited remarkable antioxidant property that could protect rats from isoproterenol-induced cardiotoxicity, ischemia-induced cardiac and cerebral damages.

Keywords: *Spondias mombin*, Antioxidant property, Cardiac damage, Neuroprotection, Antiproliferative activity

Word count: 497

ABBREVIATIONS

ADP	Adenosine diphosphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CAM	Complementary and alternative medicine
CAT	Catalase
CMC	Carboxymethyl cellulose
CNS	Central nervous system
CVD	Cardiovascular disease(s)
DOR	Deoxyribose assay for hydroxyl radical scavenging activity
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl radical
DTNB	dithiobis nitrobenzene
e	Electron
EDTA	Ethylene diamine tetraacetic acid
FRAP	Ferric reducing antioxidant power
GSH	Glutathione (reduced)
GSSG	Oxidized glutathione
h	Hour
H_2O_2	Hydrogen peroxide
HOO$^{\cdot}$	Hydroperoxyl radical
HOCl	Hypochlorous acid
Km	Michaelis constant
LDH	Lactate dehydrogenase
MCAO	Middle cerebral artery occlusion
MDA	Malondialdehyde
MES	Methanolic extract of *Spondias mombin* leaves
min	Minute
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)

NBT	Nitroblue tetrazolium chloride
NED	*N*-(1-naphthyl) ethylenediamine dihydrochloride
NO	Nitric oxide
NO^-	Nitroxyl
NO_2	Nitrogen dioxide
NOCl	Nitrosyl chloride
NOS	Nitric oxide synthase
cNOS	Endothelial Nitric oxide synthase
iNOS	Inducible Nitric oxide synthase
nNOS	Neuronal Nitric oxide synthase
$^1O_2$	Singlet oxygen
O_3	Ozone
O_2^-	Superoxide radical
$OH^·$	Hydroxyl radical
$ONOO^-$	Peroxynitrite radical
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffered saline
pH	log ($1/[H^+]$)
ppm	Part per million
PMS	Phenazine methosulphate
r	Correlation coefficient
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
s	Second
SM	*Spondias mombin*
SOD	Superoxide dismutase
SRB	Sulphorhodamine B
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TDW	Triple distilled water

TEAC	Trolox equivalent antioxidant capacity
TEP	1,1,3,3-tetraethoxypropane
TFC	Total flavonoid content
TOSC	Total oxidant scavenging capacity
TPC	Total phenolic content
TRAP	Total radical-trapping antioxidant parameter
TTC	Triphenyl tetrazolium chloride
WHO	World health organization
w/v	Weight per volume
w/w	Weight per weight

CHAPTER ONE

INTRODUCTION

Generation of free radicals in the body beyond its antioxidant capacity leads to oxidative and nitrosative stresses which have been implicated in the etiology of many diseases (Tshibangu *et al.*, 2002, Nicolescu *et al.*, 2004). In particular, reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and H_2O_2 together with reactive nitrogen species (RNS) like peroxynitrite ($ONOO^-$), nitroxyl (NO^-), nitrosyl chloride (NOCl) and nitrogen dioxide (NO_2) are important factors in the etiology of several pathological conditions such as lipid peroxidation, protein peroxidation, DNA damage and cellular degeneration related to cardiovascular, cerebrovascular and neurodegenerative diseases, diabetes, ischemia-reperfusion injury, local and systemic inflammation, cancer, and many other disorders (Clayson *et al*, 1994, Pacher *et al.*, 2007). The aberrant reactions of ROS, produced from oxygen contribute to oxidative stress whereas reactions of RNS, produced from reaction of NO are proposed to contribute to nitrosative stress. In consonance with this, mitigation of oxidative stress via scavenging of free radicals and augmentation of antioxidant defenses in living systems has been advanced to be a common route through which many drugs and natural products exercise their health-promoting effects.

Plants contain many antioxidative components which act as major defense against radical-mediated toxicity by preventing or attenuating the deleterious effects of free radicals. Inhibition of free radical generation can serve as a facile system for identifying cancer preventive, chemotherapeutic and prophylactic agents (Halliwell and Gutteridge, 1992; Bauerova and Bezek, 1999; Farombi *et al.*, 1997; Farombi *et al.*, 1998; Farombi, 2000; Finkel and Holbrook, 2000). A systematic search for useful bioactivities from medicinal plants is now considered to be a rational approach in nutraceutical and drug research. According to a conservative estimate, 300, 000 to 400,000 plant species grow on earth, only a small percentage have had their phytochemistry and biological function investigated (Kitani *et al.*, 2001). Despite the upsurge in phytochemical research, relatively little information is available

concerning the antioxidant potential and biological activity of plant species, especially in Africa which contains one of the richest biodiversity in the world and abounds in plants of economic and medicinal importance (Farombi, 2003). According to Hoareau and Dasilva (1999), interest in medicinal plants as a re-emerging health aid has been fuelled by the rising cost of prescription drugs in the maintenance of personal health and well-being, and the bioprospecting of new plant-derived drugs. It was opined that based on current research and financial investments, medicinal plants would continue to play a leading role in the global quest for a healthier world. Possible toxicity and general consumer rejection of synthetic additives have also spurred the search for plant-based alternatives (Namiki, 1990).

Over 80% of the developing world's population still depends on the complementary and alternative systems of medicine (CAM) while about half of the population in the industrialized countries uses CAM (Bodeker and Kronenberg, 2002). It has always been an "invisible mainstream" within the health care delivery system (Penson et al. 2001). The Alma-Ata declaration in 1978 stated that mobilization of traditional medicine systems is an important way to make health for all a reality (Shaikh and Hatcher, 2005). The WHO in 1970 recommended that proven traditional remedies should be incorporated within national drug policies (Wondergem *et al.*, 1989). Ethnobotanical studies carried out throughout Africa confirm that native plants are the main constituent of traditional African medicines (Oliver-Bever, 1986). These plants have to be subjected to phytochemical investigations and bioactivity studies in order to validate putative claims for them as proven remedies and to correct problems arising from their unorthodox use for medicinal and allied purposes.

As pharmaceutical and nutraceutical interest in natural antioxidants has skyrocketed in the past few years, a plethora of methods have come into common use for screening antioxidant activity of various classes of compounds. These include oxygen radical absorbance capacity (ORAC), trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), total oxidant scavenging capacity (TOSC), total radical-trapping antioxidant parameter (TRAP), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) reactivity, total phenolic content analysis among others. Typically, the method selected depends on ease of use and availability of instrumentation, and very often there is lack of correlation between activity assays and phenolic content.

between activities determined on the same material by different assays and between activities determined by the same assay in different laboratories (Schaich, 2006). Environmental influences such as temperature, grades of reagents employed in assays and source of samples used may be contributory factors to these discrepancies. Besides these, antioxidant assays do not all measure the same chemical action. Some assays measure hydrogen atom transfer capability (classical radical quenching), some measure electron transfer propensity. Therefore to adequately and fairly compare the antioxidant potentials of plant products, a series of methods should be utilized. Plants differ in the types and combinations of phytochemicals and antioxidant compounds they contain and therefore, mechanism of antioxidant action will not always be the same for all samples (McDonald-Wicks *et al.*, 2006; Kaur and Geetha, 2006).

Cardiovascular diseases include coronary heart disease (heart attacks), cerebrovascular disease, raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. Tobacco use, physical inactivity, and an unhealthy diet can lead to CVD. Globally, cardiovascular diseases are the number one cause of death and are projected to remain so into the foreseeable future. An estimated 17.5 million people died from cardiovascular disease in 2005, representing 30% of all global deaths. Of these deaths, 7.6 million were due to heart attacks and 5.7 million due to stroke (WHO, 2007).

A stroke or cerebrovascular accident occurs when the blood supply to part of the brain is suddenly interrupted or when a blood vessel in the brain bursts, spilling blood into the spaces surrounding brain cells. Neurons die when they no longer receive oxygen and nutrients from the blood or there is sudden hemorrhage into or around the brain. In 1999, stroke was the cause of death in 5.5 million people worldwide (WHO, 2003). At present, stroke is the third leading cause of death, after cardiovascular diseases and cancer, in most developed countries and the leading cause of disability in adults (Ikeda *et al.*, 2003; Bémeur *et al.*, 2007). Projections to the year 2020 indicate that the number of people suffering from cerebrovascular disease each year will increase substantially, and that the majority of these will be in developing countries (WHO, 2003). There are, to date, no effective curative treatments for stroke but plant derived antioxidants are very promising drugs in the management of ischemic stroke. (Margaill *et al.*, 2005; Bémeur *et al.*, 2007).

The present study has been designed to investigate the antioxidant, cardioprotective and neuroprotective potential of *Spondias mombin*. Ten widely used indigenous Nigerian medicinal plants were evaluated for antioxidant and free radical scavenging activities which formed the basis for the selection of *Spondias mombin* for the cardioprotective and neuroprotective studies.

CHAPTER TWO

LITERATURE REVIEW

2.1 FREE RADICALS AND OXIDATIVE STRESS

Free radicals are chemically active atoms or molecular fragments that have a single unpaired electron in an outer orbit. This unstable configuration creates energy which is released through reactions with adjacent molecules. Examples of free radicals are superoxide radical, hydroxyl radical, trichloromethyl radical ($^{\cdot}CCl_3$), ions of transition metals like iron and copper, nitric oxide and ozone. The presence of an unpaired electron makes free radicals highly unstable and consequently highly reactive since they abstract electron from adjacent molecules in order to attain a stable state. Oxygen free radicals or more generally, reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) are subsets of free radicals which are of special interest to scientists. Radicals derived from oxygen represent the most important class of radical species generated in living systems.

2.1.1 Reactive oxygen species

Reactive oxygen species are either free radicals, reactive anions containing oxygen atoms or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. They include: superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), singlet oxygen ($^1O_2$) and hydroperoxyl radical ($HOO^{\cdot}$). Other non-radical reactive oxygen species include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3). ROS are generated through a number of means including ultraviolet and ionizing radiations, chemical reactions and metabolic processes. The production of ROS is actually a normal part of life, arising from the inhalation of oxygen. Free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, by bacterial leucocytes, through xanthine oxidase activity, atmospheric pollutants, from transitional metal catalysts, drugs and xenobiotics. In addition, chemical mobilization of fat stores under various conditions such as lactation, exercise, fever, infection and even fasting can result in

increased radical activity and damage to the immune and nervous systems. Stress hormones (adrenalin and noradrenalin) secreted by the adrenal glands under conditions of continuing and excessive emotional stress, are metabolised into simpler, albeit, free radical molecules (Atawodi, 2005). The complete reduction of oxygen is summarized by the following equations (Clarkson and Thompson, 2000):

$$O_2 + e \longrightarrow O_2^{\cdot -} \text{ (Superoxide radical)}$$

$$O_2^{\cdot -} + H_2O \longrightarrow HO_2^{\cdot} + OH^- \text{ (hydroperoxyl radical)}$$

$$HOO^{\cdot} + e \longrightarrow H_2O_2 \text{ (hydrogen peroxide)}$$

$$H_2O_2 + e \longrightarrow {}^{\cdot}OH + HO^- \text{ (hydroxyl radical)}$$

Hydrogen peroxide is not a free radical but it is considered a reactive oxygen species because of its ability to generate the highly reactive hydroxyl radical through its interaction with transition metals (Aruoma *et al.*, 1991). In cellular oxidation reactions, the superoxide radical is normally formed first and is therefore considered the "primary" ROS, and can further interact with other molecules to generate other kinds of cell damaging free radicals and oxidizing agents, "secondary" ROS, either directly or prevalently through enzyme- or metal-catalyzed processes (Valko *et al.*, 2005). Various pathways of ROS formation are shown in Figure 1. The damaging action of the hydroxyl radical is the strongest among free radicals (Liu and Ng, 2000).

2.1.2 Reactive nitrogen species

The pathologically and physiologically important reactive nitrogen species (RNS) have been gaining increasing prominence in the past few decades. NO can be regarded as the primary RNS. $NO^{\cdot}$ is generated in biological tissues by specific nitric oxide synthases (NOSs), which metabolize arginine to citrulline with the formation of $NO^{\cdot}$ *via* a five electron oxidative reaction (Ghalourdar and Cadenas, 2005). Nitric oxide ($NO^{\cdot}$) is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission and synaptic plasticity in the CNS, blood pressure

regulation, defence mechanisms, smooth muscle relaxation and immune regulation (Bergendi *et al.*, 1999). Due to its extraordinary properties, $NO^{\cdot}$ was acclaimed as the "molecule of the year" in 1992 by *Science Magazine* (Koshland, 1992). In the extracellular milieu, $NO^{\cdot}$ reacts with oxygen and water to form nitrate and nitrite anions. Overproduction of reactive nitrogen species is called nitrosative stress (Klatt and Lamas, 2000; Ridnour *et al.*, 2004). This may occur when the generation of RNS in a system exceeds the system's ability to neutralise and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function. Cells of the immune system produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes. Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion ($ONOO^{-}$), which is a potent oxidizing agent that can cause DNA fragmentation and lipid oxidation (Carr *et al.*, 2000):

$$NO^{\cdot} + O_2^{\cdot -} \rightarrow ONOO^{-}$$

Thus $NO^{\cdot}$ toxicity is predominantly linked to its ability to combine with superoxide anions (Figures 1 and 2). Nitric oxide readily binds certain transition metal ions; in fact many physiological effects of $NO^{\cdot}$ are exerted as a result of its initial binding to Fe^{2+}-Haem groups in the enzyme soluble guanylate cyclase (Archer, 1993). The NO derived chemical species most routinely implicated in toxicity have been peroxynitrite ($ONOO^{-}$), nitroxyl (NO^{-}), nitrosyl chloride (NOCl) and nitrogen dioxide (NO_2). Myeloperoxidase catalyzes the formation of 3-nitrotyrosine, another RNS, from the reaction of nitrite with proteins. The reaction of NO_2 with phenols (including tyrosine) yields nitrophenols, in particular, nitrotyrosine (Reiter *et al.*, 2000). Both peroxynitrite and NO_2 have been proposed to initiate lipid peroxidation and degradation of other biomolecules (Byun *et al.*, 1999). Nitrotyrosine is used as a common marker of nitrosative stress. Nitrotyrosine readily decomposes to tyrosine and nitrite. Hence nitrosative stress produces products such as nitrosothiols and nitrosamines but nitrotyrosine and nitrotryptophan are more stable products and indicative of a more intense oxidative stress (Thacher *et al.*, 2007).

2.1.3. ROS: The Pros and the Cons

ROS, as well as reactive RNS, are products of normal cellular metabolism. ROS and RNS are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems (Valko *et al.*, 2006). The aberrant reactions of ROS, produced from oxygen contribute to oxidative stress, whereas reactions of nitric oxide are proposed to contribute to nitrosative stress (Wink and Mitchell, 1998). Generally, RNS could be considered as a subset of ROS since they usually contain oxygen and are associated with reactions producing the latter (Figure 1). Similarly, oxidative stress could be considered to be inclusive of nitrosative stress.

Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defence against infectious agents and in the function of a number of cellular signalling systems. ROS at low/moderate concentrations are also involved in the induction of a mitogenic response, the control of blood flow through the arteries and in maintaining CNS health. Some free radicals at low levels are signaling molecules responsible for turning genes on and off. Others, such as nitric oxide and superoxide, are produced in very high amounts by immune cells to destroy viruses and bacteria. Some free radicals kill cancer cells. Many cancer drugs are actually designed to increase the production of free radicals in the body. The 1998 Nobel Prize in Physiology and Medicine was awarded to the scientists who discovered nitric oxide's role as a signaling molecule in the cardiovascular system (Packer and Colman, 1999).

The harmful effect of free radicals causing potential biological damage, oxidative stress and nitrosative stress (Kovacic and Jacintho, 2001; Valko *et al.*, 2001; Ridnour *et al.*, 2005), results from a shift in the balance of the prooxidant and antioxidant homeostatic phenomenon in the body. Prooxidant conditions dominate either due to the increased generation of the free radicals or due to the poor scavenging/quenching of the free radicals in the body due to depletion of the dietary antioxidants (Dringen, 2000). Oxidative stress occurs when free radicals "go on rampage" and is associated with the development of chronic and degenerative diseases. It has been implicated in etiology of several pathological and degenerative conditions such as cardiovascular

diseases (CVD), cancer and carcinogenesis-induced mutation and tumour promotion, arthritis, diabetes, acquired immune deficiency syndrome (AIDS), Alzheimer's disease, inflammation and the ageing process; arising from their involvement in lipid peroxidation, protein peroxidation and DNA damage (Halliwell and Gutteridge, 1992; Clayson *et al.*, 1994; Knight, 1995; Bauerova and Bezek, 1999; Visioli *et al.*, 2000; Olinski *et al.*, 2003; Pcuchant *et al.*, 2004 and Neergheen *et al.*, 2006). As long as the body's antioxidant defense mechanism is still able to contain or curtail the activities of free radicals, oxidative stress and nitrosative stress will not occur. The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by mechanisms called "redox regulation". The process of "redox regulation" protects living organisms from various oxidative stresses and maintains "redox homeostasis" by controlling the redox status in vivo (Dröge, 2002).

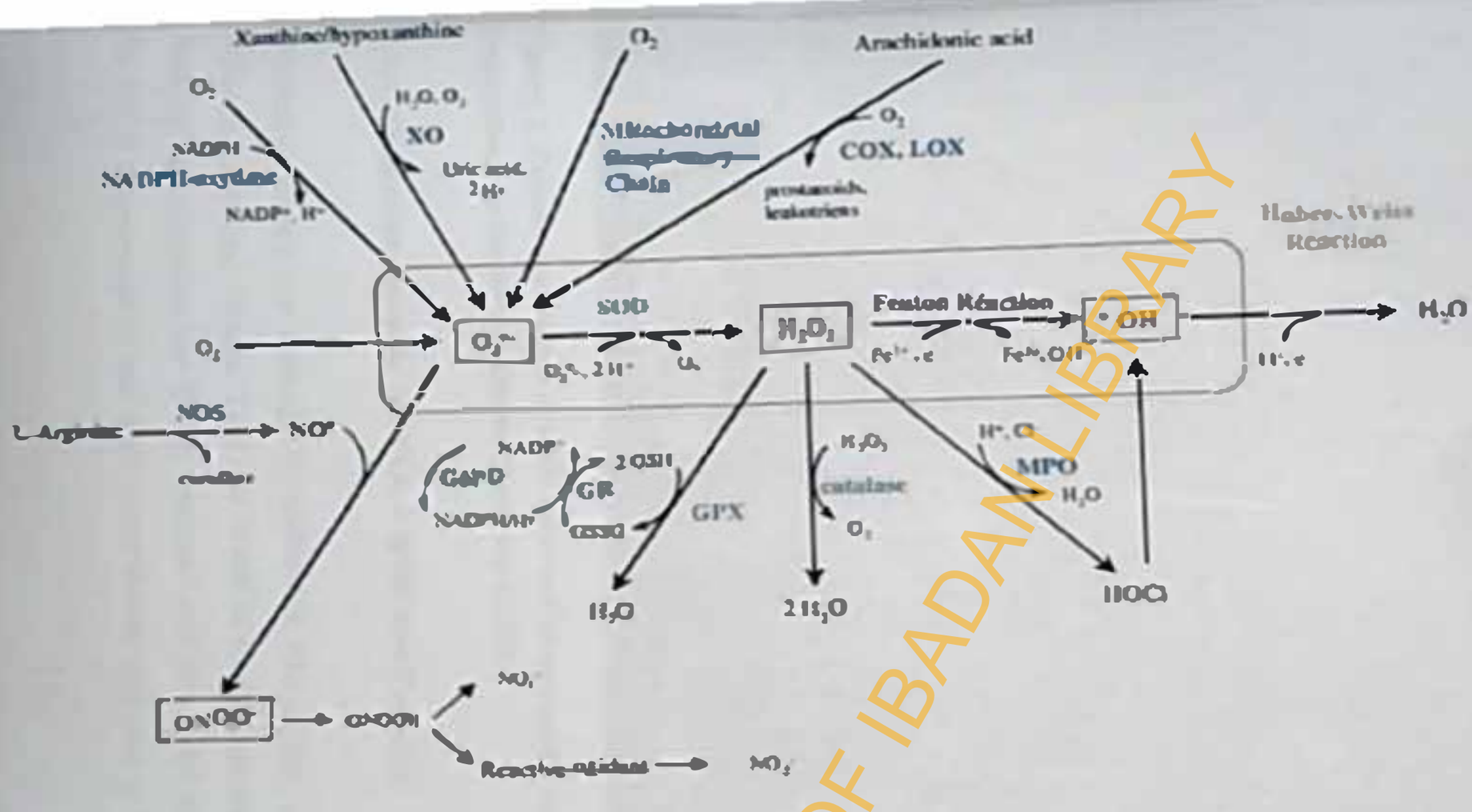

Figure 1. Main sources of free radicals. The highly reactive superoxide anions are mainly produced by (1) the arachidonic acid pathway, (2) the mitochondrial respiratory chain, (3) oxidation of xanthine and hypoxanthine by xanthine oxidase, and (4) NADPH-oxidases. Superoxide anions can either react with nitric oxide, produced by nitric oxide synthases, to generate the strong oxidant peroxynitrite, or be degraded by superoxide dismutase into the less reactive species hydrogen peroxide. Peroxide can then (1) be catabolized by glutathione peroxidase or catalase reaction, (2) react with Fe^{2+} to form hydroxyl radicals via the Fenton reaction, or (3) be degraded by the myeloperoxydase, another source of hydroxyl radicals. Abbreviations: COX, cyclooxygenase; G6PD, glucose-6- phosphate dehydrogenase; GSH, reduced glutathione; GR, glutathione reductase; GPX, glutathione peroxidase; GSSG, oxidized glutathione; HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; LOX, lipoxygenase; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NO_3^-, nitrate; NO_2^-, nitrite; NOS, NO synthase; O_2^-, superoxide anion; $OH^{\cdot}$, hydroxyl radical; $ONOO^-$, peroxynitrite anion; ONOOH, peroxynitrous acid; SOD, superoxide dismutase; XO, xanthine oxydase. (Source: Margaill, *et al*, 2005.)

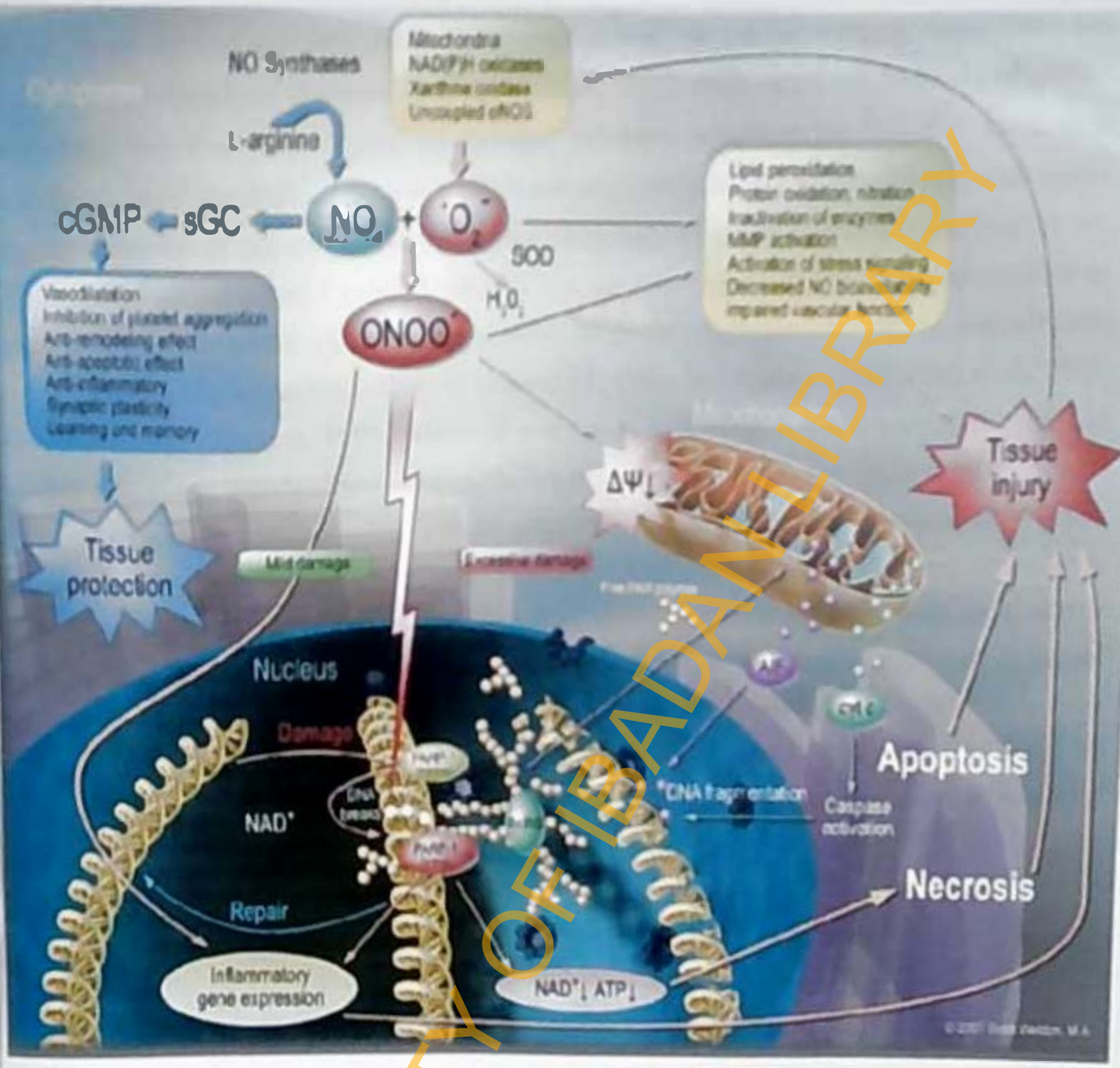

Figure. 2. Cellular metabolism of nitric oxide and peroxynitrite. ©2007 Scot Weldon. Nitric Oxide and Peroxynitrite in Health and Disease (Pacher *et al.*, 2007).

2.1.4. Oxidative Damage to DNA, Lipids and Proteins

Oxidative stress can damage biomolecules like lipids, proteins, enzymes, carbohydrates and DNA in cells and tissues. This can result in membrane damage, fragmentation or random cross linking of molecules like DNA, enzymes and structural proteins and even to cell death induced by DNA fragmentation and lipid peroxidation (Rajnam *et al.*, 2006). The hydroxyl radical reacts

with all components of the DNA molecule, damaging both the purine and pyrimidine bases and the deoxyribose backbone (Halliwell and Gutteridge, 1999). Permanent modification of genetic material resulting from these "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis, and ageing (Valko *et al.*, 2007). ROS also attacks polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Siems *et al.*, 1995). Once formed, peroxyl radicals (ROO') can be rearranged *via* a cyclization reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA) (Fedtke *et al.*, 1990; Wang *et al.*, 1996; Fink *et al.*, 1997; Mao *et al.*, 1999; Marnett, 1999). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide radicals are formed (Stadtman, 2004). The side chains of all amino acid residues of proteins, in particular cysteine and methionine residues of proteins are susceptible to oxidation by the action of ROS/RNS (Stadtman, 2004). Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups (–SH) and low molecular weight thiols, in particular GSH (S-glutathiolation). The concentration of carbonyl groups, generated by many different mechanisms is a good measure of ROS-mediated protein oxidation.

2.2. Ischemia - Reperfusion Injury

Reperfusion injury refers to damage to tissue caused when blood supply returns to the tissue after a period of ischemia. The absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function. Reperfusion injury is the leading cause of tissue damage occurring in conditions such as myocardial infarction, stroke, organ transplantation, and cardiopulmonary bypass, as well as a major mechanism of end-organ damage complicating the course of circulatory shock of various etiologies. In all these conditions, the initial trigger of the damage is the transient disruption of the normal blood supply to target organs followed by reperfusion. No effective therapy is currently available to limit

eperfusion injury, which emphasizes the importance of a better understanding of its underlying athological mechanisms, to devise potential future therapeutic strategies (Pachar *et al.*, 2007).

2.2.1. Mechanisms of Reperfusion Injury

An imbalance between oxygen supply and demand due to compromised vascular flow results in ischemia. In theory, the process is very simple; lack of adequate oxygen and metabolic substrates rapidly decreases the energy available to the cell and leads to cell injury that is of reversible or irreversible nature. In practice, the process is very complex. The extent of injury is determined by various factors; the severity of ischemia (low-flow vs. zero-flow ischemia), the duration of ischemia, the temporal sequence of ischemia (e.g. short ischemia followed by long ischemia), changes in metabolic and physical environment (hypothermia vs. normothermia, preischemic myocardial glycogen content, perfusate composition) as well as the inflammatory response. Reperfusion, generally a pre-requisite for tissue survival, may also increase injury over and above that sustained during ischemia. This phenomenon leads in turn to cell death (Pantos *et al.*, 2006). The damage of reperfusion injury is due in part to the inflammatory response of damaged tissues. White blood cells carried to the area by the newly returning blood release a host of inflammatory factors such as interleukins as well as free radicals in response to tissue damage. The restored blood flow reintroduces oxygen within cells that damages cellular proteins, DNA, and the plasma membrane. Damage to the cell's membrane may in turn cause the release of more free radicals. Such reactive species may also act indirectly in redox signaling to turn on apoptosis. Leukocytes may also build up in small capillaries, obstructing them and leading to more ischemia. Neutrophils are the principal effector cells of reperfusion injury. Under the conditions of ischemia/reperfusion, xanthine dehydrogenase is converted into xanthine oxidase which uses oxygen as a substrate. During ischemia, oversized ATP consumption leads to accumulation of the purine catabolites hypoxanthine and xanthine, which upon subsequent reperfusion and influx of oxygen are metabolized by xanthine oxidase to produce enormous amounts of superoxide radical, hydrogen peroxide and hydroxyl radical (Granger *et al.*, 2001). Xanthine oxidase also produces uric acid, which may act as both a prooxidant and as a scavenger of reactive species such as peroxynitrite. Excessive nitric oxide produced during reperfusion reacts with superoxide to produce the potent reactive species peroxynitrite. Such radicals and

reactive oxygen species attack cell membrane lipids, proteins, and glycosaminoglycans, causing further damage. They may also initiate specific biological processes by redox signaling.

2.2.2 Myocardial Ischemia

Acute myocardial ischemia (ischemic heart disease) accounts for the highest percentage of morbidity and mortality in the Western world (Lopez and Murray, 1998). Persistent ischemia can result in cardiomyocyte death and lead to congestive heart failure. It is characterized by reduced blood supply to the heart muscle, usually due to coronary artery disease (atherosclerosis of the coronary arteries). Its risk increases with age, smoking, hypercholesterolaemia (high cholesterol levels), diabetes, hypertension (high blood pressure) and is more common in men and those who have close relatives with ischaemic heart disease. Coronary reperfusion utilizing thrombolytics and coronary angioplasty can partially rescue the ischemic myocardium and limit the development of an infarct. However, reperfusion, though a prerequisite for tissue salvage, might also lead to increased cell mortality, possibly as a result of the inflammatory response, a burst of oxygen free radical production and calcium overload (Bognar *et al.*, 2006). In response to the increasing toll of ischaemic heart disease, the last 50 years have seen an enormous amount of research aimed at understanding the biology of ischaemia and developing methods to control it (Hearse, 2001). Myocardial ischemia results in ATP depletion and accumulation of toxic metabolites, whereas reperfusion leads to the production of reactive oxygen intermediates and calcium overload. The alterations in cellular metabolism and generation of toxic molecules contribute to myocardial ischemia/reperfusion injury (Marczin *et al.*, 2003). Myocardial Ischemia–reperfusion injury may occur as damage to the myocardium following blood restoration after a critical period of coronary occlusion (Goldhaber and Weiss, 1992, Dhalla *et al.*, 2000). It is now recognized that there are a spectrum of responses of the myocardium to reduced coronary perfusion and that the response of the myocardium to ischemic injury can be modulated by a number of processes, particularly reperfusion and preconditioning (Buja, 2005)

There are two main hypotheses, namely oxidative stress and Ca-overload, which have been proposed to explain the pathogenesis of myocardial ischemia–reperfusion injury (Figure 3).

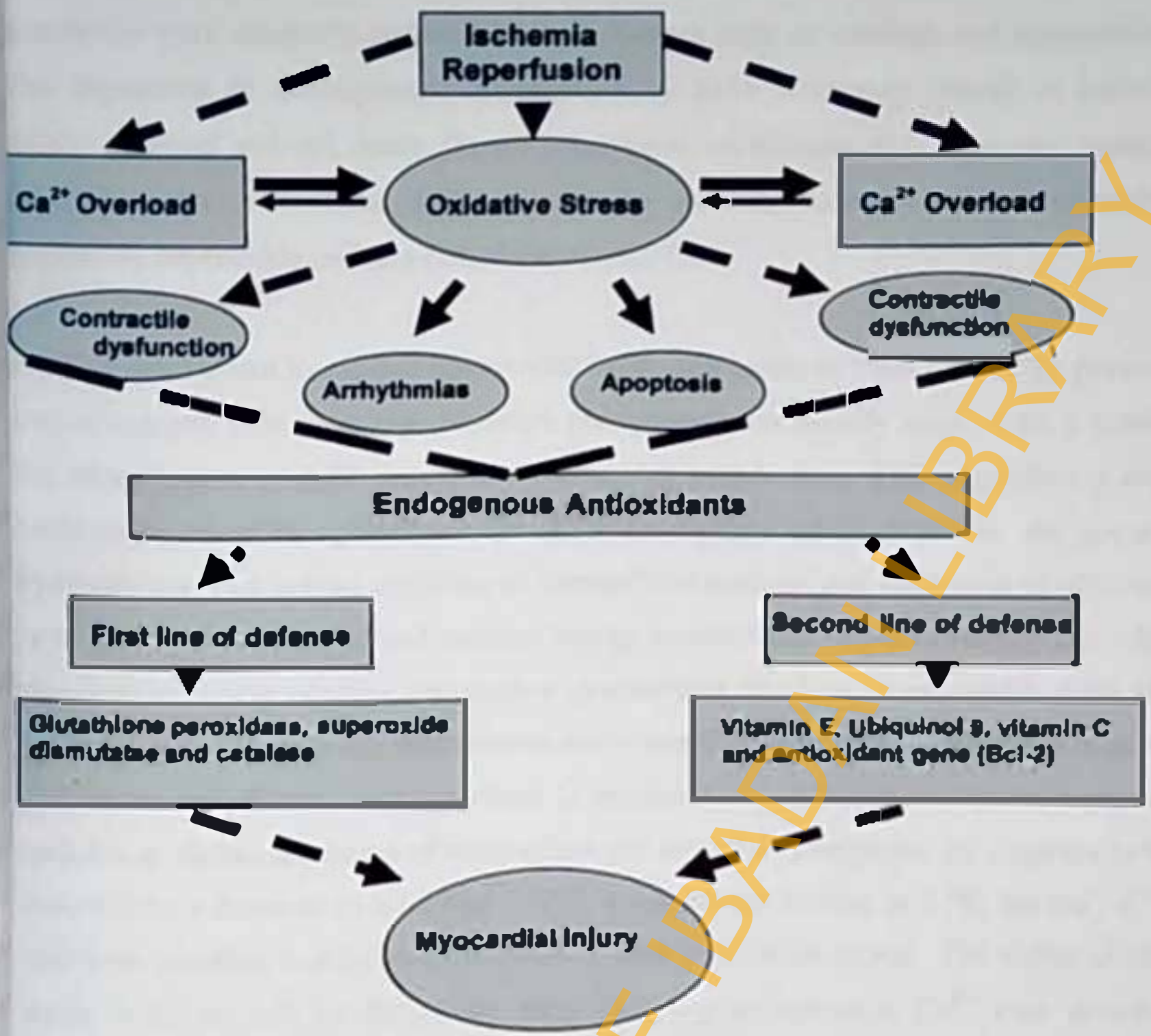

Figure 3: Schematic diagram showing pathophysiologic and therapeutic implications of oxidative stress and endogenous antioxidants in ischemia– reperfusion injury in the heart (Dhalla *et al.*, 2000).

Oxidative stress, which is usually associated with increased formation of reactive oxygen species (ROS), modifies phospholipids and proteins leading to lipid peroxidation and oxidation of thiol groups; these changes are considered to alter membrane permeability and configuration in addition to producing functional modification of various cellular proteins. Oxidative stress may result in cellular defects including a depression in the sarcolemmal (SL) Ca-pump ATPase and Na-K ATPase activities; these changes lead to decreased Ca-efflux and increased Ca-influx, respectively and the inhibition of Ca sequestration from the cytoplasm in cardiomyocytes. The oxidative stress-induced changes in the SR Ca-pump as well as SL Na-K pump are not limited to cardiomyocytes but have also been observed in the coronary artery smooth muscle cells. These

alterations were markedly reduced by antioxidants such as catalase and superoxide dismutase. The depression in Ca-regulatory mechanism by ROS ultimately results in intracellular Ca^{2+} ($[Ca]_i$) overload and cell death. On the other hand, an increase in $[Ca]_i$ during ischemia induces the conversion of xanthine dehydrogenase to xanthine oxidase and subsequently results in generating superoxide radicals (Dhalla *et al.*, 2000).

Another mechanism to explain myocardial ischaemia injury is from the energy perspective. With loss of oxygen, mitochondrial oxidative phosphorylation rapidly stops, with a resultant loss of the major source of ATP production for energy metabolism. This engenders a compensatory increase in anaerobic glycolysis for ATP production which leads to the accumulation of hydrogen ions and lactate, resulting in intracellular acidosis and inhibition of glycolysis, as well as mitochondrial fatty acid and residual energy metabolism. Impaired contraction with persistent electrical activity (excitation contraction uncoupling) develops in association with alterations in ion transport systems in the sarcolemma and organellar membranes. This establishes a milieu for ventricular arrhythmias. Initially, there is increased K^+ efflux related to an increased osmotic load due to the accumulation of metabolites and inorganic phosphate. An increase in free Mg^{2+} is followed by a decrease in total Mg^{2+}. With a substantial decline in ATP, the Na^+, K^+-ATPase is inhibited, resulting in a further decline of K^+ and an increase in Na^+. The influx of Na^+, Cl^-, and water leads to cell swelling. An early increase in cytosolic Ca^{2+} also develops due to multifactorial changes in transport systems in the sarcolemma and sarcoplasmic reticulum. Ca^{2+}-induced activation of proteases causes alterations in contractile proteins, decreased sensitivity to Ca^{2+}, and sustained impairment of contractility despite the elevated cytosolic Ca^{2+} (Figure 4). The necrosis of myocytes and nonmyocytes triggers an inflammatory reaction with subsequent organization and healing. The progression to an advanced stage of cardiomyocyte injury is mediated by progressive membrane damage involving several mechanisms. The altered metabolic milieu with a sustained increase in cytosolic Ca^{2+} leads to phospholipase activation and phospholipid degradation with release of lysophospholipids and free fatty acids. Impaired mitochrondrial fatty acid metabolism results in the accumulation of free fatty acids, long-chain acyl CoA, and acyl carnitine, and these amphiphilic molecules, together with products of phospholipid degradation, incorporate into membranes and impair their function. Toxic oxygen species and free radicals are generated from ischemic myocytes, ischemic endothelial cells, and

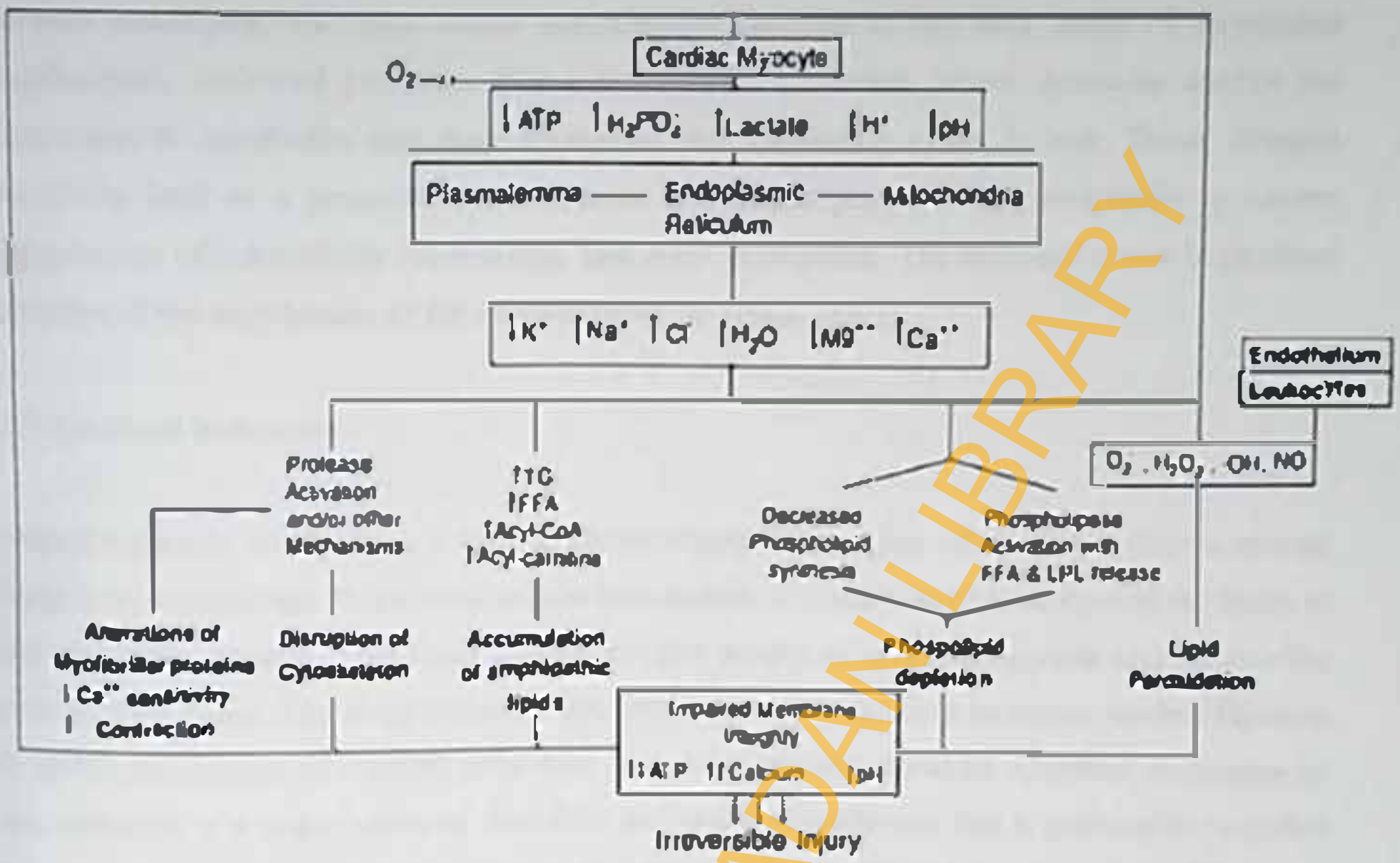

Figure 4: Postulated sequence of alterations involved in the pathogenesis of irreversible myocardial ischemic injury. Oxygen deficiency induces metabolic changes, including decreased adenosine triphosphate (ATP), decreased pH, and lactate accumulation, in ischemic myocytes. The altered metabolic milieu leads to impaired membrane transport with resultant derangements in intracellular electrolytes. An increase in cytosolic Ca^{2+} triggers the activation of proteases and phospholipases with resultant cytoskeletal damage and impaired membrane phospholipid balance. Alterations of myofibrillar contractile proteins lead to decreased Ca^{2+} sensitivity and decreased contraction despite the increased cytosolic Ca^{2+}. Lipid alterations include increased phospholipid (PL) degradation with release of free fatty acids (FFA) and lysophospholipids (LPL) and decreased phospholipid synthesis. The accumulation of amphipathic lipids alters membrane fluidity. Lipid peroxidation occurs as a result of attack by free radicals produced, at least in part, by the generation of excess electrons (e⁻) in oxygen-deprived mitochondria. Free radicals also are derived from the metabolism of arachidonic acid and catecholamines, the metabolism of adenine nucleotides by xanthine oxidase in endothelium (species dependent), and the activation of neutrophils and macrophages. The irreversible phase of injury is mediated by severe membrane damage produced by phospholipid loss, lipid peroxidation, and cytoskeletal damage (Buja, 2005).

activated leukocytes, and they induce peroxidative damage to the fatty acids of membrane phospholipids. Activated proteases cleave cytoskeletal filaments, which normally anchor the sarcolemma to myofibrils, and their anchoring and stabilizing effect is lost. These changes collectively lead to a progressive increase in membrane permeability, progressively severe derangements of intracellular electrolytes, and ATP exhaustion. The terminal event is physical disruption of the sarcolemma of the swollen myocyte (Buja, 2005).

2.2.3 Cerebral Ischemia

Cerebral ischemia results from a loss of blood supply to a region of the brain due to arterial blockage or hemorrhage. It is a condition in which there is insufficient blood flow to the brain to meet metabolic demand. This leads to poor oxygen supply or cerebral hypoxia and thus to the death of brain tissue. The most common and familiar manifestation is ischemic stroke (Flynn *et al.*, 2008) also known as cerebral infarction or cerebrovascular accident. Cerebral ischaemia or brain ischemia is a major cause of disability and death globally and has a profoundly negative impact on the individuals it affects, those that care for them and society as a whole. There are very few treatments for stroke and the development of new treatments requires a comprehensive understanding of the diverse mechanisms of ischemic brain damage that are responsible for neuronal death (Doyle *et al.*, 2008).

Stroke can be subdivided into 2 categories, ischemic and hemorrhagic. Ischemic strokes make up approximately 87% of all cases, and have been the target of most drug trials (Rosamond, 2007). A thrombosis, an embolism or systemic hypo-perfusion, all of which result in a restriction of blood flow to the brain, can cause an ischemic stroke, which results in insufficient oxygen and glucose delivery to support cellular homeostasis. Brain injury, following stroke, results from the complex interplay of multiple pathways including excitotoxicity, acidotoxicity, ionic imbalance, peri-infarct depolarization, oxidative and nitrative stress, inflammation and apoptosis (Gonzalez *et al.*, 2006). Each of the above pathophysiological processes has a distinct time frame, some occurring over minutes, others over hours and days, causing injury to neurons, glia and endothelial cells. Within the core of the ischemic area, where blood flow is most severely restricted, excitotoxic and necrotic cell death occurs within minutes. In the periphery of the

ischemic area, where collateral blood flow can buffer the full effects of the stroke, the degree of ischemia and the timing of reperfusion determine the outcome for individual cells. In this ischemic penumbra, cell death occurs less rapidly via mechanisms such as apoptosis and inflammation (Gonzalez *et al.*, 2006). The mechanism of ischemia reperfusion injury in the heart and brain are basically similar.

The human brain requires more oxygen (20% of total oxygen consumption) relative to its size (2% of body weight) (Edvinsson and Krause, 2002). This large amount of energy is needed by the brain to generate sufficient ATP by oxidative phosphorylation to maintain and restore ionic gradients. One estimate suggests that the Na^+/K^+ATPase found on the plasma membrane of neurons, consumes 70% of the energy supplied to the brain (Edvinsson and Krause, 2002). This ion pump maintains the high intracellular K^+ concentration and the low intracellular Na^+ concentration necessary for the propagation of action potentials. After global ischemia, mitochondrial inhibition of ATP synthesis leads to the residual ATP being consumed within 2 min, this causes neuronal plasma membrane depolarization, release of potassium into the extracellular space and entry of sodium into cells (Caplan, 2000). Energy failure also prevents the plasma membrane Ca^{2+} ATPase from maintaining the very low concentrations of calcium that are normally present within each cell. The extracellular calcium concentration is approximately 1.2 mM and most cellular processes regulated by calcium have a K_m value in the range of 0.1 to 1 μM. During ischemia intracellular calcium levels rise to 50 - 100 μM, activating many, if not all calcium dependent proteases, lipases and DNases (Edvinsson and Krause, 2002). This leads to many cells in the ischemic core dying from simple catabolism. Since no ATP is available for the re-synthesis of cellular constituents these catabolic enzymes cause the necrosis of essential cellular structures. Membrane depolarization also leads to neurotransmitter release, with the release of the excitatory neurotransmitter glutamate playing a critical role in ischemic pathology. A large concentration gradient of glutamate is maintained across the plasma membrane by sodium-dependent glutamate transporters located on presynaptic and postsynaptic membranes. The synaptic glutamate concentration is in the micromolar range, whereas the cytosolic concentration of glutamate is approximately 10 mM (Hsu, 1998). Membrane depolarization and accumulation of sodium inside cells during ischemia causes reversal of glutamate transporters and allows glutamate to exit cells along its concentration gradient. The

effect of an increase in synaptic glutamate concentration is the activation of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA) receptors. NMDA receptors are calcium permeable and the opening of these channels leads to further membrane depolarization and greater calcium influx, exacerbating intracellular calcium overload (excitotoxicity) (Olney, 1969). Concurrent to the induction of excitotoxicity, calcium overload is further exacerbated by acidosis, one of the hallmark neurochemical elements of the anaerobic metabolism of ischemia. Hyperglycemia increases lactate in the ischemic environment further depressing pH. Dissociated protons activate sodium-selective acid-sensing ion channels (ASICs) that are permeable to calcium leading to further calcium entry into the cell (acidotoxicity) (Simon, 2006).

Apart from excitotoxcity, acidotoxicity and ionic imbalance, peri-infarct depolarizations also contribute to neuronal death in cerebral ischemia. Cortical spreading depression (CSD) is a self-propagating wave of electrochemical activity that progresses through cortical tissue in intact brain. CSD causes sustained (1-5 min) cellular depolarization, depressed neuro-electrical activity, increased glutamate release and loss of membrane ionic gradients (Gonzalez *et al.*, 1992). Peri-infarct depolarizations (PIDs) are spontaneous waves of depolarization with all of the characteristic features of CSD that propagate through the penumbra following focal stroke. PIDs may be caused by the release of potassium and excitatory amino acids from the ischemic core. Although CSD in the normally perfused brain does not lead to cell death, recurrent PIDs in the ischemic brain are associated with increased ischemic injury. Repeated depolarization in the penumbra may mediate tissue damage by allowing calcium to accumulate within neurons. A critical threshold of calcium could be reached in the case of PID due to the compromised energy supply of the tissue, thus causing damage in the case of PID but without evidence of lasting damage in the case of CSD. PIDs are known to occur in animal stroke models, where the incidence and duration of spreading depression correlates with infarct maturation (Gill *et al.*, 1992; Strong *et al.*, 2000). Recently Fabricius and colleagues demonstrated the existence of PIDs in the acutely injured human brain, which suggests that inhibition of spreading depression using a therapeutic approach such as hypothermia or glutamate receptor antagonism could be an important strategy to limit development of ischemic injury within the penumbra (Chen *et al.*, 1993; Fabricius *et al.*, 2006).

Oxidative and nitrative stress is another key contributor to brain damage in ischemia. High levels of intracellular Ca^{2+}, Na^{+} and ADP cause mitochondria to produce deleterious levels of reactive oxygen species. Unlike other organs the brain is especially vulnerable to reactive oxygen species due to neurons having relatively low levels of endogenous antioxidants (Coyle and Puttfarcken, 1993). Overly abundant oxygen radicals cause the destruction of cellular macromolecules and participate in signaling mechanisms that result in apoptotic cell death (Halliwell, 1994; Sugawara and Chan, 2003). Nitric oxide synthase (NOS) is activated in ischemia and increases the generation of nitric oxide (NO), which combines with superoxide to produce peroxynitrite. The Production of NO and oxidative stress is also linked to over-activation of poly (ADP-ribose)polymerase- 1 (PARP-1), a DNA repair enzyme. In response to DNA strand breaks PARP-1 catalyzes the transformation of β-nicotinamide adenine dinucleotide (NAD^{+}) into nicotinamide (NA) and long polymers of poly(ADP-ribose). When PARP-1 is over-activated it depletes cells of NAD^{+}, impairing NAD^{+} dependent processes such as anaerobic glycolysis and mitochondrial respiration, which leads to ATP starvation, energy failure and neuronal death (Gonzalez *et al.*, 2006). Following reperfusion there is a surge in production of superoxide, NO and peroxynitrite. Formation of these radicals in the vicinity of blood vessels plays an important role in reperfusion-induced injury. These radicals activate matrix metalloproteases (MMPs), which degrade collagen and laminins in the basal lamina, which disrupts the integrity of the vascular wall and increases blood brain barrier (BBB) permeability. Oxidative and nitrative stress also triggers recruitment and migration of neutrophils and other leukocytes to the cerebral vasculature, which release enzymes that further increase basal lamina degradation and vascular permeability. These events can lead to parenchymal hemorrhage, vasogenic brain edema and neutrophil infiltration into the brain (Crack and Taylor, 2005). The surge in production of free radicals associated with delayed reperfusion brings a second wave of oxidative and nitrative stress that increases the risk of brain hemorrhage and edema (Doyle *et al.*, 2008). The oxidative burst caused by reperfusion after a period of cerebral ischemia exacerbate these injuries.

Another critical metabolic event in cerebral ischemia is the activation of phospholipase A_2 (PLA_2) occasioned by excess glutamate release and the stimulation of its receptors which results in the activation of phospholipases, hydrolysis of membrane phospholipids and release of free

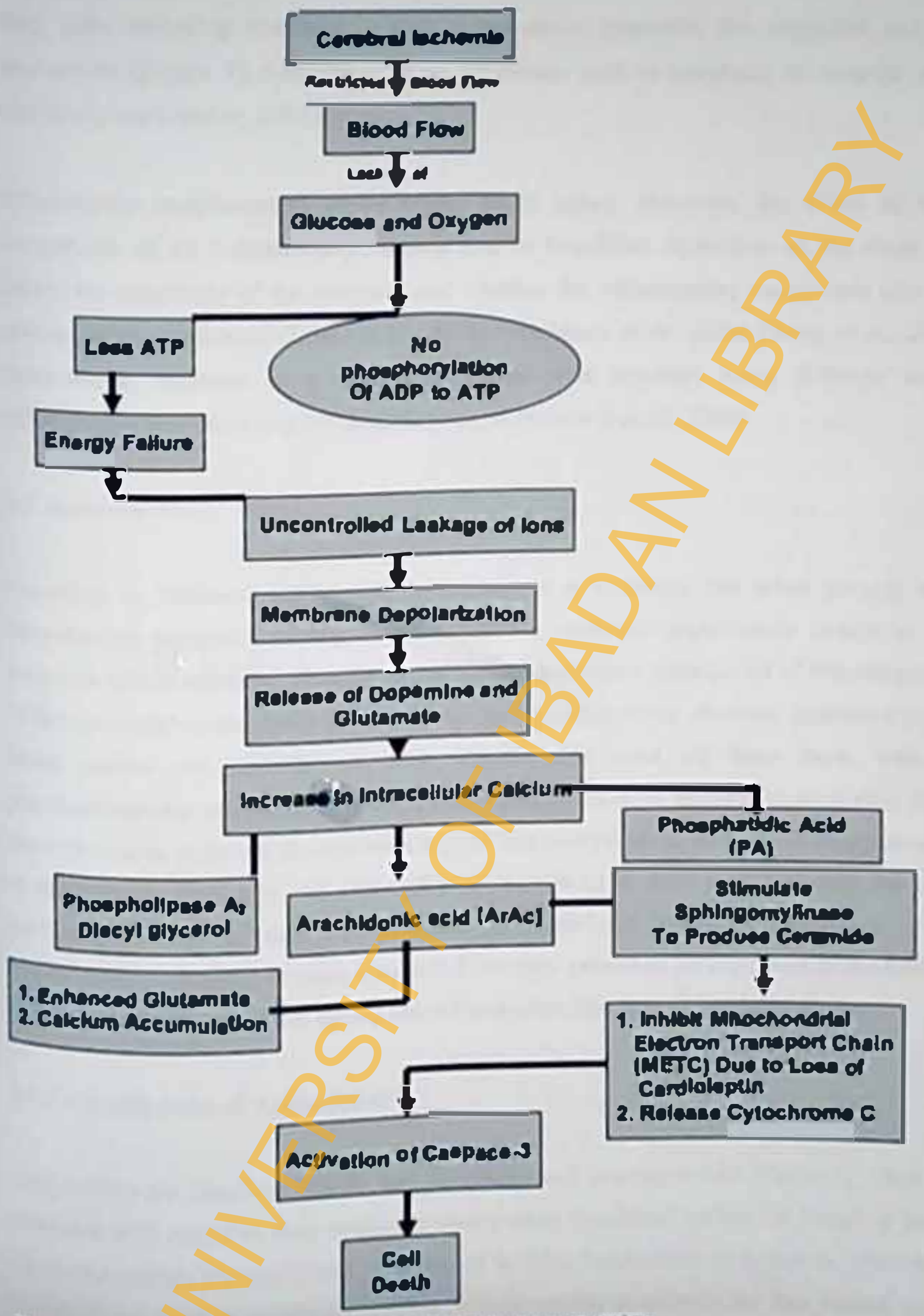

Figure 5. Cerebral ischemia induced death cascade (Saeed *et al.*, 2007).

fatty acids including arachidonic acid, a metabolic precursor for important cell-signaling eicosanoids (Figure 5). Ultimately these processes lead to apoptotic or necrotic cell death (Adibhatla and Hatcher, 2006).

Inflammation contributes to stroke-related brain injury. However, the effect of individual components of the inflammatory cascade can be beneficial depending on the stage of tissue injury, the magnitude of the response and whether the inflammatory component also activates neuroprotective pathways (Bruce *et al.*, 1996; Nawashiro *et al.*, 2000; Zhang *et al.*, 2000). The inflammatory response is a composite process that involves many different cell types, inflammatory mediators and extracellular receptors (Doyle *et al.*, 2008).

2.3. Antioxidants

According to Halliwell (1990), an antioxidant is a substrate that when present at a low concentration compared to that of an oxidisable substrate, significantly delays or prevents oxidation of that substrate. In recent years, antioxidants have gained a lot of importance because of their potential as prophylactic and therapeutic agents in many diseases. Extensive research is being carried out globally on these agents, and most of them have been proven pharmacologically active. Traditionally, herbal medicines with antioxidant properties have been used for various purposes and epidemiological data also points at widespread acceptance and use of these agents. Presently, the active phytochemicals from these herbal sources are extracted, purified and tested for their activities and the results are promising (Ratnam *et al.*, 2006). Antioxidant constituents of plant materials have been proved to be important in the maintenance of health and protection from ageing-related and other free radical mediated diseases.

2.3.1. Classification of Antioxidants

Antioxidants are classified chiefly into enzymatic and non-enzymatic (Table 1). They are also classified with regard to their source as endogenous (Produced within the body) or exogenous (produced outside the body) and with regard to their mechanism of action as primary (inhibit oxidation via chain terminating reactions through proton transfer to the free radical species) or

secondary (decompose hydroperoxides into non-radical, non-reactive, and thermally stable products). Many non-enzymatic antioxidants are obtained from dietary sources and could be referred to as dietary or nutritional antioxidants. The main dietary antioxidants are the phytochemicals called polyphenols.

2.3.2. Mechanism of Action of Antioxidants

Although the exact mechanisms and interactions among various anti-oxidants are not fully understood, it is possible that one antioxidant may equilibrate with another to establish a cellular redox potential and thus all endogenous anti-oxidants may act in concert to protect against oxidative insult. Nonetheless, it has been suggested that antioxidants can act through several mechanisms such as: (a) scavenging ROS or their precursors, (b) inhibiting the formation of ROS, (c) attenuating the catalysis of ROS generation via binding to metals ions, (d) enhancing endogenous anti-lipoprooxidant generation and (e) reducing apoptotic cell death by upregulating the anti-death gene (*Bcl*-2) (Dhalla *et al.*, 2000).

SOD catalyzes the dismutation of superoxide anion ($O_2^{\cdot-}$) to H_2O_2. Subsequently H_2O_2 is reduced to H_2O and O_2 by peroxidases such as glutathione peroxidase or catalase. SOD is present in the cytoplasm as well as on the endothelial cell surface with either copper or zinc (CuSOD, ZnSOD) and in the mitochondria with manganese (MnSOD). Glutathione peroxidase catalyzes the peroxidation of H_2O_2 in the presence of reduced glutathione (GSH) to form H_2O and oxidized glutathione (GSSG). The GSSG is recycled to give GSH by glutathione reductase, which requires NADPH from the hexose monophosphate shunt. Catalase is a membrane bound enzyme which is present in peroxisomes but its activity has also been observed in the mitochondrial matrix. Vitamin E is a fat soluble substance and is mainly associated with plasma lipoproteins. It acts as a potent peroxyl radical scavenger via breaking the lipid peroxidation chain reaction (Dhalla *et al.*, 2000).

Table 1: Important enzymatic and non-enzymatic physiological antioxidants

Antioxidants	Location	Properties
Enzymatic		
Superoxide dismutase (SOD)	Mitochondria, cytosol	Dismutate superoxide radicals
Glutathione peroxidase (GSH)	Mitochondria and cytosol	Removes hydrogen peroxide organic hydroperoxide.
Catalase (CAT)	Mitochondria and cytosol	Removes hydrogen peroxide
Non-enzymatic		
Vitamin C	Aqueous phase of cell	Acts as free radical scavenger and recycles vitamin E
Vitamin E	Cell Membrane	Major chain-breaking antioxidant in cell membrane
Uric acid	Product of purine metabolism	Scavenger of OH radicals
Glutathione	Nonprotein thiol in cell	Serves multiple roles in cellular antioxidant defense
α-lipoic acid	Endogenous thiol	Effective in recycling vitamin C, may also be an effective glutathione substitute
Carotenoids	Lipid soluble antioxidants, located in membrane tissue	Scavengers of reactive oxygen species, singlet oxygen quencher
Bilirubin	Product of heme metabolism in blood	Extracellular antioxidant
Ubiquinones	Mitochondria	Reduced forms are efficient antioxidants
Transferrin, ferritin, lactoferrin		Chelating of metal ions responsible for Fenton's reaction
Ceruloplasmin		Chelating Copper ion

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Ceruloplasmin		Chelating Copper ion

2.3.3. Methods for assessment of antioxidant activity

There are numerous antioxidant assay methods and their modifications for evaluation of antioxidant activity. These include oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity TEAC), ferric reducing antioxidant power (FRAP), total oxidant scavenging capacity (TOSC), total radical-trapping antioxidant parameter (TRAP), DPPH reactivity, croton bleaching, LDH oxidation, liposome oxidation and total phenolic analysis (Schaich, 2006). Of these, total antioxidant activity, reductive potential, DPPH assay, metal chelation, active oxygen species quenching are most commonly used (Chang *et al*., 2002; Gulcin *et al*., 2002). For the most part, assay selection is based on ease of use and availability of instrumentation, and very often there is lack of correlation between activities determined on the same material by different assays, and between activities determined by the same assay in different laboratories (Schaich, 2006). This is because assays do not all measure the same chemical action. Some assays measure hydrogen atom transfer capability (classical radical quenching), some measure electron transfer propensity while some evaluate the chelating ability.

2.4. Phytochemicals

Plants show enormous versatility in synthesizing complex materials which have no immediate obvious growth or metabolic functions. These complex materials are referred to as secondary metabolites. Some biological roles of plant secondary metabolites include ability to act as phytotoxins, phytoalexins (defensive chemicals produced by plant), animal repellants, animal attractants (for pollination and seed dispersal) and insect hormones (to coordinate growth of the insect with that of the plant). Plant secondary metabolites can also be used by humans as drugs and medicines (for cardiovascular and neurological disorders, and as antitumor agents), narcotics, stimulants, hallucinogens and poisons (insecticides, rodenticides). Secondary metabolites are also important for development of traits such as flower colour, flavor of food and resistance against pests and diseases.

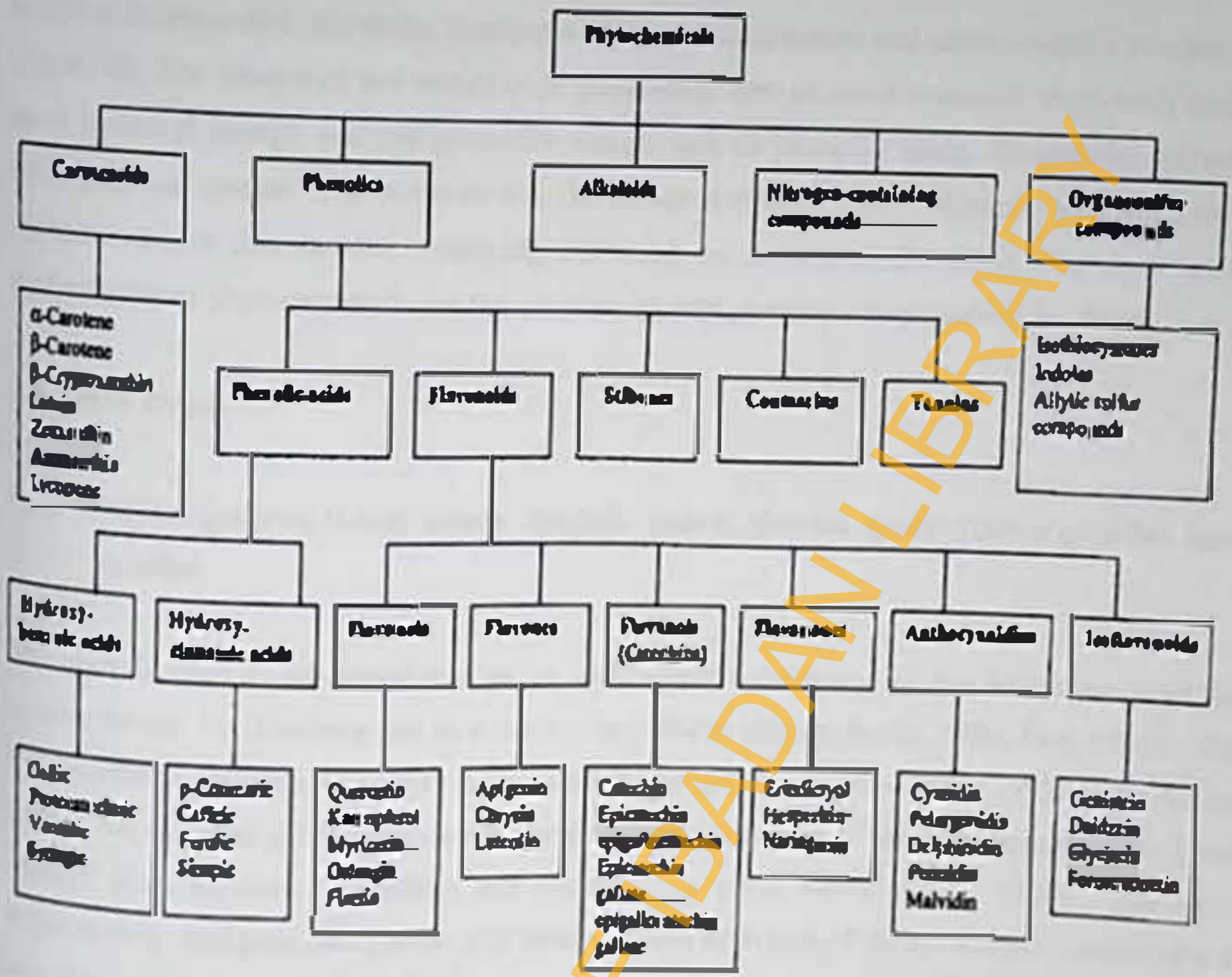


Figure 6: Classification of dietary phytochemicals (Liu, 2004).

Plant secondary metabolites have recently been referred to as phytochemicals which have been defined as bioactive nonnutrient plant compounds in fruits, vegetables, grains and other plant foods that have been linked to reducing the risk of major diseases (Liu, 2004). New Phytochemicals are being discovered on a daily basis, and it is estimated that plants contain hundreds of thousands of different Phytochemicals (Liu, 2004). Researchers have long known that phytochemicals provide health benefits for plants, but it is only recently that certain phytochemicals have been recommended for the purpose of treating various diseases in humans. It is believed that phytochemicals may be effective for combating or preventing disease due to their antioxidant effect (Farombi and Britton, 1999a; Farombi and Britton, 1999b; Farombi, 2000). The medicinal values of plants lie in their component phytochemcals, which produce a definite physiological action on the human body. Phytochemicals can be classified as

carotenoids, phenolics, alkaloids, nitrogen-containing compounds and organosulphur compounds (Figure 6). The phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and are generally categorized as phenolic acids, flavonoids, stilbenes, coumarins and tannins. It is estimated that flavonoids account for approximately two thirds of the phenolics in our diet and the remaining one third are from phenolic acids. The most widely studied of these phytochemicals are the carotenoids and phenolic compounds (Liu, 2004).

2.5. Study Plants

2.5.1. *Psidium guajava* (Local names: English: guava; Yoruba: guafa; Hausa: gwaaba; Igbo: ugwoba)

The plant is used in ethnomedicine as an antimalarial. Infusions of the leaves are used for treating fevers, for diarrhoea and as a tonic in psychiatry (Oliver-Bever, 1986; Iwu, 1993). The hydroalcoholic extract was shown to decrease motor activity in mice (Iwu, 1993). The leaves contain an essential oil rich in cineol, tannins and triterpenes. Three flavonoids have been isolated from the leaves (Khadem and Mohammed, 1958; Oliver-Bever, 1986). The anti-inflammatory, analgesic, antipyretic and antidiarrhoeal activities of the methanolic extract of the leaves have been reported. Its CNS depressant activity has also been reported (Olajide *et al*., 1999).

2.5.2. *Cassia alata* (Local names: English: ringworm plant, candle bush, Yoruba: esunwon; Igbo: ogala)

It is used for many ailments and disease conditions. The dried leaves are taken internally as a laxative and also in cases of constipation. The sap of the leaves is a well-known remedy for ringworm, scabies, ulcers, swellings or inflammatory conditions and other parasitic skin diseases (Elujoba, 1989; Abatan, 1990). An infusion of the leaves and flowers is used for asthma and bronchitis. *Cassia alata* is reputed to prevent or cure hepatic diseases. It is also used for the treatment of dropsy, poisonous bites, venereal eruptions and as a vermifuge (Quisumbing, 1951; Palanichamy *et al*., 1991; Wijayakusuma, 1996). It has been reported to possess wound-healing,

hypoglycaemic, analgesic and antifungal properties as well as antibacterial, antimicrobial, diuretic and cholerctic activities. Anthraquinones, anthracene derivatives, flavonoids, tannins, saponins, sesquiterpenes and phenolic compounds have been discovered in extracts from leaves and fruits of *Cassia alata* (Rai, 1978; Elujoba, 1989). A probable antimicrobial agent, chrysophanol has also been detected in the leaf extract (Ibrahim and Osman, 1995).

2.5.3. *Newbouldia laevis* (Local names: Yoruba: Akoko; Hausa: aduruku; Igbo: ogbu)

It is used for the treatment of various diseases including epilepsy, convulsions, rheumatism, arthritis and fever. The bark and leaves are used for the treatment of breast tumors. Extracts of the leaf, stem bark and root exhibited antimicrobial activities. The plant has been reported to possess anti-inflammatory, analgesic, anti pyretic and anticonvulsant activities. The leaf extract was shown to possess antimalarial activity against *Plasmodium falciparum in vitro* (Dalziel, 1937; Ogunlana and Ramstad 1975; Burkill, 1985; LeGrand *et al.*, 1988; Gbeassor *et al.*,, 1990). Phytochemical screening gave positive tests for flavonoids (Olajide *et al.*, 1997).

2.5.4. *Alstonia boonei* (Local names: Yoruba: awun, ahun; Others: egbu, akpi)

The plant has been reported to possess anti-venom, anti-inflammatory, antihypertensive, antipyretic and analgesic properties (Kweifo-Okai, 1991a, b; Kweif-Okai *et al.*, 1995). Ojewole (1984) and Asuzu and Anaga (1991) reported that the stem bark is used for treating ailments such as malaria, painful micturition and rheumatic conditions. Extracts of the plant has also been reported to possess antimicrobial property against *E. coli, Salmonella paratyphi and Shigella dysenteriae*. Kweifo-Okai *et al.* (1995) remarked that the anti-inflammatory activity of *A boonei* could be due to alpha-amyrin palmitate as the active ingredient. However, Olajide *et al.* (2000) submitted that no report on phytochemical studies on *A. boonei* has shown the presence of this compound. Phytochemicals established to be present in the extract of the plant include echitamine, an alkaloid (Ojewole, 1984); saponins and alkaloids, triterpenes and indole alkaloids for example, alstonine, prophyine and astonidine.

2.5.5. *Chromolaena odorata* (Local names: English: siam weed; Yoruba: akintola, awolowo; Igbo: obiatakara, ahihia eliza)

The antispasmodic, antiprotozoal, antititrypanosomal, antibacterial, antidiarrhoeal, antihypertensive, anti-inflammatory, astringent, diuretic and hepatotropic properties of the plant have been reported (Watt and BreyerBrandwijik, 1962; Feng *et al.*, 1964; Weniger and Robineau, 1988, Iwu, 1993). It is used ethnomedically in wound dressing, to treat skin infection and to stop bleeding. Phytochemicals shown to be present in the plant include the flavonoids quercetin, isosakuranetin and sakuranetin (Metwally and Ekejuba, 1981), limonene found in the leaves and flowers, phenolic compounds found in extracts from the leaves, flavonols (flavones and chalcones), linoleic acid, carnosine and pyrroloquinoline quinone (from the leaves). *C. odorata* causes skin irritations and rashes in people with allergic reactions to it. Toxin from it has allelopathic effect on tomatoes (*Lycopersicum esculentum* Mill).

2.5.6. *Globimetula cupulata* (Local names: English: mistletoe; Yoruba: afoma; Hausa: kauchi; Igbo: apari, awuruse)

Globimetula cupulata is used for treating hypertension. Certain components of the plant also have anticancer activity but the value of the whole plant in cancer treatment is not fully accepted (Brown, 1985; Chevallier, 1996). The leaves and young twigs are antispasmodic, cytostatic, diuretic and hypotensive. *Globimetula cupulata* has a reputation for curing epilepsy and other convulsive nervous disorders. It has also been employed in checking internal hemorrhages and to treat arthritis, rheumatism, chilblains, leg ulcers and varicose veins (Brown, 1985).

2.5.7. *Securidaca longepedunculata* (Local names: English: violet tree; Yoruba: ipeta; Hausa: uwarmagunguna, sanya; Igbo: ekoogwu, tshi-venda mpesu)

It is used as traditional medicine in many parts of Africa and against a number of invertebrate pests of stored grains. Main volatile component is methyl salicylate. The root bark has 2-hydroxy-6-methoxy benzoic acid methyl ester. The xanthones 1,7-dimethoxy-2-hydroxy-xanthone and 1,4 -dihydroxy-7-methoxy-xanthone isolated from the root have activity against

erectile dysfunction (Rakuambo *et al.*, 2004). The plant is used to treat erectile dysfunction, coughs, colds, fever, backache, toothache, sleeping sickness, venereal disease, malaria, inflammation, rheumatism, snake bite, tuberculosis, ulcers and pneumonia and as a contraceptive.

2.5.8. *Ocimum gratissimum* (Local names: English: basil; Yoruba: efinrin, efinrin-nla, oromobo; Hausa: Daidoya; Igbo: Nchanwu)

The plant is used for the treatment of rheumatism, paralysis, epilepsy, high fever, diarrhoea, and mental illness. Extracts from the plant have been found to possess antipyretic, antidiarrhoeal and hepatoprotective properties (Dhawan, *et al.*, 1977; Abdulrahman, 1992; Sofowora, 1993). Essential oil from the plant inhibited protozoan growth. The essential oil contains Eugenol, linalool, methyl cinnamate, camphor and thymol. The eugenol content has antibacterial and antihelminthic activities (Holets, 2003). The plant also contains phenolic acids with antibacterial and molluscicidal properties. The essential oils have antifungal activity. The plant extracts is used against gastrointestinal helminths of animals and man and inhibits glutathione-S-transferases from parasitic nematodes. It is also used as an emetic and for the treatment of haemorrhoids, stomach problems and eye/throat inflammation.

2.5.9. *Morinda lucida* (Local names: English: brimstone plant; Yoruba: oruwo, eruwo; Igbo: nuke, eze ogu, njisi)

Morinda lucida is used for the treatment of malaria and typhoid fever. Currently, Japan imports over fifty million dollars worth of *Morinda lucida* per year to treat people with chronic malaria and typhoid. Although the modern community has given the health benefit of *Morinda lucida* limited official standing. There have been numerous worldwide scientific studies by authoritative and respected medicinal researchers regarding the ubiquitous health benefits for people and animals as well. It was noted that whole leaf extracts could bring complete resolution of the following; typhoid, malaria and jaundice. *Morinda lucida* is hypoallergenic but has side effect when taken in large dose. It is also used in treating wound infections, abscesses and chancre (Hartill, 1997). It has been discovered also that treatment of experimental animals with essential

oil of *Morinda lucid* lower the plasma level of dienic conjugates and ketones. The hypoglycaemic effect of *Morinda lucida* has been reported.

2.5.10. *Spondias mombin* (Local names: hog plum; Yoruba: iyeye; Hausa: tsadar lamarudu; Igbo: ijikara)

Spondias mombin is a tree that is native to Africa (Duvall, 2006) and also found in other continents of the world. It is a tropical genus of the family *Anacardiaceae* with about 14 species worldwide. *Spondias mombin* is the only widespread species in Africa.

Various medicinal properties of the plant have been described. These include antioxidant, antimicrobial, antitumour, abortifacient, antidiabetic, sedative, antiepileptic and antipsychotic properties (Ayoka *et al*, 2006). The fruit juice is drunk as a diuretic and febrifuge. The decoction of the astrigent bark serves as an emetic, a remedy for diarrhoea, dysentery, hemorrhoids, gonorrhea and leucarrhea. *Spondias mombin* leaf extract has wide spectrum antibacterial effect comparable to those of ampicillin and gentamycin. It is used as an anti-infective agent in traditional medicine. Aqueous extract of the leaves possesses abortifacient property. Extracts from the bark have antitumour property and are used for the treatment of malignancies. The plant contains antibacterial and molluscicidal phenolic acids and a sizable amount of vitamin C.

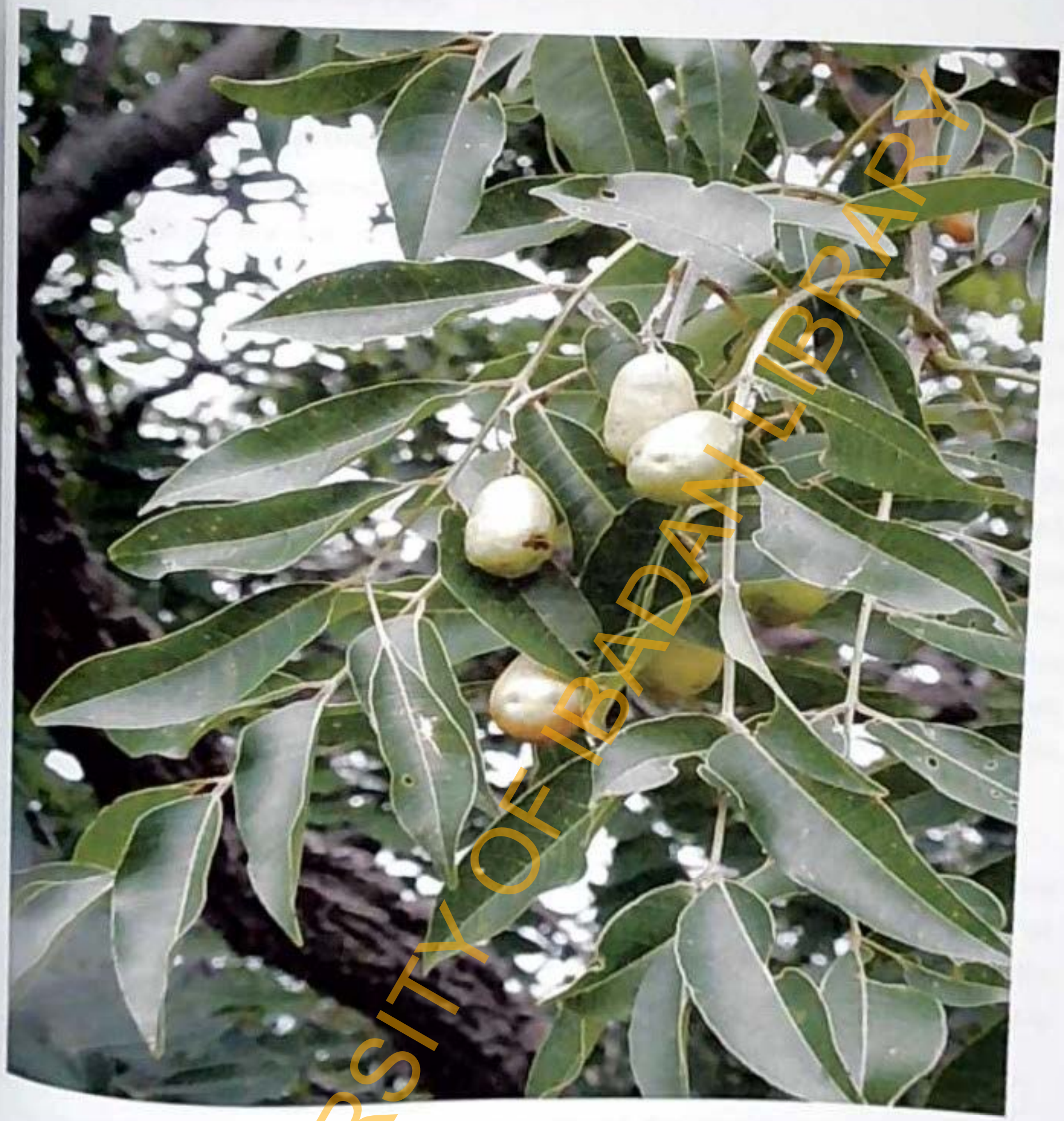


Figure 7: Leaves and fruits of *Spondias mombin*

2.6. Aims and objectives of the study

Plant extracts and plant-based foods are important sources of antioxidants. Antioxidants help protect cells from oxidative stress, a potentially damaging physiological process, which has been associated with the development of chronic diseases such as cancer, myocardial infarction and cerebrovascular accident or stroke (Farombi, 2000; Liu, 2004).

Phytochemicals are responsible for the antioxidant and free radical scavenging activities of plants. Other biological properties of many phytochemicals from different plants have been documented. There are also standard tests for evaluating bioactivities of novel phytochemicals (Farombi, 2000; Farombi, 2003; Sun et al., 2008; Akinmoladun *et al*., 2009). Knowledge of the types of phytochemicals present in a given plant could therefore be a useful guide towards elucidating the basis for their ethnomedical or traditional use and also offer insight into new therapeutic applications. Phenolics, especially flavonoids, have been mostly credited with the antioxidant property demonstrated by plant extracts. A direct proportional relationship between phenolic content and antioxidant activity of plant extracts has been reported. Accordingly, it will be necessary to screen the study plants for phytochemical constituents, determine their phenolic contents and investigate relationships among phenolic contents and other antioxidant indices.

Controversy currently exists as to the reproducibility and reliability of results obtained from assays for antioxidant activity (German, 1999; Schaich, 2006). Different assay methods will be employed for the assessment of the antioxidant and free radical scavenging activities of the extracts. A comparison of the various assay protocols will be carried out to determine whether or not there is a basis for such controversy.

Results of the first phase of this investigation showed that *Spondias mombin* possessed a remarkable antioxidant and free radical scavenging activity among the ten study plants. It was therefore selected for further studies. *Spondias mombin* is popularly and widely used in ethnomedicine all over the world. Pharmacological properties that have been reported for the plant include antimicrobial, antitumour, abortifacient, antidiabetic, sedative, anticonvulsant and

antipsychotic properties (Ayoka *et al.*, 2006). Its antioxidant, antiproliferative, cardioprotective and neuroprotective properties have not been investigated to any significant extent.

These properties have been examined in this study. Antioxidant and antilipoproliferative activities of the crude extract and fractions from the leaves of the plant will be further evaluated with a view to knowing the most active fraction and isolating some bioactive components. Furthermore, the cardioprotective and neuroprotective properties of the leaf extracts will be investigated.

The objectives of the present study are:

1. To qualitatively confirm the phytochemical groups present in extracts from the ten plants under investigation
2. To examine the correlation between total phenolic content and other antioxidant indices
3. To ascertain from results of the various antioxidant assay methods whether or not there is a basis for the present controversy regarding the reliability and reproducibility of results from antioxidant studies
4. To evaluate the cardioprotective property of the crude extract of *Spondias mombin* using *ex vivo* and *in vivo* model systems
5. To evaluate the neuroprotective property of the crude extract from *Spondias mombin* using the middle cerebral artery occlusion induce focal cerebral ischemia model
6. To evaluate the antioxidant and antiproliferative activities of the crude extract and fractions from the leaves of *Spondias mombin* with a view to characterizing bioactive compounds from the most active fraction(s).

CHAPTER THREE

MATERIALS AND METHODS

(A). Antioxidant and free radical scavenging activities of extracts from ten selected Nigerian medicinal plants

3.1. Chemicals

Folin-Ciocalteu reagent, sodium carbonate, gallic acid, aluminium chloride hexahydrate, sodium nitrate, quercetin, ascorbic acid, 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical, methanol, ethanol, thiobarbituric acid (TBA), tricholoroacetic acid (TCA), Iron (III) Chloride, Iron (II) sulphate, acetic acid, sodium dodecyl sulphate, nitroblue tetrazolium (NBT), potassium ferricyanide ($K_3Fe(CN)_6$), ethylene diamine tetraacetic acid (EDTA), riboflavin, deoxyribose, ascorbic acid, sodium cyanide, hydrogen peroxide (H_2O_2), butan-1-ol, sodium nitroprusside, sodium chloride, Griess reagent, butylated hydroxyl toluene (BHT), potassium acetate, rifampicin, nifedipin, dithiobis nitrobenzene (DTNB), reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide (NADH), triphenyl tetrazolium chloride (TTC), sulphosalicylic acid, 1,1,3,3-tetraethoxypropane (TEP), bovine serum albumin (BSA), sodium potassium tartrate, sodium pyruvate, *N*-(1-naphthyl) ethylenediamine dihydrochloride (NED), sulphanilamide, orthophosphoric acid, silica gel (230 – 400 mesh), and all other reagents used were of analytical grade and obtained from standard suppliers such as Sigma-Aldrich (USA), BDH (UK) and Spectrochem Pvt. Ltd., Mumbai, India.

3.2. Study plants and parts used

The plants and the specific parts investigated are listed in Table 2. Authentication was done at the department of Crop, Soil and Pest Management, Federal University of Technology, Akure, Nigeria and the Department of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria.

Table 2: Study plants and parts used

SCIENTIFIC NAME	LOCAL NAME	PART USED	VOUCHER NUMBER
Psidium guajava	Guava	Leaves	PG-L-001-06
Cassia alata	Asuuwon	Leaves	CA-L-001-06
Newbouldia laevis	Akoko	Stem bark	NL-SB-001-06
Alstonia boonei	Ahun	Stem bark	AB-SB-001-06
Globimetula cupulata	Afomo	Leaves	GC-L-001-06
Chromolaena odorata	Akintola	Leaves	CO-L-001-06
Securidaca longepedunculata	Ipeta	Root	SL-R-001-06
Spondias mombin	Iyeye	Leaves	SM-L-001-06
Ocimum gratissimum	Elinrin	Leaves	OG-L-001-06
Morinda lucida	Oruwo	Leaves	ML-L-001-06

3.2.1. Extraction of plant parts

Plant materials were obtained from farmlands in Akure, Ondo State, Nigeria. They were dried under active ventilation at room temperature, packed in paper bags and stored. The materials were later pulverized and extracted in 80% methanol by maceration for 72 h. The methanolic extract was concentrated in a rotary evaporator, lyophilized and preserved for further use. Parts of the pulverized samples were also extracted in water for the purpose of comparative phytochemical screening.

3.3. Phytochemical Screening

The aqueous and methanolic extracts were screened for the presence of alkaloids, saponins, tannins, phlobatannins anthraquinones, steroids, terpenoids flavonoids and cardiac glycosides.

3.3.1. *Test for alkaloids*

Extract (5 ml) was added to 5 ml of aqueous HCl (1%) in a steam bath. The solution was filtered and the filtrate treated with a few drops of Dragendorff's reagent. Turbidity or precipitate showed the presence of alkaloids (Trease and Evans, 2002).

3.3.2 *Test for saponins*

Extract (1 ml) was mixed with 5 ml of water in a test tube and warmed. Frothing indicated the presence of saponins.

3.3.3. *Test for tannins*

Extract (5 ml) was stirred with 10 ml of distilled water. The mixture was filtered and the filtrate treated with ferric chloride. A blue-green – black-green precipitate indicated the presence of tannins (Trease and Evans, 2002).

3.3.4. *Test for phlobatannin*

Extract (5 ml) was boiled with 5 ml of 1% aqueous HCl. A red precipitate showed the presence of phlobatannins (Trease and Evans 2002).

3.3.5. *Test for Anthraquinones*

Extract (5 ml) was mixed with 10 ml of benzene and filtered. Five ml of 10% ammonia solution was added to the filtrate. The mixture was vortexed. The presence of pink, red or violet colour in the ammoniacal lower phase indicated the presence of free anthraquinones.

3.3.6. *Test for steroids*

Acetic acid (2 ml) was added to 0.5 ml of extract. Two ml of H_2SO_4 was thereafter added. A violet to blue-green colour showed the presence of steroids (Edeoga *et al.*, 2005).

3.3.7. *Test for terpenoids*

Extract (5 ml) was mixed with 2 ml of chloroform. Three ml of concentrated H_2SO_4 was then carefully added to form a thin layer. A reddish brown colornion at the interface indicated positive result for terpenoids (Edeoga *et al.*, 2005).

3.3.8. *Test for flavonoids*

Dilute ammonia solution was added to the extract followed by the addition of concentrated H_2SO_4. A yellow coloration which disappeared on standing indicated the presence of flavonoids (Sofowora, 1993).

3.3.9. *Tests for cardiac glycosides*

3.3.9.1. *Salkowski test*

The extract was dissolved in chloroform and 1 ml of H_2SO_4 was carefully added to from a lower layer. A reddish brown colour at the interface showed the presence of cardiac glycosides with steroidal ring (Trease and Evans, 2002).

3.3.9.2. *Keller-Killiani test*

Extract was added to 2 ml glacial acetic acid containing a drop of ferric chloride solution. This was underplayed with 1 ml concentrated H_2SO_4. A brown ring at the interface indicated a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring while

in the acetic acid layer and a greenish ring may form just gradually throughout a thin layer (Trease and Evans, 2002).

3.4. Estimation of total phenolic content

The assay is based on the reduction of Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate) by the phenolic compounds. The reduced Folin-Ciocalteu reagent is blue and thus detectable with a spectrophotometer in the range of 500-750 nm (Singleton *et al.*, 1999; McDonald *et al.*, 2001).

Reagents

1. Ethanol
2. Folin – Ciocalteu reagent (Sigma-Aldrich)
3. Gallic acid standard (5 g/L)
   This was prepared by dissolving 0.5 g of dry gallic acid in 10 ml ethanol in a 100 ml volumetric flask and then making up the volume to mark with distilled water.
4. Sodium carbonate (15%)
   This was prepared by dissolving 15 g of sodium carbonate in distilled water and making up the volume to 100 ml.

Procedure

Serial dilutions of 50 mg/L, 100 mg/L 150 mg/L and 250 mg/L were prepared from the gallic acid standard solution. Gallic acid solution (0.1 ml) or solution of the extracts (0.1 ml, 20 mg/ml) was added to 0.2 ml Folin-Ciocalteu reagent (diluted ten-fold) and 2 ml of distilled water. After a few minutes, 1 ml of 15% Na_2CO_3 was thoroughly mixed with the solution. The solutions were incubated at 40 °C for 30 min after which the absorbance was read at 760 nm. Total content of phenolic compounds in plant methanolic extracts was expressed in mg/L gallic acid equivalent (GAE).

3.5. Estimation of total flavonoid content

Total flavonoid content of extract was estimated using the aluminium chloride colorimetric method of Chang *et al.* (2002).

Reagents

1. Methanol
2. Aluminium chloride (10%)

   Ten gram of aluminium chloride was dissolved in distilled water and made up to 100 ml.
3. Sodium acetate (1 M)

   This was prepared by dissolving 9.3 g of sodium acetate in distilled water and making it up to 100 ml. Sodium acetate was used in place of potassium acetate in the original method.
4. Quercetin (100 µg/ml)

   This was prepared by dissolving 1mg of quercetin in methanol to get a final volume of 10 ml.

Procedure

Each plant extract (0.5 ml, 1 mg/ml) in methanol were separately mixed with 0.1 ml of 10% $AlCl_3.6H_2O$, 0.1 ml of 1 M sodium acetate and 2.8 ml of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm. The calibration curve was plotted by preparing quercetin solutions at concentrations 12.5-100 µg/ml. Total flavonoid content was expressed as µg/mL quercetin equivalent (QE)

3.6. DPPH free radical scavenging activity

DPPH free radical scavenging activity of extracts was determined using the DPPH spectrophotometric method (Mensor *et al.*, 2001).

Principle

When DPPH reacts with an antioxidant compound which can donate hydrogen it is reduced. The change in colour from deep violet to golden/light yellow can be measured at 518 nm.

Reagents

1. DPPH (0.3 mM)
   Prepared by dissolving 0.03 g of DPPH in methanol and making up the volume to 250 ml.
2. Gallic acid (300 µg/ml)
   This was prepared by dissolving 3 mg of gallic acid in methanol to get a final volume of 10 ml.
3. Ascorbic acid (300 µg/ml)
   This was prepared by dissolving 3 mg of ascorbic acid in methanol to get a final volume of 10 ml.

Procedure

DPPH methanol solution (1 ml, 0.3 mM) was added to 1 ml of extract, gallic acid or ascorbic acid and allowed to react at room temperature. The absorbance values were read after 30 min and converted into percentage antioxidant activity using the formula

$$AA\% = 100 - \frac{[(Abs_{sample} - Abs_{blank}) \times 100]}{Abs_{control}}$$

Methanol (1 ml) added to 1 ml of extract served as blank. DPPH (1 ml, 0.3 mM) added to 1 ml of methanol served as negative control. The positive controls were solutions ascorbic acid and gallic acid.

3.7. Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was determined as described by Babu and Padikkala (2001).

Principle

Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction.

Reagents

1. Sodium nitroprusside (10 mM)
   Sodium nitroprusside (0.66 g) was dissolved in phosphate buffered saline and made up to 200 ml.
2. Griess reagent (Sigma-Aldrich)
3. Phosphate buffered saline (pH 7.4)
   This was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4 and 1.06 g of Na_2HPO_4 in distilled water and making up the volume to 1 l.

Procedure

The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the extract (1 mg/ml) was incubated at 25 °C for 150 min. After incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was evaluated at 546 nm and expressed in percentage.

3.8. Hydroxyl radical scavenging activity (Deoxyribose assay)

The hydroxyl radical scavenging activity was determined as described by Noorbees et al. (2006).

Principle

The method is based on studying the competition between deoxyribose and the test compounds (extracts) for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system.

Reagents

1. Deoxyribose (15 mM)
   Prepared by dissolving 0.2 g of deoxyribose in distilled water and making up the volume to100 ml.
2. $FeCl_3$ (500 µM)
   Prepared by dissolving 0.008 g $FeCl_3$ in distilled water and making up the volume to 100 ml.
3. EDTA (1 mM)
   Prepared by dissolving 0.3 g of EDTA in distilled water and making up the volume to 100 ml.
4. H_2O_2 (10 mM)
   Prepared by diluting 1.13 ml of 30% H_2O_2 to 1000 ml with distilled water.
5. Ascorbic acid (1 mM).
   Prepared by dissolving 0.18 g of ascorbic acid in water and making up the volume to 100ml.
6. TBA (1%)
   Prepared by adding 1 g of TBA to distilled water and making up the volume to 100 ml.
7. TCA (2.8%)
   Prepared by adding 2.8 g of TCA to distilled water and making up the volume to 100 ml.
8. Butan-2-ol
9. KH_2PO_4-KOH buffer (100 mM, pH 7.4)
   A 100 mM solution of KH_2PO_4 was prepared and the pH was adjusted to 7.4 with 1M KOH solution

Procedure

The reacting mixture contained 200 µL KH_2PO_4 – KOH, 200 µL deoxyribose, 200 µL $FeCl_3$, 100 µL EDTA, 100 µL sample (1500 µg/ml), 100 µL H_2O_2 and 100 µL ascorbic acid. Reaction

mixtures were incubated at 37 °C for 1 h. At the end of the incubation period, 1 ml 1% (w/v) TBA was added to each mixture followed by the addition of 1 ml 2.8% (w/v) TCA. The solutions were heated in a water bath at 80 °C for 20 min to develop the pink coloured MDA–(TBA)$_2$ adduct which was extracted into 2 ml butan-1-ol and the absorbance measured at 532 nm.

3.9. Lipid peroxidation inhibitory activity

A modified TBARS assay was used to measure the lipid peroxide formed using egg yolk homogenate as lipid-rich media (Ruberto *et al.*, 2000).

Reagents

1. $FeSO_4$ (0.07 mM)
   Prepared by dissolving 0.001 g of $FeSO_4$ in distilled water and making up the volume to 100 ml.
2. Acetic acid (pH 3.5, 20 %)
   This was prepared by mixing 20 ml of 100% acetic acid with distilled water and making up the volume to 100 ml. The pH was adjusted to 3.5.
3. SDS (1.1 %)
   This was prepared by dissolving 1.1 g of SDS in distilled water and then making up the volume to 100 ml.
4. TBA (0.8 % w/v)
   This was prepared by dissolving 0.8 g of TBA in SDS (1.1%) and making up the volume to 100 ml with same.
5. Butan – 1 – ol

Procedure

Egg homogenate (0.5 ml, 10% w/v) was added to 0.1 ml of extract (1 mg/ml). The volume was made up to 1 ml with distilled water. Thereafter, 0.05 ml of $FeSO_4$ was added and the mixture was incubated for 30 min. Then, 1.5 ml of acetic acid was added followed by 1.5 ml of TBA to

SDS. The resulting mixture was vortexed and heated at 95 °C for 60 min. After cooling, 5 ml of butan – 1 – ol was added and the mixture was centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm and converted to percentage lipid peroxidation inhibition using the fonnula

$$(1- E/C) \times 100$$

Where C = absorbance of fully oxidized control, and

E = absorbance in the presence of extract.

3.10. Evaluation of the reductive potential

The method of Oyaizu (1986) was employed in determining the reducing power of the extracts.

Reagents

1. Phosphate buffer (0.2 mM, pH 6.6)
   This was prepared by mixing 35.2 ml of 1M Na_2HPO_4 with 64.8 ml of NaH_2PO_4 and diluting the combined volume to 500 ml with distilled water.
2. Potassium ferricyanide (1 %)
   Prepared by dissolving 1g of potassium ferricyanide in distilled water and making up the volume to 100 ml with same.
3. TCA (10%)
   Prepared by dissolving 10 g of TCA in water and making up the volume to 100 ml.
4. $FeCl_3$ (1%)
   Prepared by dissolving 1 g of $FeCl_3$ in distilled water and making up the volume to 100 ml.

Procedure

Extract (150 µg/ml) in 1 ml distilled water was mixed with 2.5 ml each of phosphate buffer and potassium ferricyanide. The mixture was incubated at 50°C for 20 min. TCA (2.5 ml) was then added and the mixture was centrifuged at 1000 g for 10 min. Thereafter, 2.5 ml of the upper layer of solution was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$. The absorbance was read at 700 nm. Higher absorbance of reaction mixture indicates greater reductive potential.

(B). Studies on extract of *Spondias mombin*

3.11. Experimental Animals

Adult male Sprague-Dawley (SD) rats weighing 150-200 g for cardioprotective studies and 250 ± 20 g for neuroprotective studies were procured from National Animal Laboratory Centre (NALC) of Central Drug Research Institute (CDRI), Lucknow. Animal experiments were conducted after approval and in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC). Rats were housed in an air – conditioned room and kept in standard laboratory conditions under 12 h light-dark cycle.

3.12. Preparation of extract of *Spondias mombin*

Spondias mombin leaf extract was prepared as described in section 3.2.1. and was used for the assessment of cardioprotective and neuroprotective properties.

3.13. Cardioprotective studies

3.13.1. Inotropic, chronotropic and anti-ischemic effects of *Spondias mombin* extract on isolated rat heart preparation

Methanolic extract of *Spondias mombin* (MES) was investigated for per se (intrinsic) and anti – ischemic effects on isolated hearts of male Sprague-Dawley rats (150 – 200 g) using the

Langendorff non-recirculating technique. Ramipril (10 µM) and Nifedipine (1 µM) were used as control standard drugs.

Reagents

Normal HEPES Tyrode (NHT) Buffer

The composition of the physiological salt solution NHT buffer in mM was: NaCl 137, KCl 5.4, HEPES (N-[2-Hydroxyethyl] piperazine-N'-2-ethanesulphonic acid) buffer 3.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0 and glucose 11.1. For the preparation of 1 L of NHT buffer, $CaCl_2$ and $MgCl_2$ were dissolved separately in distilled water. The remaining reagents were dissolved separately also in distilled water. The two solutions were mixed and the volume was made up to 1 L. The pH was adjusted to 7.4 using 1 M NaOH. Fresh buffer was prepared on each day of the experiment. Both buffer and solution of extract were filtered through a 0.22 µm Millipore filter before use.

Experimental Procedure

The animals were anaesthetized with chloral hydrate and exsanguinated. Hearts were rapidly excised, rinsed in ice cold perfusion buffer and perfused retrogradely through an aortic canula in the Langendorff mode in a non-recirculating manner at a constant pressure of 80-90 mmHg with continuously oxygenated NHT buffer at a constant temperature of 37 °C. The perfusing solutions were led through glass coils enclosed within glass jackets through which warm water was pumped by a recirculating water bath. Spontaneously beating hearts were given a resting tension of 2 g and contractions were recorded through a force displacement transducer (FT 03, GRASS Instruments Company) on a GRASS Polygraph. For the evaluation of inotropic and chronotropic effects, after 30 min of equilibration in which perfusion was done with the NHT buffer, the perfusion medium was switched to the NHT buffer containing the extract or standards. Values of amplitude and heart rate (HR) for test compounds were measured and compared with the values for the NHT buffer and expressed as percentage value. For the anti-ischemic study, 30 min of equilibration was followed by 45 min of global ischemia. Reperfusion was done for 30 min with NHT buffer with or without MES (Figure 8).

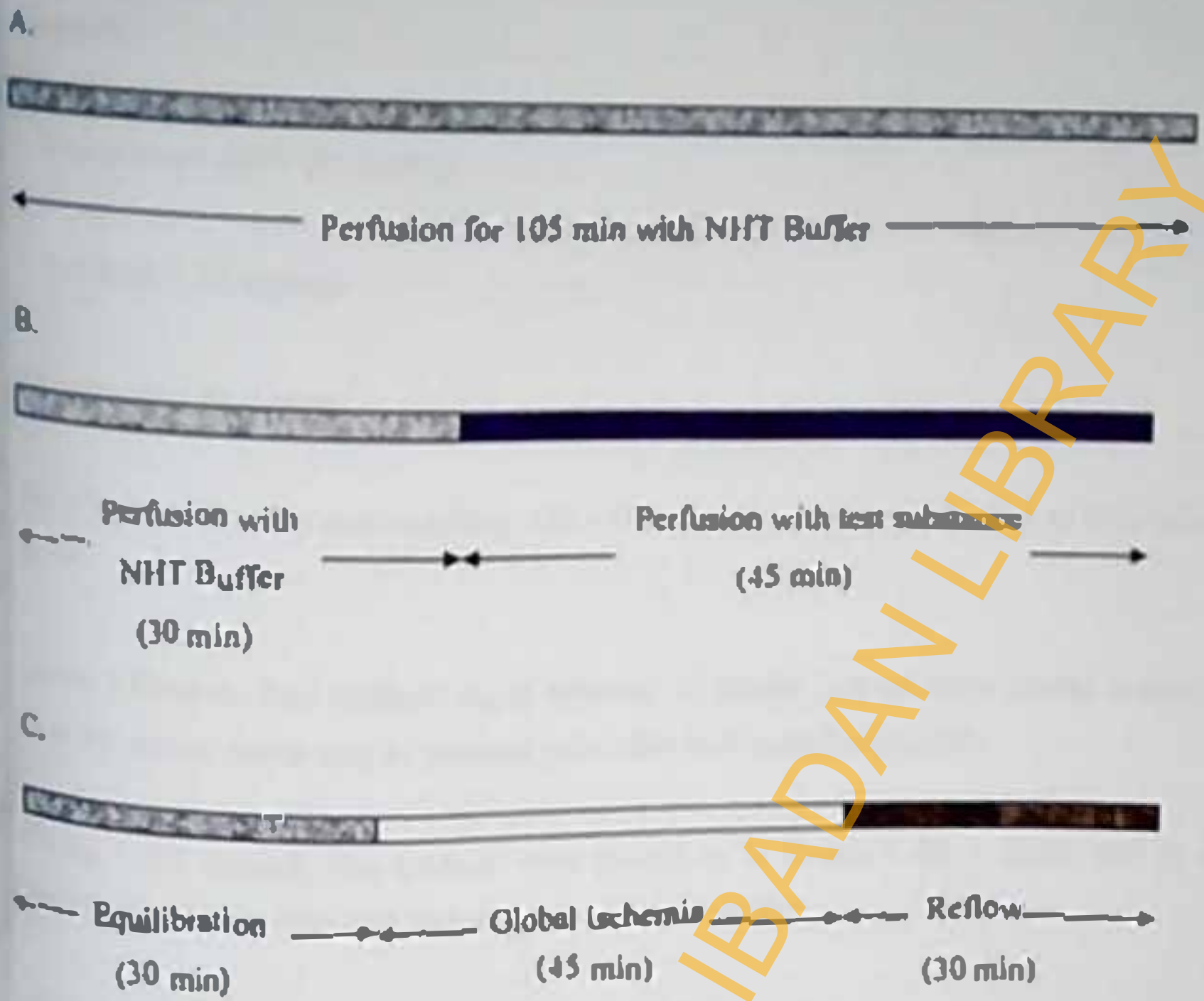


Figure 8: Experimental protocol for ex vivo cardioprotective studies using the Langendorff technique. A. Per se effect of NHT buffer; B. Per se effect of test substances; C. Anti-ischemic effect of test substances

3.1.3.2. Protective effect of *Spondias mombin* leaf extract against isoproterenol-induced myocardial infarction

Spondias mombin was investigated for *in vitro* cardioprotective property using the model of isoproterenol (ISP) induced myocardial infarction. Ramipril (1.25 mg/kg) was used as the control standard drug.

Reagents

1. Isoproterenol (ISP) (85 mg/kg)

2. Ramipril (1.25 mg/kg)

Experimental procedure

Male Sprague-Dawley rats weighing 150 - 200 g were randomly allocated to five main groups (n=9).

Group 1 Control: Rats received equal volumes of vehicle and no other special treatment other than the normal free access to standard pellet diet and water for a month.

Group 2 ISP control: The animals were treated as in Group 1 for a month and in addition received ISP on the 29th and 30th day at an interval of 24 h.

Group 3: MES treated: This was sub-divided into two groups

Group 3a (SM100), administered 100 mg/kg MES
Group 3b (SM 250), administered 250 mg/kg MES

The respective dose of MES was suspended in 0.2 % carboxy methyl cellulose (CMC) and orally fed to the animals once daily for a month.

Group 4: ISP – challenged, MES-treated group: This group also had two subgroups

Group 4a (SM 100 mg/kg + ISP), administered 100 mg/kg MES and ISP
Group 4b (SM 250 mg/kg + ISP), administered 250 mg/kg MES and ISP

In addition to receiving the treatment given to animals in Group 3, animals in this group received ISP (85 mg/kg) on the 29th and 30th day.

Group 5: ISP – challenged, Ramipril treated group: Animals in this group were administered Ramipril (1.25 mg/kg) for two weeks and also ISP on the 29th and 30th day.

Twenty four h after the second dose of ISP, animals were anaesthetized. Blood was withdrawn by retroorbital vein puncture and used for the estimation of glucose and for serum cholesterol, phosphate, MDA, LDH and plasma GSH levels. Rats were sacrificed, hearts excised, frozen in liquid nitrogen and stored at -85 ^{o}C until used for biochemical analysis.

Hearts stored in liquid nitrogen were weighed. A 10% homogenate was prepared in phosphate buffer (50 mM, pH 7.4). An aliquot was used for the assay of MDA. The homogenate was centrifuged at 15 000 x g at 4 ^{o}C for 15 min and the supernatant was used for the estimation of SOD, CAT and protein.

3.13.2.1. Biochemical parameters evaluated

1. Blood Glucose

Blood glucose was estimated using a glucometer (Accu-check® Active) with strips supplied by the manufacturer. The test strip gave a colour change on application of a drop of blood by Glucose dye oxidoreductase mediated reaction.

2. Serum Cholesterol and Phosphate

Serum cholesterol and phosphate were analyzed using the Beckman Coulter Synchron CX9 Pro clinical system with kits supplied by the manufacturer.

3. Plasma/tissue Glutathione

Glutahione was estimated according to the method of Ellman (1959) and Anderson, (1985). The assay is based on the reaction of GSH with 5, 5'- dithiobis (2-nitrobenzoic acid) (DTNB) (also known as Ellman's reagent) that produces the 2-nitro-5-thiobenzoic acid (TNB) chromophore which is measured at 412 nm.

Reagents

i. Sodium citrate (3.8% w/v) – This was prepared by dissolving 3.8 g of sodium citrate in distilled water and making up the volume to 100 ml.

ii. Acetic acid (6% v/v) – This was prepared by mixing 6 ml of acetic acid with distilled water and making up the volume to 100 ml.

iii. Sulphosalicylic acid (10% w/v) – Sulphosalicylic acid (10 g) was dissolved in distilled water and the volume was made up to 100 ml.

iv. DTNB [5,5'-Dithio-bis(2-nitrobenzoic acid)] (1.98 mg/ml) – DTNB (19.8 mg) was dissolved in GSH buffer and the volume was made up to 10 ml.

v. Phosphate buffer (50 mM, pH 7.4) (homogenizing buffer) – 77.4 ml of 1M $Na_2HPO_4 \cdot 2H_2O$ (prepared by dissolving 17.8 g in distilled water and making up the volume to 100 ml) was mixed with 22.6 ml of 1M $NaH_2PO_4 \cdot H_2O$ (prepared by dissolving 6.9 g in distilled water and making up the volume to 50 ml) and the volume was made up to 1 L. Appropriate volumes were further diluted with distilled water to give a 50 mM concentration.

vi. (Phosphate buffer (0.1 M, pH 8) (GSH Buffer) – 93.2 ml of 1M $Na_2HPO_4 \cdot 2H_2O$ (prepared by dissolving 17.8 g in distilled water and making up the volume to 100 ml) was mixed with 6.8 ml of 1M $NaH_2PO_4 \cdot H_2O$ (prepared by dissolving 6.9 g in distilled water and making up the volume to 50 ml) and the volume was made up to 1 L.

Procedure

Blood was prepared by collecting 0.5 ml of blood into 2 ml eppendorf tubes containing trisodium citrate (0.1 ml). Acetic acid (0.1 ml. 6% v/v) and sulphosalicylic (0.4 ml. 10% w/v) were added and the tubes were centrifuged at 13,000 rpm for 10 min. Heart tissue was weighed and homogenized in 10 volumes of phosphate buffer (50 mM, pH 7.4) immediately. to 100 µl of [illegible] equal volume of sulphosalicylic acid was added and the mixture was centrifuged at 10,000 rpm for 10 min. To 0.5 ml of plasma/100 µl of supernatant was added 2 ml/400 µl of GSH buffer followed by the addition of 20 µl/4 µl of DTNB and 480 µl/96 µl of triple distilled water. The mixtures were vortexed and then incubated at 37°C for 10 min. The absorbance was read at 412 nm in a Shimadzu UV – visible spectrophotometer. GSH concentrations were interpolated from a standard curve prepared using authentic GSH (Sigma-Aldrich) (0-400µg/ml).

Superoxide dismutase

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radical into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Superoxide radical converts NBT to NBT - diformazan (formazan dye) which is estimated at 560 nm. SOD was estimated using the method described by Kakkar *et al.* (1984).

Reagents

i. Pyrophosphate buffer (0.052 M, pH 8.3) – This was prepared by dissolving 0.576 g of sodium pyrophosphate in distilled water. The pH was adjusted to 8.3 and the volume was made up to 50 ml.

ii. Phenazine methosulphate (PMS) (186 µM) – This was prepared by dissolving 6 mg of PMS in triple distilled water (TDW) and making the volume up to 10 ml.

iii. Nitroblue tetrazolium (NBT) (300 µM) - Prepared by dissolving 36 mg of NBT in TDW and making the volume up to 30 ml.

iv. NADH (780 µM) – This was prepared by dissolving 30 mg of NADH in TDW and making up the volume to 10 ml.

v. n- Butanol

vi. Acetic acid (AR grade) – glacial.

Procedure

PMS (100 µl) and 300 µl of NBT were sequentially added to 1.4 ml of pyrophosphate buffer. Thereafter 1 ml of sample was added to the system and it was incubated at 37°C for 5 min. The reaction was started by the addition of 200 µl of NADH and the mixture was vortexed. The reaction mixture was incubated at 37°C for 90 s. The reaction was stopped by the addition of 1 ml glacial acetic acid. To this system was added 4 ml of n-butanol and the mixture was vortexed. The system was centrifuged at 4000 rpm for 20 min. The absorbance of the butanol – rich layer was read at 560 nm.

SOD activity (unit/min/mg protein) was calculated using the formula:

$$\text{Activity} = 1/0.5Z \times \text{Abs of sample/mg protein} \times 1.5$$

Where Z = Absorbance of blank sample

3. Catalase

Catalase catalyzes the decomposition of H_2O_2 into water and oxygen. The assay for the enzyme was carried out by continuous spectrophotometric rate determination.

Reagents

i. Phosphate buffer (50 mM, pH 7.0) – Disodium hydrogen phosphate (2.8392 g) and 2.72 g of potassium dihydrogen phosphate were separately dissolved in TDW. The combined volume of the solutions was made up to 200 ml with TDW.

ii. Buffered substrate – Hydrogen peroxide (34 µl, 30% w/v) was mixed with a few ml of TDW and the volume was made up to 10 ml with phosphate buffer.

Procedure

Buffered substrate (2.95 ml) was pipetted into a 3 ml cuvette. The reaction was started at 37^{o}C by the addition of 50 µl of sample. Absorbance was followed for 5 min at 240 nm. The change in Absorbance per minute was calculated.

Change in absorbance per minute/0.041 (coefficient factor) = Z

Catalase activity = (Z / mg protein in 50 µl enzyme source) x 3

6. Lactate dehydrogenase(LDH)

Serum LDH was estimated according to the method of Seth *et al.* (1994).

Reagents

i. Phosphate buffer (50 mM, pH 7.4) – 25 ml of 0.1M NaH_2PO_4 was prepared by dissolving 0.39 g of the salt in TDW. 100 ml of Na_2HPO_4 was prepared by dissolving 1.58 g of salt in TDW. 19 ml of Na_2HPO_4 was then mixed with 81 ml of Na_2HPO_4.

ii. Sodium pyruvate (10 mM stock) – 11 mg of sodium pyruvate was dissolved in 10 ml of phosphate buffer.

iii. NADH – 3mg of NADH was dissolved in 1 ml of TDW.

Procedure

Sodium pyruvate (1.44 ml) was added to 10 µl of the sample and the mixture was incubated at 37 ^{0}C for 10 min. Then 50 µl of NADH was added and the change in absorbance was followed for 3 min at 340 nm.

$$\text{LDH (µmole/min/ml)} = \frac{\text{Change in OD/min x dilution factor x volume of assay}}{\text{6.22 x enzyme aliquot used/assay}}$$

7. Malondialdehyde (MDA)

MDA was estimated according to the method of Colado *et al.* (1997).

Reagents

i. Phosphate buffer (50 mM, pH 7.4) – This was prepared as described in section 3.13.2.1.

ii. Trichloroacetic acid (TCA) (30 %) – 30 g of TCA was dissolved in TDW and the volume was made up to 100 ml.

iii. HCl (5 N) – This was prepared by appropriate dilution of the 11 M stock HCl with TDW.

iv. Thiobarbituric acid (TBA) (2 % in 0.5 N NaOH) – 2 g of TBA was dissolved in 0.5 N NaOH solution and the volume was made up to 100 ml.

Procedure

Serum was prepared by the centrifuging 1.5 ml of blood at 13, 000 rpm for 10 min. A 10% tissue homogenate was also prepared in phosphate buffer. To 500 µl of the serum/homogenate was added 300 µl of TCA and the mixture was vortexed. Then 150 µl of HCl was added and the mixture was vortexed. This was followed by the addition of 300 µl of TBA and then 1750 µl of TDW. The mixture was boiled at 90 ^{o}C for 20 min. 1.5 ml of the pink upper layer was centrifuged at 3000 rpm for 10 min and the absorbance of the supernatant obtained was read at 532 nm. The values obtained were read on a standard curve prepared with an MDA standard (0-0.4 mg/ml).

8. Nitrite Level

Nitric oxide (NO) is a molecular mediator of many physiological processes including vasodilation, inflammation, thrombosis, immunity and neurotransmission. A number of methods exist for measuring NO in biological systems. One of these methods involves the use of the Griess diazotization reaction to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under physiological conditions.

Reagent

Griess reagent: To 0.04 g of *N*-(1-naphthyl) ethylenediamine dihydrochloride (NED) dissolved in 1 ml of orthophosphoric acid was added 0.4 g of sulphanilamide. The volume was made up to 40 ml with TDW and stored away from light.

Procedure

Sample and Griess reagent were mixed in a 1:1 ratio and incubated for 30 min at 37 ^{o}C. The absorbance was read at 548 nm and the nitrite concentrations were extrapolated from a standard curve prepared by using $NaNO_3$.

3.13.2.2 Histopathology

Myocardial tissue was perfused with saline and then 4 % paraformaldebyde. It was then fixed in 4 % paraformaldehyde for 24 h. The tissues were routinely processed and embedded in paraffin. Serial sections were cut and each section was stained with hematoxylin and eosin. The stained sections were examined under a microscope and photomicrographs were taken.

3.14 Neuroprotective Studies

3.14.1 Middle Cerebral Artery Occlusion (MCAO)

Focal cerebral ischemia was induced in male SD rats by occlusion of the middle cerebral artery (MCA) using a modification of the intraluminal technique of Longa *et al* (1989). Animals were anesthetized with chloral hydrate (350 mg/kg i.p.). The body temperature of the animals was maintained at 37°C during and after surgery by the use of a thermoregulated dissecting surgical table. The left common carotid artery (CCA) was exposed through a midline incision in the neck region. The neck muscles were carefully separated further to expose the internal carotid artery (ICA) and external carotid artery (ECA). A 3.0 monofilament nylon suture (Ethicon, Johnsons & Johnsons Ltd. Mumbai) was introduced into the ECA lumen through a small nick and gently advanced from the ECA to the ICA lumen (about 20-22 mm from the CCA bifurcation) to block blood supply to the MCA. The ECA nick was tightened by thread around the intraluminal nylon suture to prevent bleeding. Recirculation of cerebral blood flow was allowed by removing the monofilament carefully after 1 h of ischemia followed by 24h of reperfusion. In sham-operated animals, all the procedure except for the insertion of the nylon filament was carried out. Animals in the vehicle group received 0.2 % CMC while the treated groups were administered 100 mg/kg *Spondias mombin* extract suspended in 0.2 % CMC.

3.14.2 Assessment of neurological deficit

On recovery from anaesthesia, rats were examined for neurological deficit on a ten – point scale (Table 3).

Table 3: Scoring for neurological assessment

Neurological Deficit	Neurological Score	Description
No neurological deficit	0	Normal
Flexion	1	Mild
Circling	2	Moderate
Hemi paresis	3	Severe
Non-spontaneous movement	4	Severe

3.14.3 Quantification of infarct size

Rats were anaesthetized with ether and the brains were taken out. Each brain was cut into seven 2mm thick slices and incubated with 1% TTC (dissolved in 0.1M phosphate buffered saline) at 37°C for 30 min. The slices were scanned and analyzed by using computerized image analysis system (Biovis Image Plus). The infarct area of all brain slices of each rat was multiplied by the slice thickness to give the infarct volume.

3.14.4 GSH and MDA estimation in the brain

These were estimated as described under the cardioprotective studies.

3.14.5 Western blot analyses

The sham operated rats and rats subjected to 1 h of MCAO followed by 24 h of reperfusion, were sacrificed by overdose of anesthetic ether. The ipsilateral portion of brain tissue subjected to ischemia/reperfusion was quickly excised and homogenized in ten volumes of ice cold lysate buffer (200 mmol/l HEPES (pH 7.5), 250 mmol/l sucrose, 1 mmol/l dithiothreitol, 1.5 mmol/l MgCl2, 10mmol/l KCl, 1mmol EDTA, 1mmol EGTA, 1mmol Phenyl methyl sulfonyl chloride, 10µg/ml leupeptin, 5µg/ml pepstatin, 2µg/ml aprotinin) using a teflon homogenizer.

Sub cellular fractionation was performed as follows. The homogenates were spun at 800 x g for 10 min. The 800 x g supernatants were spun at 20000 x g for 20 min. The resultant supernatants were used for the evaluation of gp91phox, p22phos, eNOS, nNOS and SOD. The protein concentration of each sample was determined using the Lowry method (Lowry *et al.*, 1951). An aliquot of 20 µg of protein was subjected to 10% or 12% Sodium dodecylsulfate polyacrylamide gel electrophoresis. The separated proteins were transferred onto nitrocellulose membrane. For immunoblotting, goat polyclonal primary antibodies were used. The secondary antibodies used were HRP conjugated anti-IgG. The immunoreactive bands were visualized by enhanced chemiluminiscence (ECL) detection. The band intensity was measured using spot densitometry analysis software of Alphamnger ™ 2200.

(C). Studies on fractions from *Spondias mombin* and the isolation of bioactive compounds

3.15 Preparation of Fractions

Extract of *Spondias mombin* was prepared as described in section 3.2.1. The crude methanolic extract was then fractioned sequentially into water, butanol, ethyl acetate, dichloromethane and hexane (Figure 9). Antioxidant and antiproliferative tests were carried out on the crude extract and the fractions.

3.16 *In vitro* free radical scavenging activities of fractions

3.16.1 *DPPH free radical scavenging activity*

DPPH free radical scavenging activity of extracts was determined using the DPPH photometric method of Mensor *et al.* (2001) as described in section 3.6.

3.16.2 *Nitric oxide scavenging activity*

Nitric oxide scavenging activity was carried out as described in section 3.7.

Reagents

Griess reagent was prepared as described in section 3.13.2.1.

3.16.3 *Superoxide radical scavenging activity*

Superoxide anions were generated non - enzymatically by phenazine methosulphate and nitroblue tetrazolium in the absence or presence of compounds in 100 mM phosphate buffer (pH 8.2). The reaction mixtures were incubated at 37 ^{o}C and after 30 min the reaction was stopped by adding 0.5 ml glacial acetic acid. The amount of formazone formed was measured at 560 nm on a spectrophotometer.

3.16.4 *H_2O_2 scavenging activity*

This was assessed by the method of Ruch *et al.* (1989). H_2O_2 (2 nM/L) was prepared in phosphate buffered saline (PBS, pH 7.4). One ml of extract was added to H_2O_2 solution (0.6 ml) and the absorbance was read at 240 nm against a blank solution containing extract (1 ml) in PBS without H_2O_2.

3.16.5 *Inhibition of lipid peroxidation in rat brain*

A modified method of Oliknwa *et al.* (1979) was used to evaluate lipid peroxidation inhibitory activity. Homogenate of rat brain (0.5 ml, 10 % v/v) in phosphate buffered saline (50 mM, pH 7.4) was added to 0.1 ml of various concentrations of extract or fractions. The volume was made up to 1ml with distilled water. Thereafter, 0.05 ml of 0.07 mM $FeSO_4$ was added and the mixture incubated for 30mins. Then, 1.5 ml acetic acid acid (20%, pH 3.5) was added followed by 1.5 ml of 0.8 % w/v TBA in 1.1 % SDS. The resulting mixnue was vortexed and heated at 95°C for 60

min. After cooling, 5ml of butan – 1 –ol was added and the mixture centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm and converted to percentage inhibition using the formula (1- E/C) x 100, where C is the absorbance of fully oxidized control and E is the absorbance in the presence of extract, fractions or standards.

3.17 Screening for Antiproliferative Activity

A colorimetric sulphorhodamine B (SRB) (Sigma) assay was used for measurement of cell proliferation (Houghton *et al.*, 2007). Briefly, 10^4 cells (in 180 µl) were added to each well of a 96-well plate incubated overnight to allow for cell attachment. Cells were then treated with 50 µg/ml of MES or its fractions. Untreated cells, receiving the same volume of medium served as control. After 48 h exposure time, cells were fixed with ice-cold 50% TCA followed by staining with 0.4% (w/v) SRB in 1% acetic acid, washed and air dried. Bound dye was solubilized with 150 µl of 10 mM Tris base. The plates were read at 540 nm absorbance. The cytotoxic effect of the extract and fractions was assessed as the percentage of inhibition of cell growth, where untreated cells were taken as 100 % viable. Percentage cell growth inhibition was determined using the formula [100 - (Absorbance of treated cells/Absorbance of untreated control cells)] x 100. Four cell lines were used: KB (Oral cancer), C – 33A (Cervical cancer), MCF – 7 (Breast cancer) and A – 549 (Lung cancer). NIH3T3 (Mouse fibroblast) was used as control cell line. Cell lines were sourced from ATCC.

3.18 Characterization of Bioactive Compounds

The protocol followed is shown in Figure 9.

Column chromatography

Silica gel (230 – 400 mesh) was used for column chromatography. The solvent system used for column chromatography was Chloroform:methanol:water (65:25:20). The solvents were mixed in the stated ratio, the upper aqueous layer was removed and the remaining mixture was clarified with some methanol.

Thin layer chromatography

TLC was run on precoated silica gel 60 F_{254} and RP – 18 F (Sigma – Aldrich). Detection was done under UV light, by iodine vapour, spraying either with cerric sulphate in 1M H_2SO_4 or spraying with 10% methanolic sulphuric acid followed by heating at 110 ^{o}C. The TLC profiles of hexane and dichloromethane fractions were obtained using the solvent system hexane:ethylacetate (70:30) while those of the ethyl acetate and n-butanol fractions were obtained using the solvent system Ethyl acetate:methanol:water (8:1:1). The solvent system used for the TLC of fractions obtained from column chromatography was chloroform:methanol (80:20).

High performance liquid chromatography

HPLC was run on Shimadzu, UV SPD-10 AVP system, using RP-18 (Slim-Pack RRC-ODS 20 mm x 25 cm) columns.

UV spectra

The UV spectra were recorded on Perkin Elmer λ – 15 UV/Visible spectrophotometer using methanol as solvent. The UV spectra of the flavonoids were obtained as methanol as well as by adding sodium methoxide, aluminium chloride and sodium acetate as diagnostic reagents.

IR spectra

IR spectra were recorded on a Perkin-Elmer RX-I spectroplot using either KBr pellets or in neat.

MS and NMR

The EIMS were recorded on a Jeol-JMS-D_300 spectrophotometer at70 eV with direct inlet system. The FABMS were recorded using a beam of Argon (2-8 eV) on Jeol SX 102/DA-6000 mass spectrophotometer. The NMR spectra were run on an AVANCE DPX 200, Bruker DRX

300 FTNMR and 600 MHz Varian Inova spectrophotometer. The chemical shifts are reported in δ (ppm) downfield from TMS which was used as internal standard. The optical rotation was determined on an Autopol III automatic polarimeter using sodium D-line (c in g/100 ml). Elemental analyses were obtained in a Carlo-Erba 1108 CHN elemental analyzer.

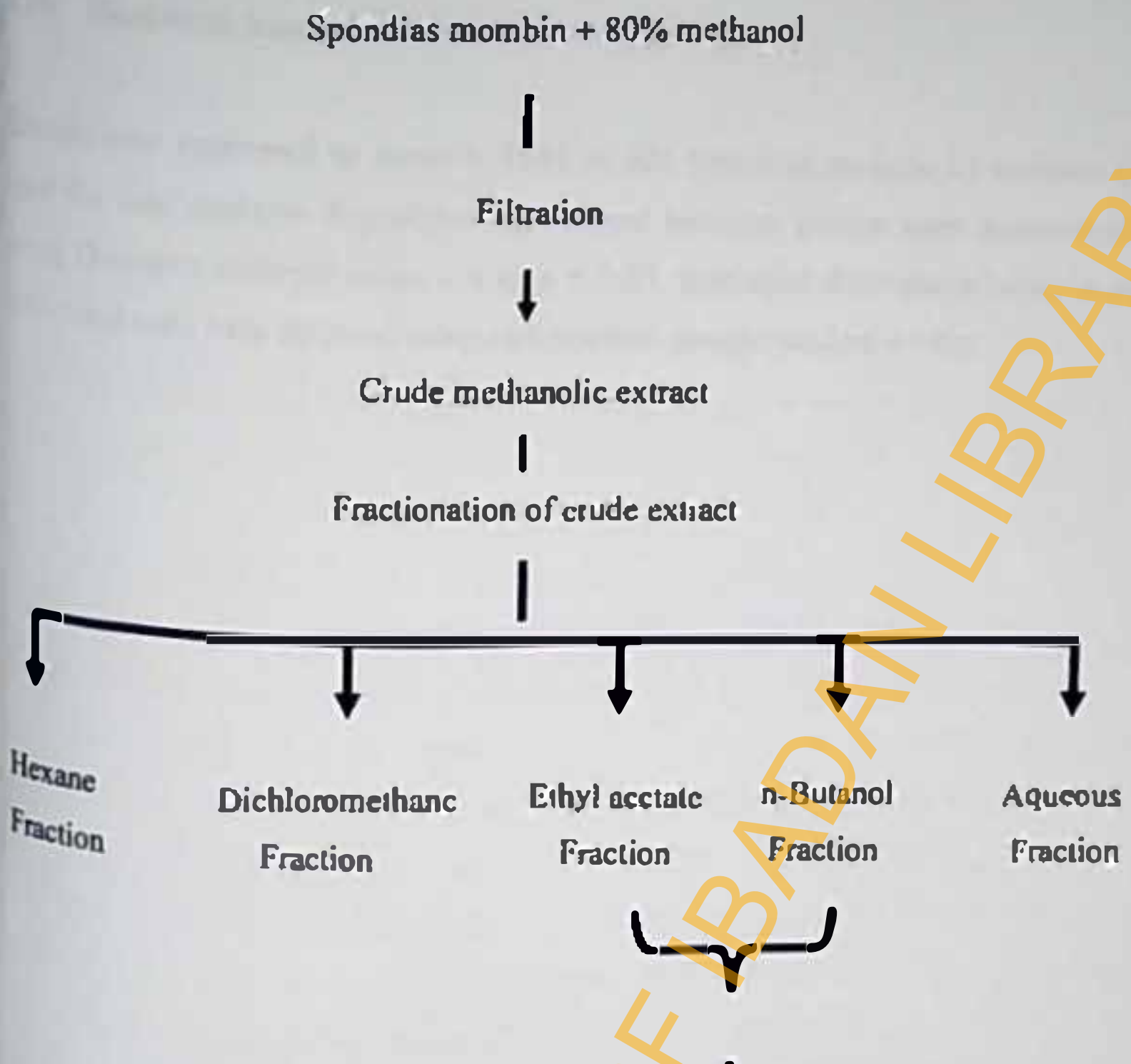


Figure 9: Protocol for isolation of bioactive compounds from *Spondias mombin*

3.19 Statistical Analysis

Results were expressed as mean ± SEM or SD. One-way analysis of variance (ANOVA) was used for data analysis. Significant differences between groups were detected in the ANOVA using Duncan's multiple range test at $p < 0.05$. Statistical differences between mean values of individual tests were detected using independent-sample Student's *t*-test.

CHAPTER FOUR

EXPERIMENTS AND RESULTS

INVESTIGATION ONE

4.1. Antioxidant and free radical scavenging activities of extracts from ten selected Nigerian medicinal plants

INTRODUCTION

Plants contain many bioactive compounds which counteract free radical mediated toxicity through the prevention or attenuation of damages caused by the radical species. Inhibition of free radical generation is now being employed as a facile system to carry out the primary screening for chemotherapeutic agents. A systematic search for useful bioactivities from medicinal plants is considered a rational approach in nutraceutical and drug research. Bioprospecting for new plant-derived drugs has been on the increase in recent times because these drugs have fewer side effects than the synthetic ones (Farombi, 2003) and many important leads are continuously being discovered (Negi *et al.*, 2008). Despite the upsurge in medicinal plant research, relatively little information is available concerning the antioxidant potential and biological activity of plant species, especially in Africa which contains one of the richest biodiversity in the world and abounds in plants of economic and medicinal importance (Farombi, 2003).

4.1.1. EXPERIMENT 1: Phytochemical Screening

INTRODUCTION

Phytochemicals are plant secondary metabolites with medicinal and health benefits. It is believed that phytochemicals may be effective for combating or preventing disease due to their antioxidant effect (Farombi and Britton, 1999a; Farombi and Britton, 1999b; Farombi, 2000). Certain phytochemicals have been recommended for the purpose of treating various diseases in

humans. Phytochemical screening involves performing simple qualitative chemical tests on plant extracts for the purpose of detecting/identifying the different chemical groups present in the plant.

PROCEDURE

The aqueous and methanolic extracts were screened for the presence of alkaloids, saponins, tannins, phlobatannins anthraquinones, steroids, terpenoids flavonoids and cardiac glycosides using the methods described in section 3.3.

RESULT

Phytochemical screening gives an overview of the major chemical classes in the extracts and an indication of their potential medicinal properties. Phytochemical screening gave positive results for steroids, terpenoids, and cardiac glycosides in all extracts. Alkaloids, tannins, and flavoooids were also detected in many of the extracts (Table 5). Some chemical groups which were not detected in one solvent were detected in the other. This explains why hydroalcoholic solvents are often employed in preparing herbal extracts. The results show that the studied plants are rich in diverse phytochemicals, which are probably responsible for their medicinal properties (Table 4). Herbal extracts often demonstrate multiple bioactivities because of the presence of varied phytochemicals with distinct or overlapping medicinal properties. This synergistic or antagonistic interaction among phytochemicals have been shown to modulate the bioefficacy of plant extracts

Table 4: Folkloric use of study plants

Plant species	Common name	Part used	Traditional use
Psidium guajava	Guava	Leaves	Used for treating fevers and diarrhea and as a tonic in psychiatry
Cassia alata	Asunwon	Leaves	Laxative, remedy for parasitic skin diseases, ulcers, asthma, and bronchitis
Newbouldia laevis	Akoko	Stem bark	Febrifuge, used for the treatment of epilepsy, convulsion, rheumatism, and arthritis
Alstonia boonei	Ahun	Stem bark	For treating malaria, painful micturation, and rheumatic conditions, antivenom, and antihypertensive
Globimetula cupulata	Afomo	Leaves	Antihypertensive, for treating epilepsy, internal hemorrhages, arthritis, rheumatism, chilblains, leg ulcers, and varicose veins
Chromolaena odorata	Akintola	Leaves	For wound dressing, to treat skin infection and stop bleeding
Securidaca longepedunculata	Ipeta	Root	For erectile dysfunction, coughs, colds, fever, backache, toothache, sleeping sickness, and venereal disease
Spondias mombin	Iyeye	Leaves	Diuretic, emetic, febrifuge, and abortifacient; also used for diarrhea, dysentery, hemorrhoids, and gonorrhea
Ocimum gratissimum	Efinrin	Leaves	Used for the treatment of rheumatism, paralysis, epilepsy, high fever, diarrhea, and mental illness; as an emetic and for hemorrhoids, stomach problems, and eye/throat inflammation
Morinda lucida	Oruwo	Leaves	Used for malaria, typhoid fever, and jaundice and for treating wound infections, abscesses, and chancre

Table 3: Phytochemical profile of extracts of selected plants

Plants	Methanolic extract										Aqueous extract									
	Alk	Sap	Tan	Phl	Anth	Ster	Terp	Flav	CG1	CG2	Alk	Sap	Tan	Phl	Anth	Ster	Terp	Flav	CG1	CG2
A. boonei	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+
N. latifolia	+	-	+	-	-	+	+	+	+	+	+	-	-	-	+	+	-	+	-	+
P. guajava	-	+	+	-	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
S. longepedunculata	+	+	-	-	-	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+
C. odorata	+	-	+	-	-	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+
O. gratissimum	+	-	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
C. alata	+	-	+	+	+	+	+	-	+	+	-	-	+	+	-	+	+	-	+	+
S. senegalia	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	-	+
M. lucida	-	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
G. capsulare	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+

KEY

- = absent
+ = present

Alk = alkaloids. Sap = saponins, Tan = tannins, Phl = Phlobatannins, Anth = anthraquinones, Ster = steroids, Terp = terpenoids, Flav = flavonoids, CG1 = Cardiac glycoside with steroidal ring, CG2 = Cardiac glycoside with deoxy sugar

4.1.2. EXPERIMENT TWO: Evaluation of antioxidant and free radical scavenging activities.

INTRODUCTION

Numerous assays exist for the in vitro evaluation of antioxidant and free radical scavenging capacity of extracts from plants. The total antioxidant activity, reductive potential, DPPH scavenging activity, metal chelation ability and active oxygen species quenching activity are often evaluated (Chang *et al.*, 2002; Gulcin *et al.*, 2002). Evaluation is usually done using multiple assays because each assay evaluates a different aspect of the antioxidant action. In this experiment, seven tests for antioxidant and free radical scavenging capacity of the study plants were carried out.

PROCEDURES

Total phenolic content and total flavonoid content were estimated as described in sections 3.4 and 3.5 respectively. DPPH free radical, nitric oxide radical and hydroxyl radical scavenging activities were determined as described in sections 3.6, 3.7 and 3.8 respectively. Lipid peroxidation inhibitory activity and reductive potential were determined as described in section 3.9 and 3.10 respectively.

RESULTS

The results of the *in vitro* antioxidant tests are shown in Figure 10 to 16. *P. guajava* extract showed consistently high values in all assays except NO (21.68 ± 1.51%), where it had the least value among all the studied plants. It had the highest values for TPC (380.08 ± 4.40 mg/L GAE), LPIA (70.82 ± 0.90%), and RP (0.79 ± 0.04). In the DPPH and TFC assays, its values were not significantly different from those of the extracts of *S. mombin* (88.58 ± 3.04%) and *C. alata* (275.16 ± 1.62 µg/mL QE), which recorded the highest values, respectively (Table 6).

S. mombin and *G. cupulatus* were second and third, respectively, behind *P. guajava* in order of ranking. *C. alata* and *O. gratissimum* also have high values of antioxidant ladica in many of the assays. The NO (44.88 ± 0.55%) value for *A. boonei* is

remarkably higher than the values for the other plant extract. *C. odorata* showed a remarkably high value for TFC (272.12 ± 2.32 µg/mL QE) and hydroxyl radical scavenging activity (56.53 ± 0.86%). The NO value for *S. longepedunculata* was high (43.90 ± 0.04%). Only *M. lucida* appears to have consistently low values in the assays.

The correlation coefficients confirm that there is a high level of agreement between pairs of some of the assays (Figs 17-20). DPPH assay had an extremely significant correlation with total phenolic content ($r = 0.76$, $P = .001$) and RP ($r = 0.81$, $P < .05$) (Fig. 17) and a significant correlation with LPIA ($r = 0.41$, $P < .05$) (Figure 19A). There was also an excellent significant correlation between TPC and RP ($r = 0.79$, $P = 0.0006$) (Figure 18A) and a significant correlation between TPC and LPIA ($r = 0.55$, $P = .01$) (Figure 18B). A significant correlation was also observed between TPC and TFC content ($r = 0.43$, $P < 0.05$) (Table 7). A fair correlation was observed between LP and DPPH ($r = 0.50$, $P < .05$) and LPIA and RP ($r = 0.40$, $P < 0.05$) (Figure 19), whereas the level of correlation observed between DOR and LPIA ($r = 0.31$) and DOR and TPC ($r = 0.33$; $P > .05$) was somewhat low (Figure 20). The values of *P. guajava*, *S. monbin*, *G. cupulara*, *C. olaia*, and *O. gratissimum* for DPPH free radical scavenging capacity, TPC, LPIA, and RP reflect these observations. The trend of the results in the four assays for the five plants is apparently the same. However, the levels of agreement between some other pairs of assay methods are insignificant (Table 7).

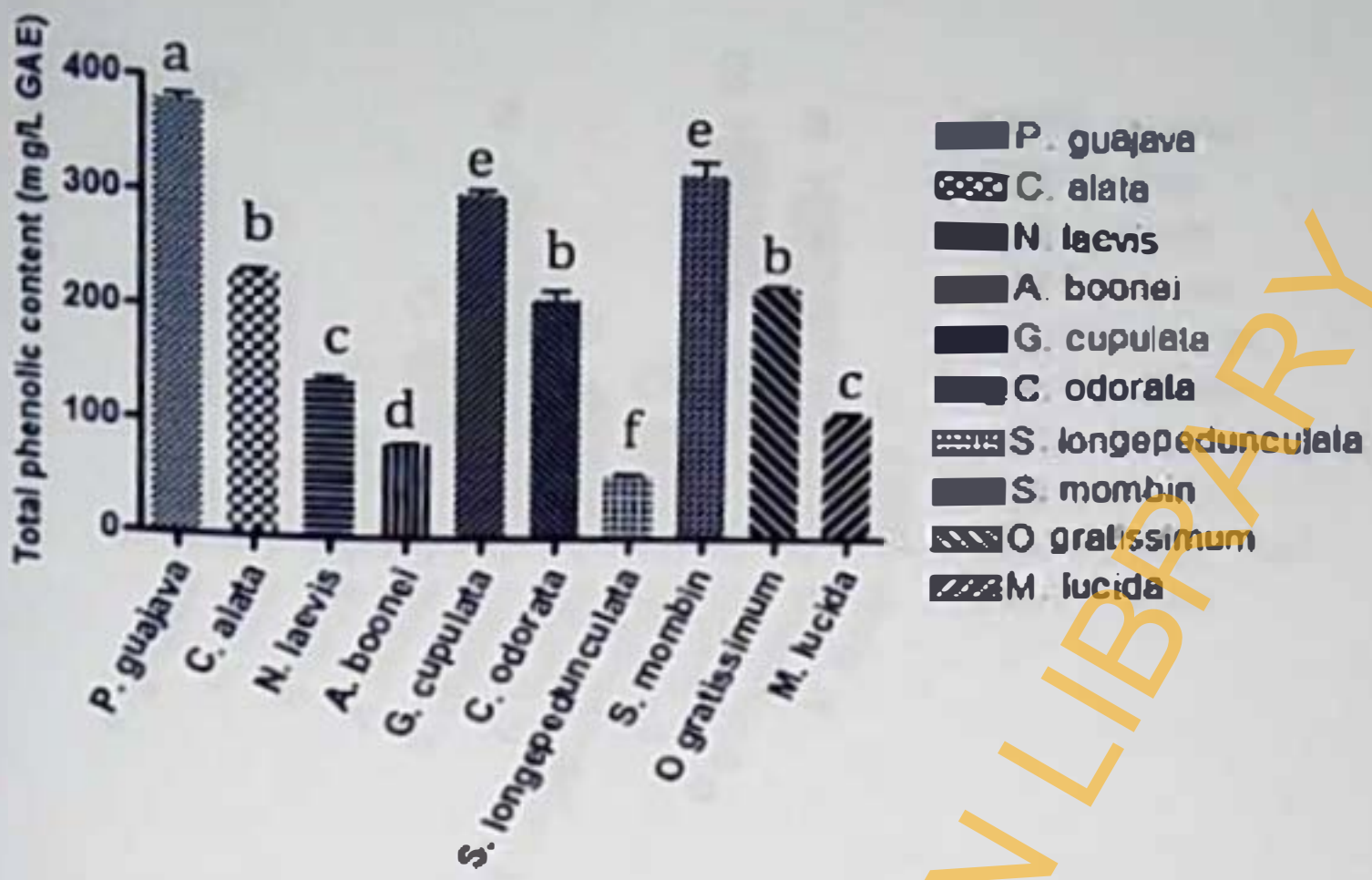


Figure 10: Total phenolic content of extracts. Results are presented as mean ± SEM (n=3). Bars with different lower case letters are significantly different (P<0.05)

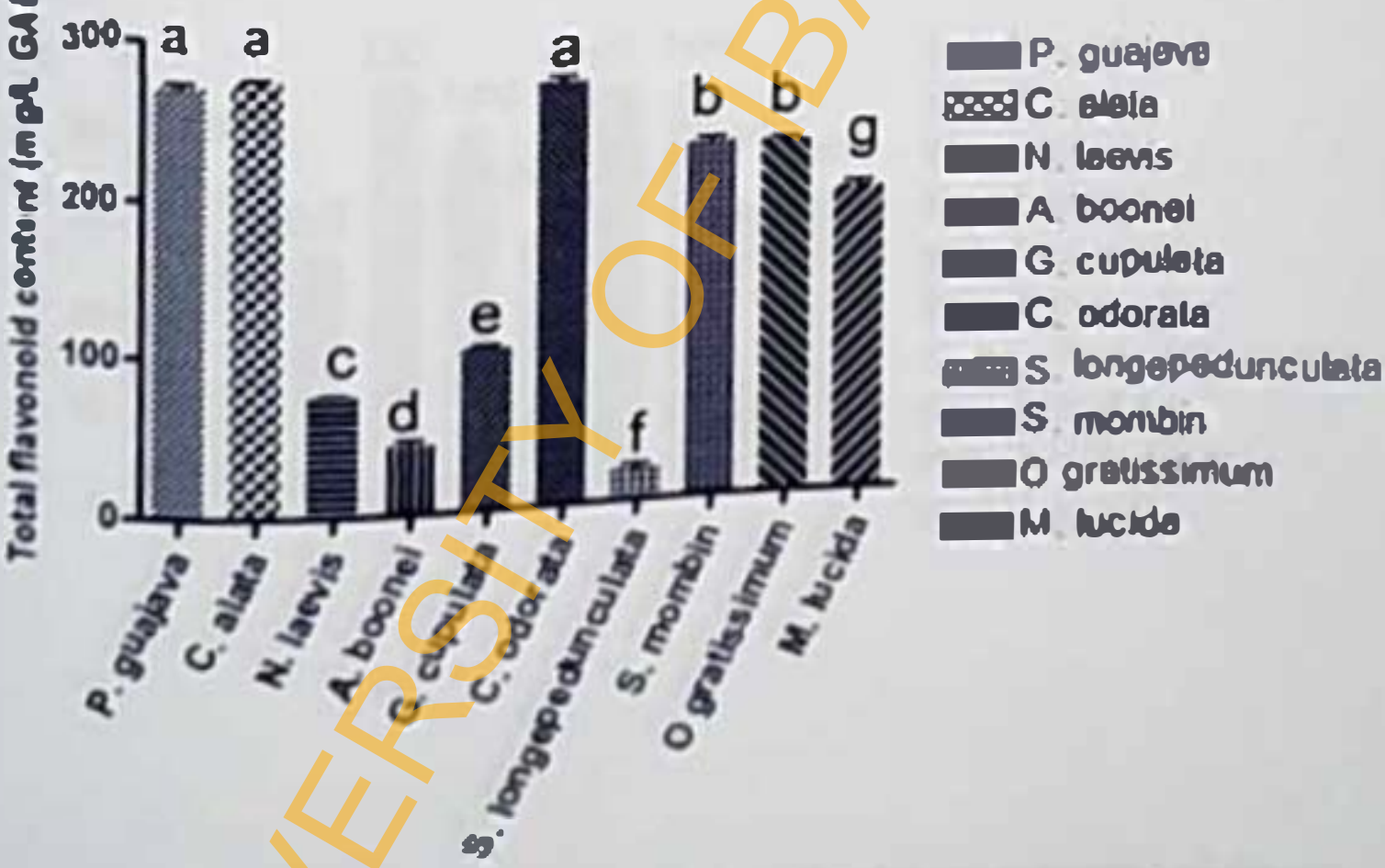


Figure 11: Total flavonoid content of extracts. Results are presented as mean ± SEM (n=3). Bars with different lower case letters are significantly different (P<0.05)

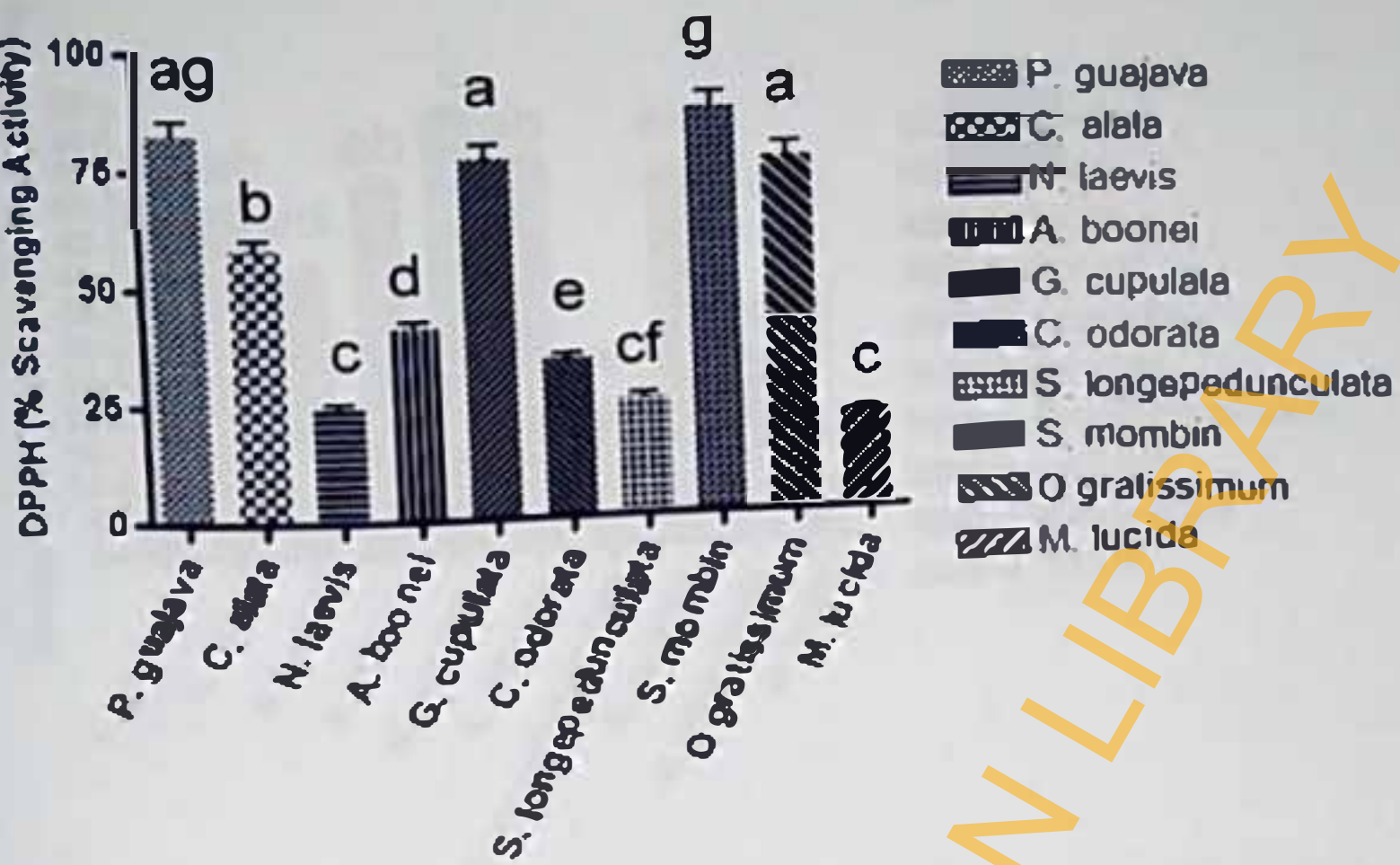


Figure 12: DPPH scavenging activity of extracts. Results are presented as mean ± SEM (n=3). Bars with different lower case letters are significantly different (P<0.05)

Figure 13: NO scavenging activity of extracts. Results are presented as mean ± SEM (n=3). Bars with different lower case letters are significantly different (P<0.05).

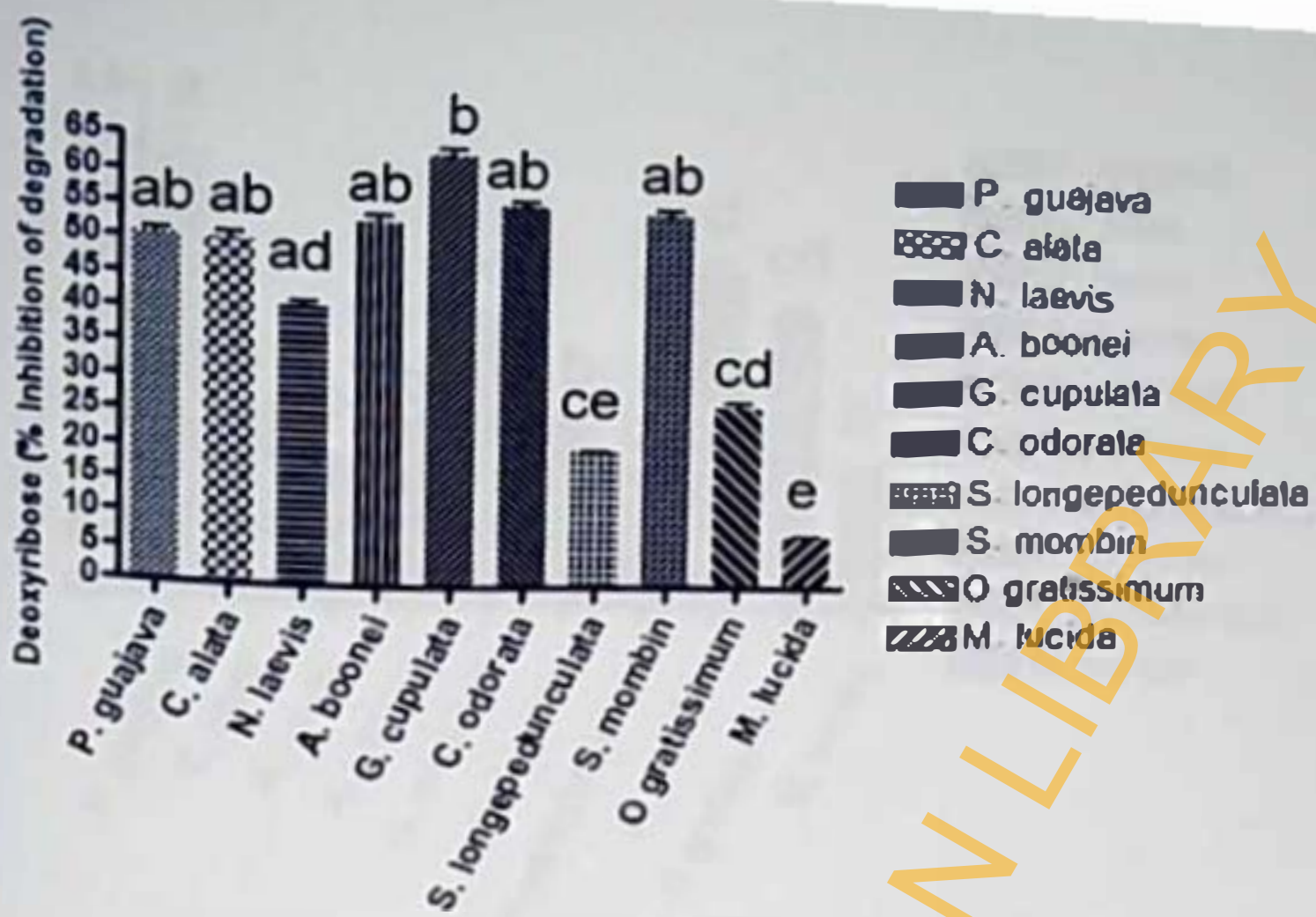


Figure 14: Hydroxyl radical scavenging activity of extracts. Results are presented as mean ± SEM (n=3). Bars with different lower case letters are significantly different (P<0.05).

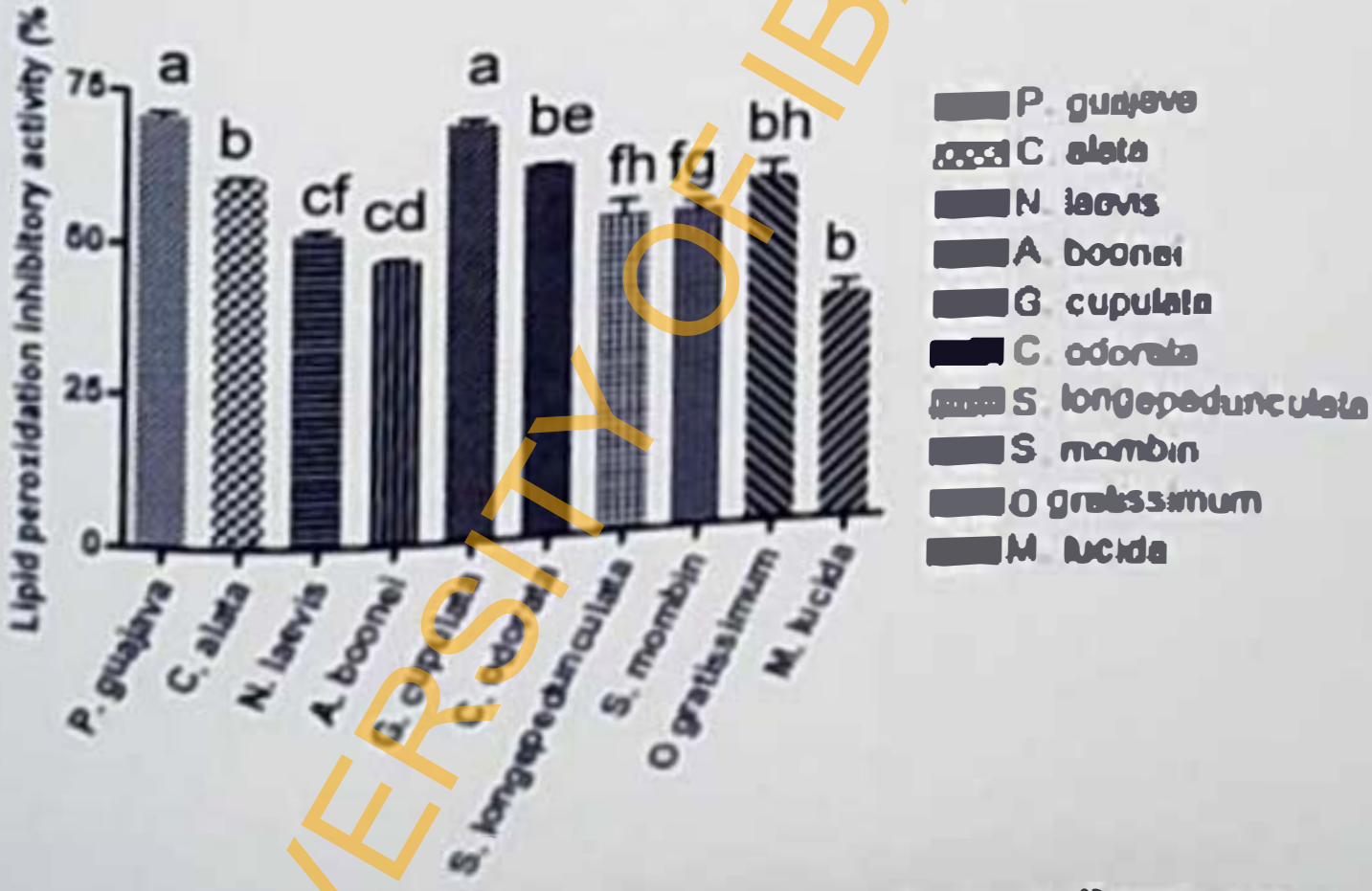


Figure 15: Lipid peroxidation inhibitory activity of extracts. Results are presented as mean ± SEM (n=3). Bars with different lower case letters are significantly different (P<0.05).

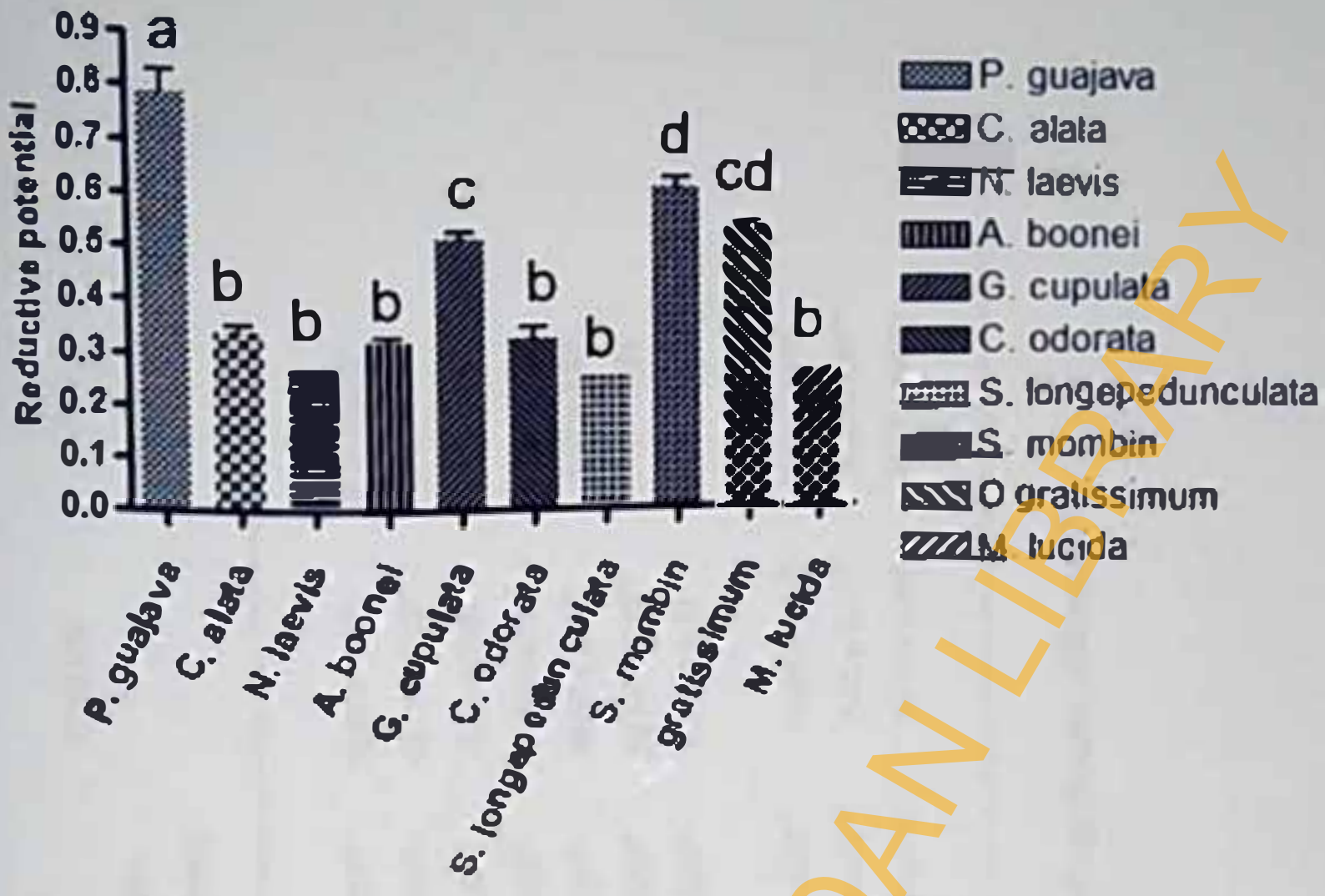


Figure 16: Reducing property of extracts: Bars with different lower case letters are significantly different ($P<0.05$).

Table 6: Summary chart of the results of antioxidant assays

Plant	TPC (mg/L GAE)	TFC (µg/mL QE)	DPPH (% scavenging Activity)	NO (% scavenging activity)	DOR (% inhibition of degradation)	LPIA (%)	RP
P. guajava	380.08 ± 4.40^{a}	269.72 ± 2.78^{a}	82.79 ±2.84^{ab}	21.68 ± 1.51^{b}	50.60 ± 0.77^{ab}	70.82 ± 0.90^{a}	0.79 ± 0.04^{a}
C. alata	232.68 ± 2.54^{b}	275.16 ± 1.62^{a}	58.80 ± 2.02^{b}	35.81 ± 1.91^{ab}	58.53 ± 0.77^{ab}	61.15 ± 0.13^{b}	0.34 ± 0.01^{b}
N. latifo	139.17 ± 3.49^{e}	78.00 ± 0.93^{e}	34.86 ± 0.85^{e}	26.57 ± 0.37^{cd}	41.89 ± 0.62^{cd}	51.91 ± 0.48^{cd}	0.27 ± 0.00^{b}
A. boonei	83.65 ± 1.49^{g}	44.43 ± 2.02^{g}	41.58 ± 1.43^{d}	44.88 ± 0.55^{ab}	53.84 ± 0.83^{ab}	47.16 ± 0.59^{cd}	0.32 ± 0.01^{b}
G. cupulata	306.20 ± 4.99^{a}	103.99 ± 1.32^{c}	77.79 ± 2.67^{ab}	38.69 ±1.01^{bcd}	63.84 ± 0.97^{b}	69.97 ± 0.68^{a}	0.52 ± 0.01^{c}
C. edoensis	213.35 ± 8.43^{b}	272.12 ± 2.32^{a}	34.62 ± 1.19^{e}	28.37 ± 1.87^{cd}	56.53 ± 0.86^{ab}	62.60 ± 0.25^{bc}	0.33 ± 0.02^{b}
S.	55.72 ± 2.43^{f}	20.65 ± 2.16^{f}	25.66 ± 0.88^{ef}	43.90 ± 0.84^{abc}	20.37 ± 0.31^{de}	53.69 ± 2.21^{de}	0.26 ± 0.00^{b}
Longepedunculata							
S. [redacted]	328.56 ± 11.37^{a}	227.96 ± 3.46^{b}	88.58 ± 3.04^{a}	42.95 ± 0.85^{abc}	55.81 ± 0.85^{ab}	54.03 ± 1.30^{de}	0.62 ± 0.02^{d}
Q. gratissimum	227.35 ± 2.57^{b}	228.84 ± 1.43^{b}	77.81 ± 2.67^{b}	30.57 ± 1.61^{cde}	27.50 ± 0.42^{cd}	9.12 ± 2.23^{fg}	0.55 ± 0.01^{cd}
M. [redacted]	113.90 ± 2.54^{f}	196.00 ± 3.11^{g}	21.37 ± 0.73c	31.33 ± 0.41ade	7.76 ± 0.12e	38.74 ± 1.99b	0.27 ± 0.00b

Data are mean ± SEM values (n = 3). Data with the same superscript letters in a column are not significantly different (P > .05).

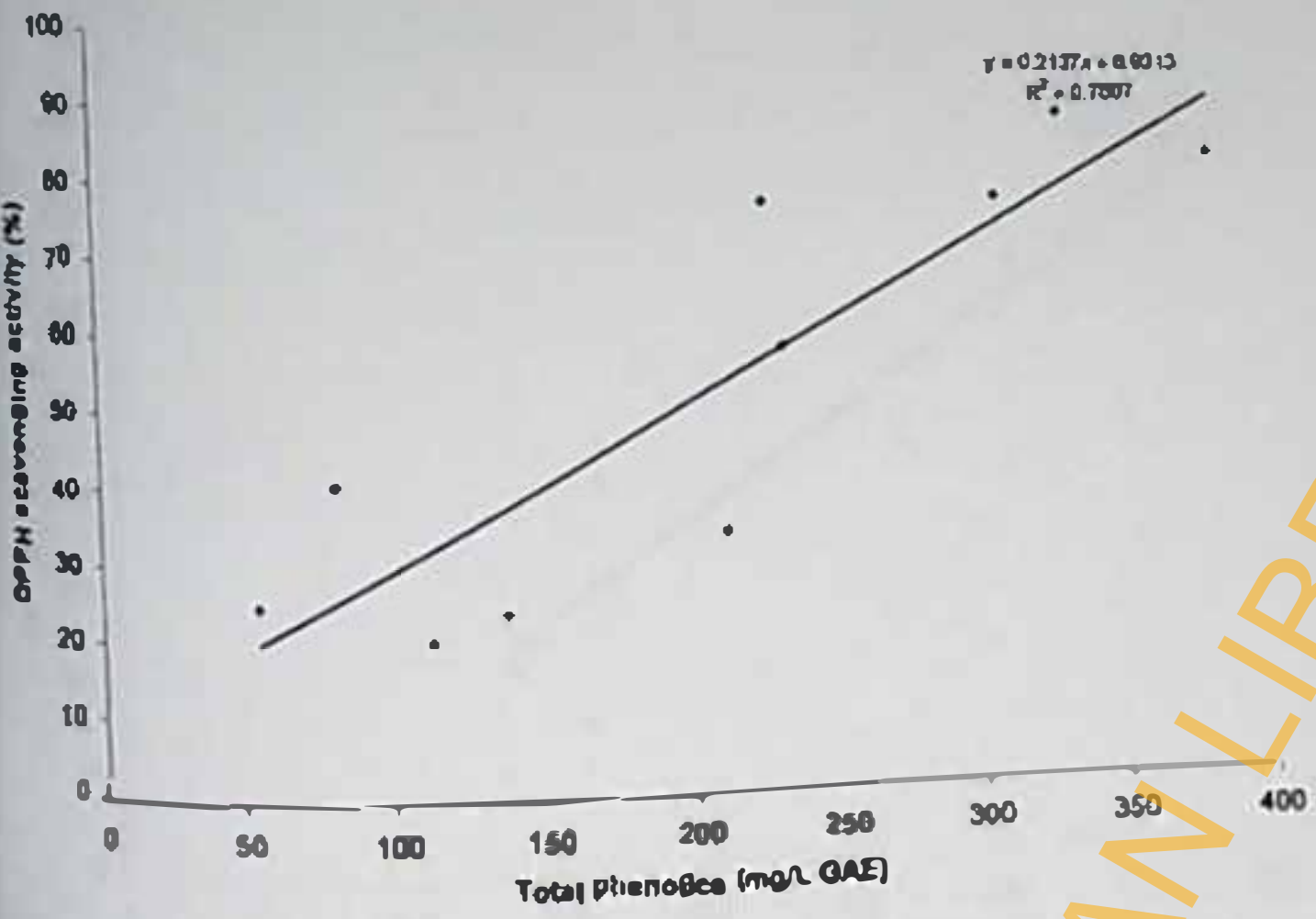


Figure 17 A

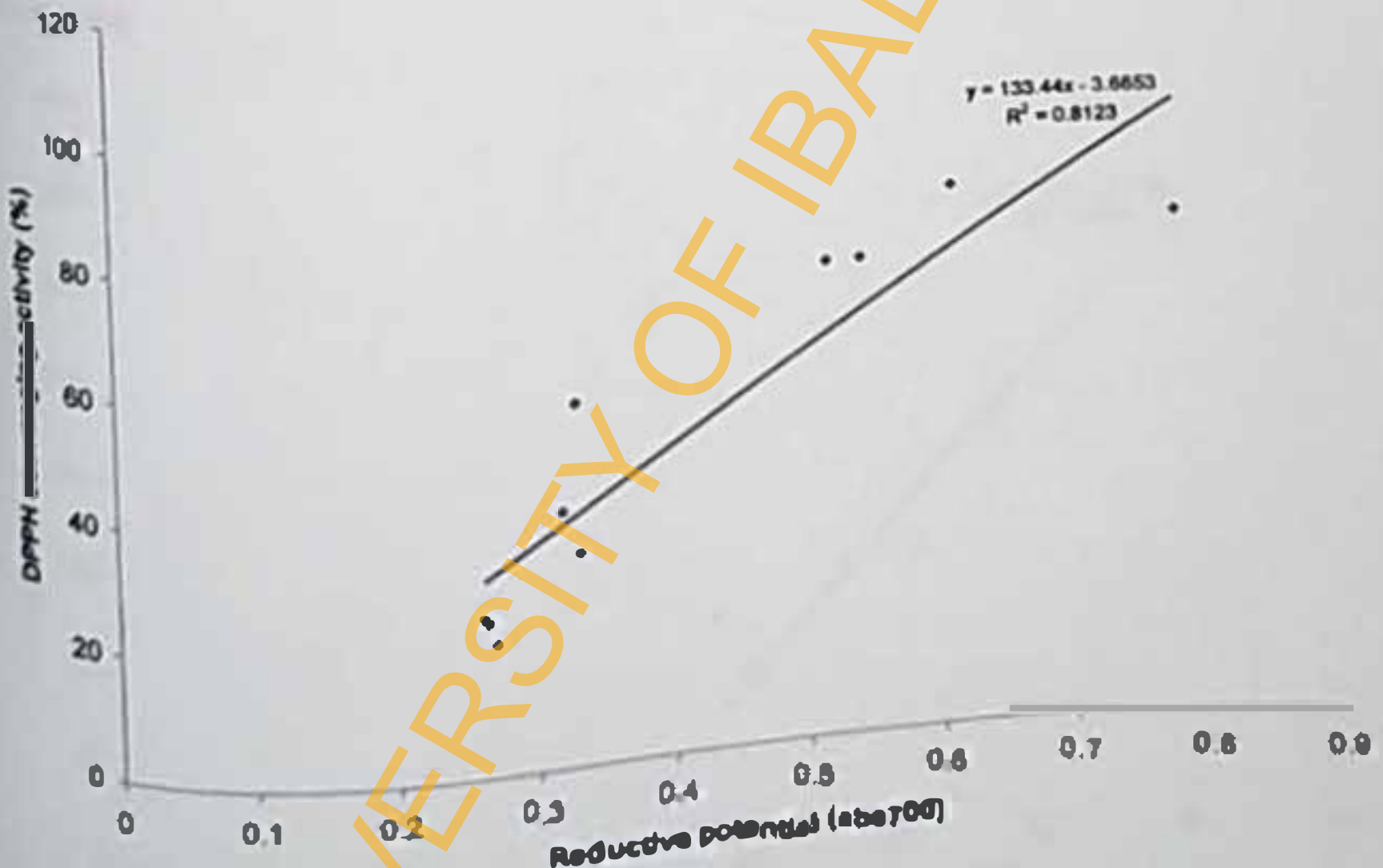


Figure 17 B

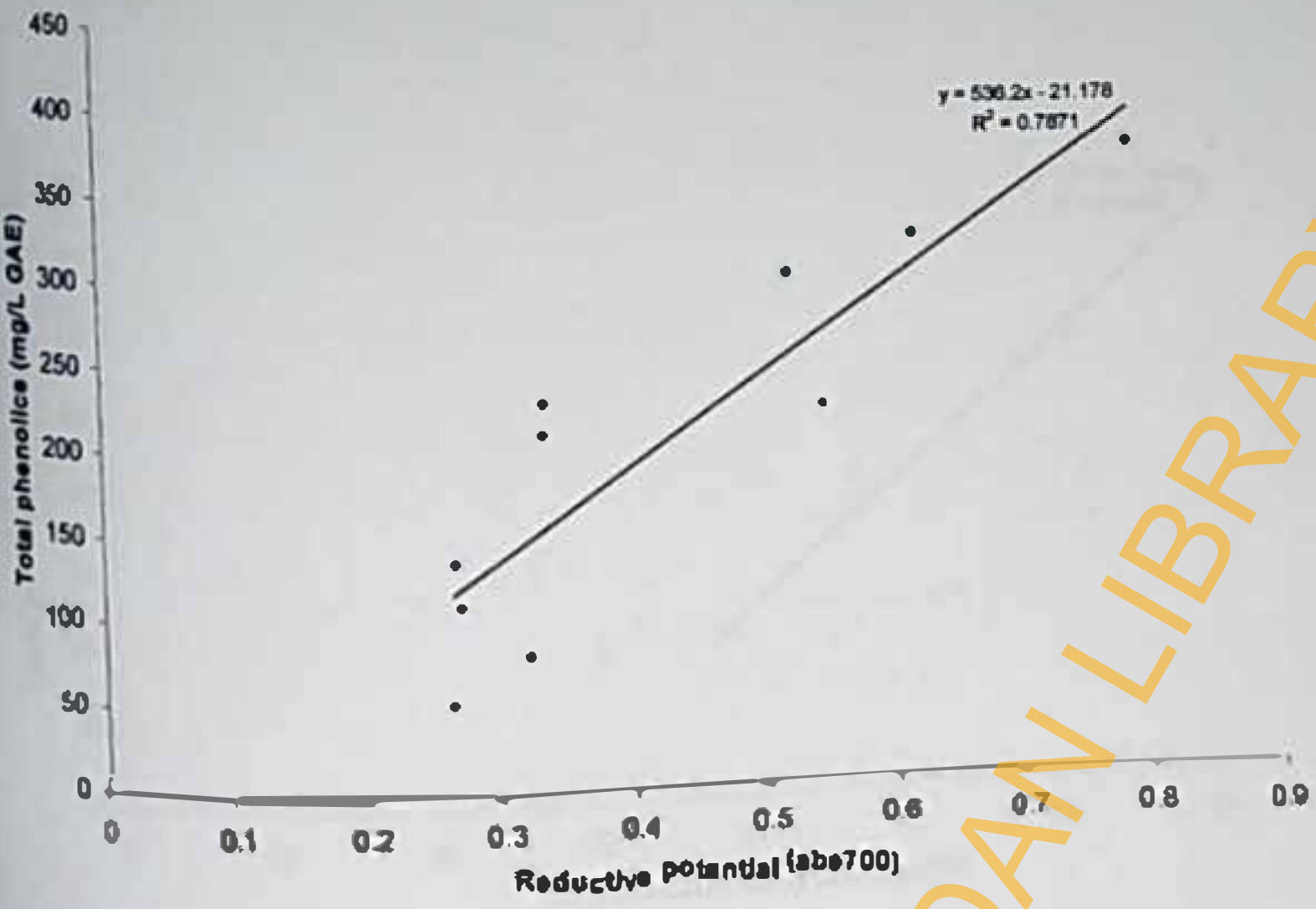


Figure 18A

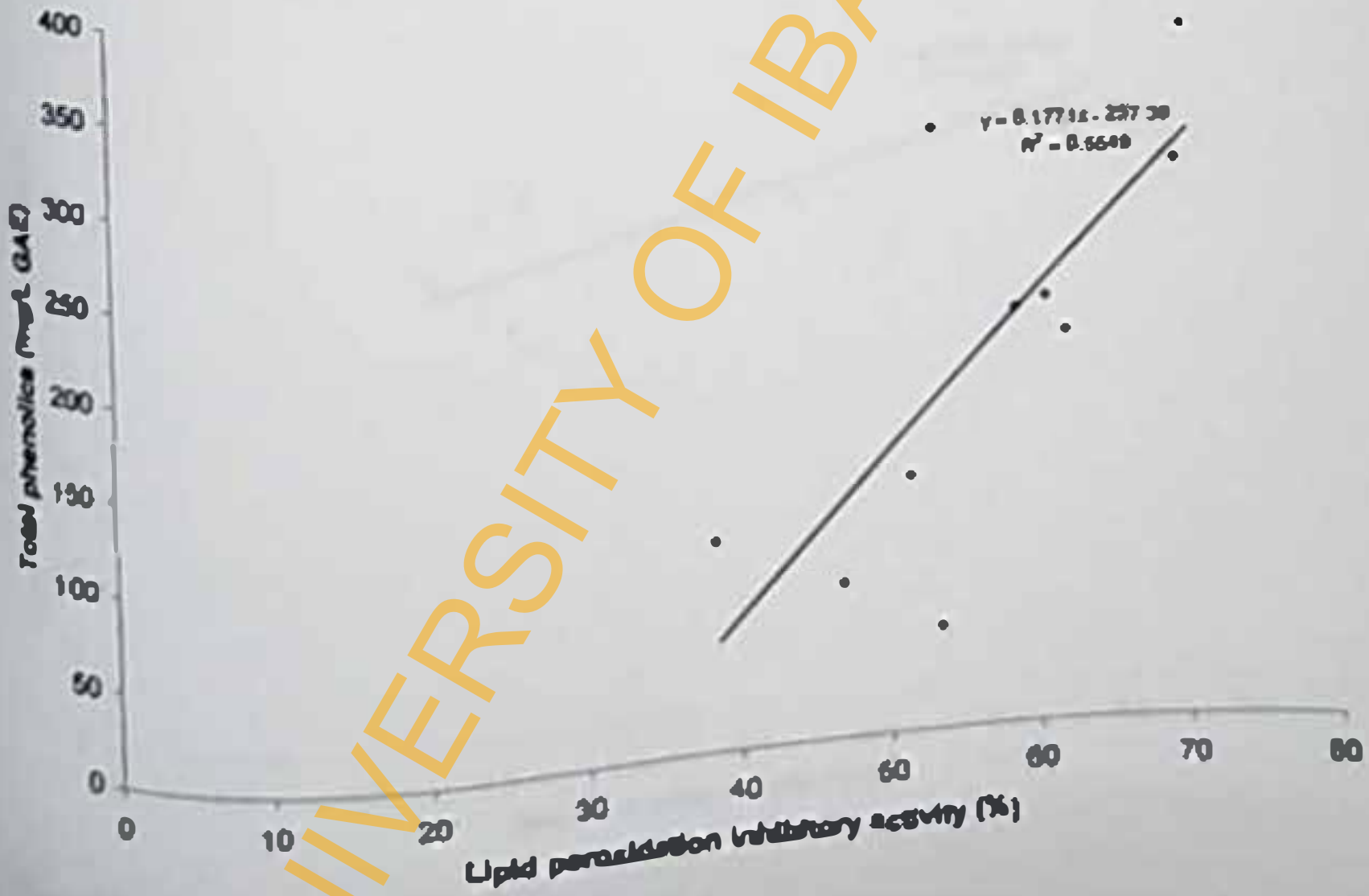


Figure 18B

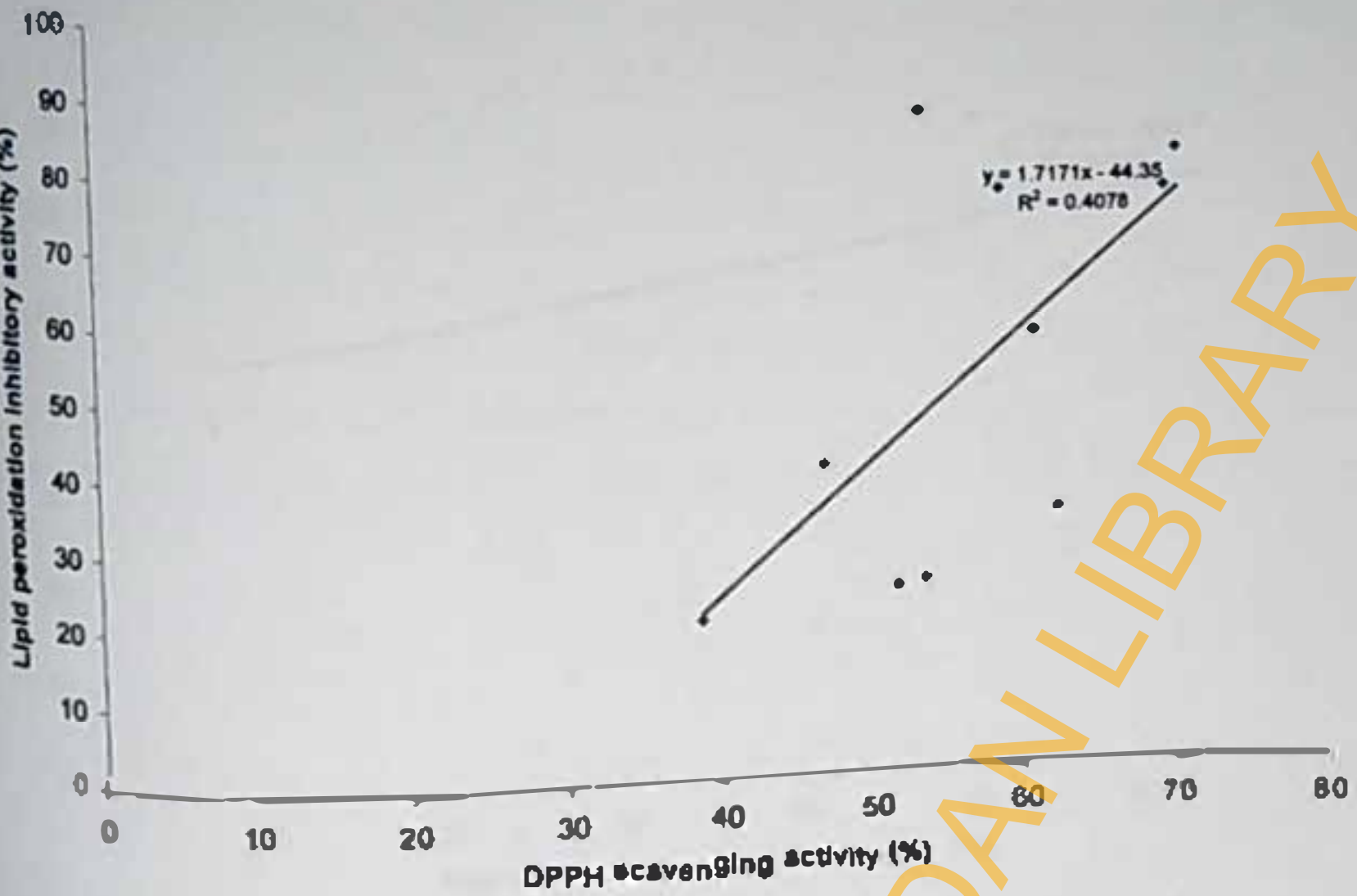


Figure 19A

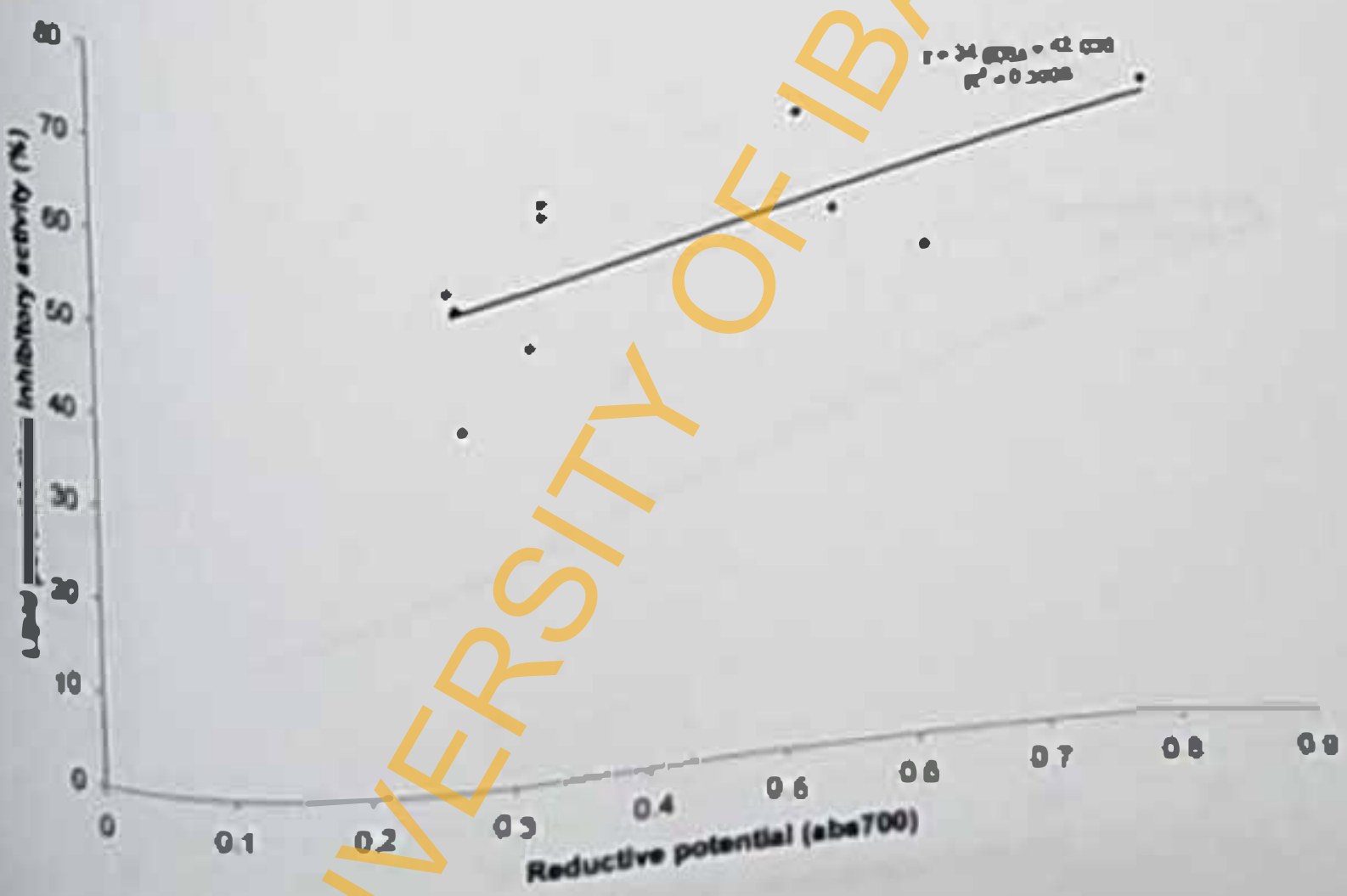


Figure 19B

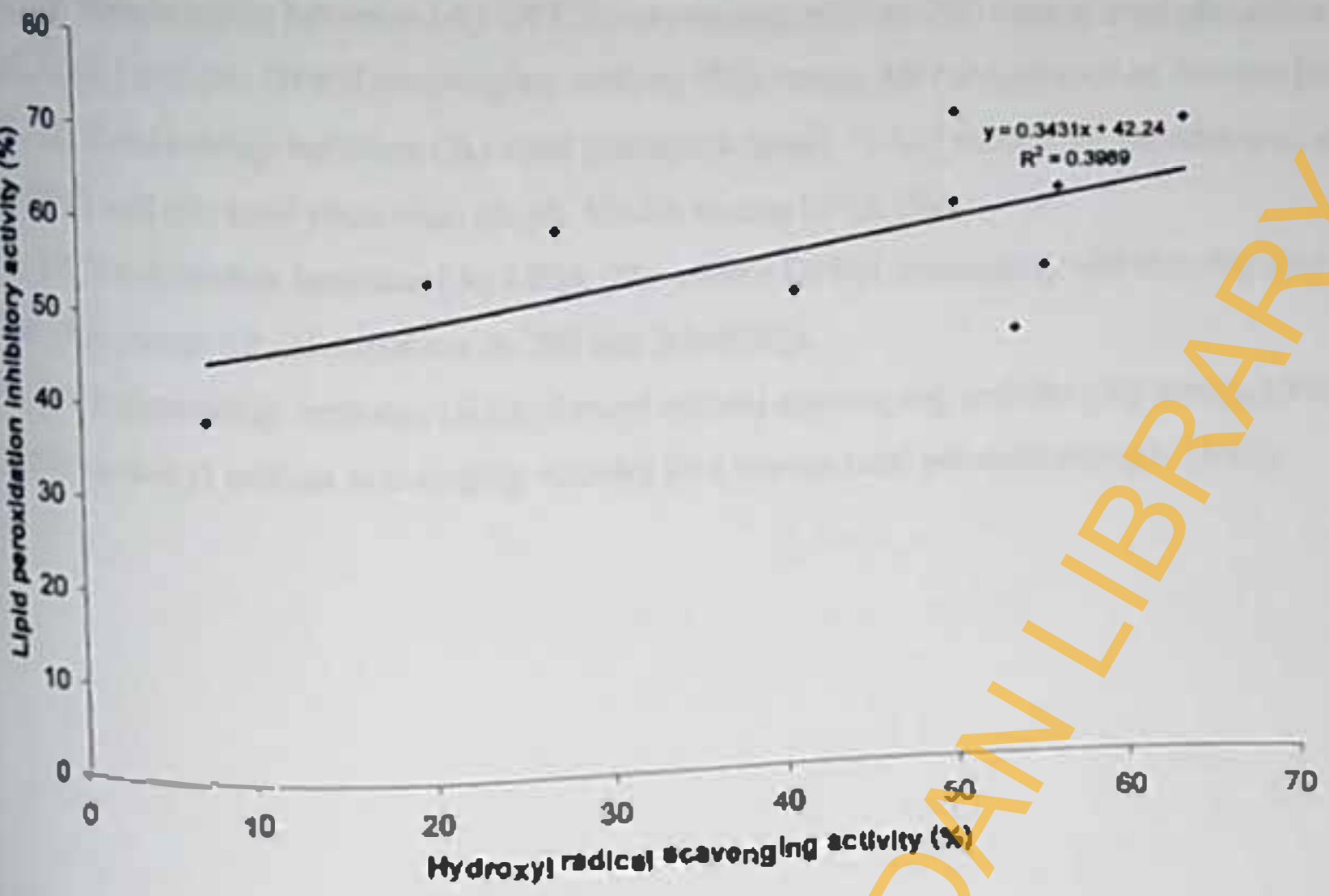


Figure 20A

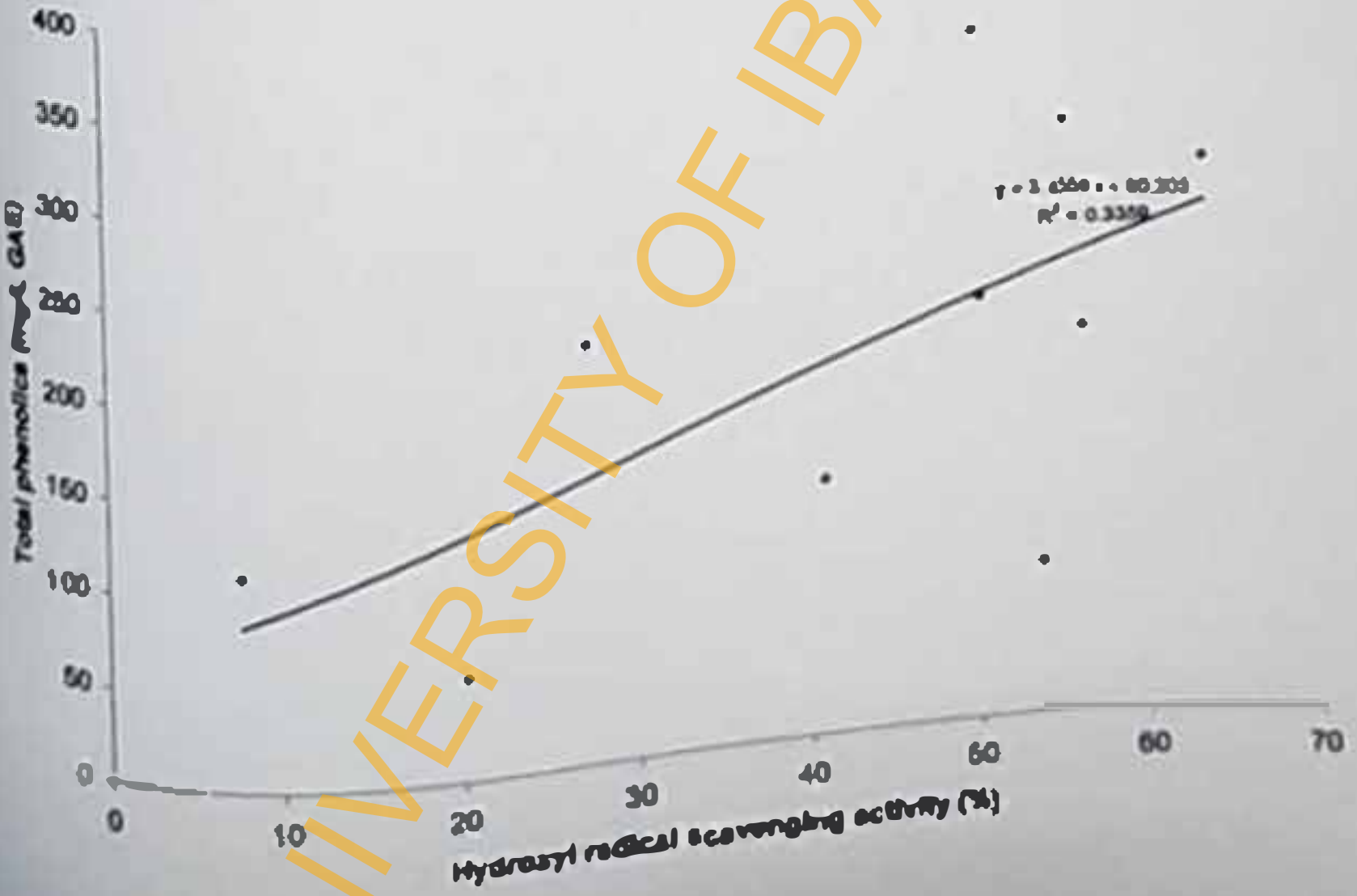


Figure 20B

FIG. 17. Relationship between (A) DPPH scavenging activity (%) versus total phenolics (in mg/L GAE) and (B) DPPH scavenging activity (%) versus RP (absorbance at 700 nm [abs700]).

FIG. 18. Relationship between (A) total phenolics (mg/L GAE) versus RP (absorbance at 700 nm [abs700]) and (B) total phenolics (mg/L GAE) versus LPIA (%).

FIG. 19. Relationship between (A) LPIA (%) versus DPPH scavenging activity (%) and (B) LPIA (%) versus RP (absorbance at 700 nm [abs700]).

FIG. 20. Relationship between (A) hydroxyl radical scavenging activity (%) versus LPIA (%) and (B) hydroxyl radical scavenging activity (%) versus total phenolics (mg/L GAE).

Table 7: Level of Correlation between assay methods

Assays	Correlation Coefficient (*r*)
TPC/TFC	0.43
DPPH/TPC	0.76
TPC/NO	0.12
TPC/DOR	0.34
TPC/LPIA	0.55
TPC/RP	0.79
DPPH/TFC	0.21
TFC/NO	0.29
TFC/DOR	0.03
TFC/LPIA	0.13
TFC/RP	0.22
DPPH/NO	3.0×10^{-5}
DPPH/DOR	0.27
LPIA/DPPH	0.41
DPPH/RP	0.81
NO/DOR	0.01
LPIA/NO	0.10
LPIA/RP	0.40
LPIA/DOR	0.40
RP/NO	0.07
RP/DOR	0.15

INVESTIGATION TWO

4.2. Inotropic, chronotropic and anti-ischemic effects of *Spondias mombin* extract on isolated rat heart preparation using the Langendorff technique

INTRODUCTION

Myocardial ischemia results in ATP depletion and accumulation of toxic metabolites, whereas reperfusion leads to the production of reactive oxygen intermediates and calcium overload. The alterations in cellular metabolism and generation of toxic molecules contribute to myocardial ischemia/reperfusion injury (Marczin et al., 2003). The Langendorff technique is a popular method for studying the modulatory effects of drugs and other variables on partial or global ischemia and reperfusion in organs *ex vivo*. In this experiment, the non-recirculating Langendorff technique was used to evaluate the *per se* and anti-ischemic effects of MES on excised rat hearts.

PROCEDURE

Experiments were performed as previously described (section 3.13.1).

4.2.1. EXPERIMENT 3: Evaluation of inotropic (force of heart contraction) and chronotropic (heart rate) effects of MES

RESULTS

Perfusate effect of buffer (Control)

Table 8 shows the intrinsic effects (evaluated as amplitude and heart rate) of the NIIT buffer on perfused isolated rat hearts. The results indicate that test substances (which would be dissolved in the perfusion medium) would be free of interference from the perfusion medium. The results also show that excised hearts retrogradely perfused in the Langendorff mode with NIIT buffer can maintain the same force and rate of contraction for more than 90 minutes. This implies that

deviation from this pattern in subsequent tests may be attributed mainly to the modification of experimenta. conditions or the introduction of extracts/compounds into the perfusion medium.

Table 8: Perfusion with NHT buffer for 90 min

	Time (min)				
	30	45	60	75	90
Amp (%)	100±0	100±0	98±0	102±2	100±3
HR (%)	100±0	99±1	99±1	97±2	98±1

Amp: Amplitude; HR: Heart rate. Results are presented as mean ± SEM (n = 6)

Inotropic and chronotropic effect of MES

Table 9 shows the inotropic (force of heart contraction or amplitude) and the chronotropic (heart rate) effects of the extract and standards on the isolated rat hearts. The results indicate that *Spondias mombin* had no significant deleterious or cytotoxic effect on the isolated rat hearts. The results obtained for the extract is comparable to that of ramipril while nifedipine on the other hand significantly decreased the amplitude.

Table 9: Effect of extract and standards

Reperfusion Medium (conc.)	Control		Test Compounds					
			15'		30'		45'	
	AMP	HR	AMP	HR	AMP	HR	AMP	HR
SM (0.001 g/L)	100	100	87±9	92±6	78±9	86±5	84±11	86±1
SM (0.005 g/L)	100	100	110±2	93±4	118±1	88±3*	---	---
SM (0.01 g/L)	100	100	119±6	90±5	121±7	85±5	121±7	80±4*
SM (0.02 g/L)	100	100	89±4*	104±3	98±5	95±5	110±4*	100±7
Ramipril (10 µM)	100	100	101±3	92±6	105±5	86±5	107±9	80±5
Nifedipine (1 µM)	100	100	47±14	81±10	57±13*	83±11	54±13	88±19

AMP: Amplitude, HR: Heart rate, 15', 30', 45': 15th, 30th, 45th minute of reperfusion; SM: *Spondias mombin*. Results are presented as mean ± SEM (n = 6). *Significantly different from control ($p<0.05$).

4.2.2. EXPERIMENT 4: Evaluation of the anti-ischemic effect of MES

RESULTS

Forty five minutes of global ischemia followed by reflow resulted in the significant reduction of the amplitude of hearts perfused with the NHT buffer alone (control) at both 15 and 30 min post ischemia (Table 10). MES was effective in reversing the decline in the force of heart contraction (amplitude) produced by global ischemia especially at 0.005 g/L.

Table 10: Global ischemia followed by reflow with buffer or extract

Compound used for reflow	Pre-Ischemic value		Post-Ischemic value			
			15'		30'	
	Amp	Rate	Amp	Rate	Amp	Rate
NHT Buffer	100±0	100±0	66±6*	133±10	73±8*	109±3
SM (0.005g/L)	100±0	100±0	122±3[a]	110±19	122±7[a]	104±10
SM (0.01g/L)	100±0	100±0	87±28	105±4	100±17	104±7
SM (0.02g/L)	100±0	100±0	84 ±2	141±7	121±4[a]	112±9

Results are presented as mean ± SEM (n = 6). *Significantly different from pre – ischemic value; [a]Significantly different from NHT Buffer ($p < 0.05$)

INVESTIGATION THREE

43. Protective Effect of *Spondias mombin* extract against Isoproterenol-Induced Myocardial Infarction

43.1. EXPERIMENT 5: Modulatory effect of MES on antioxidant indices and markers of tissue damage in isoproterenolchallenged rats

INTRODUCTION

Spondias mombin was investigated for in vivo cardioprotective property using the model of isoproterenol (ISP)-induced myocardial infarction. Ramipril (1.25 mg/kg) was used as the control standard drug. Isoproterenol, a synthetic β-adrenoceptor agonist, has been found to induce myocardial infarction in rat as a result of disturbance in physiological balance between production of free radicals and antioxidative defense system. It is well known to generate free radicals and stimulate lipid peroxidation, which is a causative factor for irreversible damage to the myocardium. It also increases the levels of serum and myocardial lipids, which in turn leads to coronary heart disease (Nair and Devi, 2006; Zhou *et al.*, 2008).

PROCEDURE

Male Sprague-Dawley rats weighing 150 - 200 g were randomly allocated to five main groups with six animals in each group. The groups were treated as described in section 3.13.2. On day 31, blood was withdrawn by retroorbital vein puncture and used for the estimation of glucose (Accu-check® Active glucometer) and for serum cholesterol, phosphate (Beckman Coulter Synchron CX9 Pro clinical system), MDA (Colado *et al.*, 1997), LDH (Seth *et al.*, 1994) and plasma GSH (Anderson, 1985) levels. Rats were sacrificed, hearts excised, frozen in liquid nitrogen and stored at -85 °C until used for biochemical analysis. Hearts stored in liquid nitrogen were weighed. A 10% homogenate was prepared in phosphate buffer (50 mM, pH 7.4). An aliquot was used for the assay of MDA. The homogenate was centrifuged at 15 000 x g at 4 °C for 15 min and the supernatant was used for the estimation of nitrite (Griess diazotization

reaction), SOD (Kakkar *et al.*, 1984), CAT and protein (Lowry *et al.*, 1951) as described in section 3.13.2.1.

RESULTS

Heart weight/Body weight ratio

Figure 21 shows that ISP administration led to an increase in the heart weight/body weight ratio indicating that it possibly caused inflammation of the heart MES extract and Ramipril were able to reduce the elevated heart weight/body weight ratio. Administration of the plant extract alone had no adverse effect on the heart weight/body ratio.

Biochemical estimations

Figure 21 to 34 illustrate the disruption caused by ISP administration on the levels of different blood, serum, plasma and tissue metabolites and endogenous systems. The endogenous antioxidant GSH was depicted (from 1.72 ± 0.09 for control group to 1.26 ± 0.01 µM for the ISP-treated group in the plasma and from 3.53 ± 0.16 for control group to 2.85 ± 0.17 units/mg protein for the ISP-treated group in the heart tissue) while production of MDA was exacerbated (from 1.91 ± 0.21 for the control group to 3.00 ± 0.12 µM for the ISP-treated group in the serum and from 4.45 ± 0.94 for the control group to 8.52 ± 2.00 units/mg protein for the ISP-treated group in the heart tissue (Figures 22 and 23). Administration of SM extract and Ramipril significantly reversed these deleterious trends in the serum as well as the heart tissue. In the ISP +250 mg/kg SM group, plasma and tissue levels of GSH were increased to 1.73 ± 0.12 µM and 3.47 ± 0.13 units/mg protein respectively compared to values for ISP-challenged group while serum and tissue levels of MDA were reduced to 2.11 ± 0.05 µM and 5.77 ± 0.28 units/mg tissue respectively compared to the ISP-challenged group. These values were comparable to those obtained for the ramipril-treated groups. Moreover, decreases in tissue SOD activity (from 4.30 ± 0.1 units/mg protein for the control to 3.52 ± 0.08 units/mg protein) and catalase activity (from 2.50 ± 0.37 for the control to 1.08 ± 0.06) occasioned by ISP intoxication were significantly corrected by treatment with extracts and Ramipril (Figures 24 and 25). SOD and

catalase activity for the ISP + 250 mg/kg SM group were 4.39 ± 0.09 and 2.05 ± 0.08 units/mg protein respectively. The increase in tissue nitrite level observed in the ISP intoxicated group was significantly decreased in the 250 mg/kg extract-treated group by about 33.3% which is comparable to the 36.1% reduction in the Ramipril-treated groups (Figure 26).

ISP administration probably caused disturbances in glucose metabolism resulting in hyperglycaemia (Figure 27). The SM extract seemed to offer protection against the ISP – induced hyperglycaemia only at 250 mg/kg. Blood glucose level was not lowered in animals administered SM extract alone. Ramipril seemed to correct the imbalance in blood glucose level caused by treatment with ISP. Serum cholesterol level elevated as a result of ISP administration was reduced by SM at the dose of 250 mg/kg ($p<0.05$) (Figure 28) while the elevated phosphate level in ISP challenged group was reduced by SM at the two doses employed ($p<0.05$) (Figure 29).

ISP intoxication led to the massive leakage of LDH into the serum (Figure 30). Both SM extract and Ramipril were effective in decreasing the elevated LDH levels ($p<0.001$). The 250 mg/kg extract-treated group decreased the elevated LDH level by over 60%.

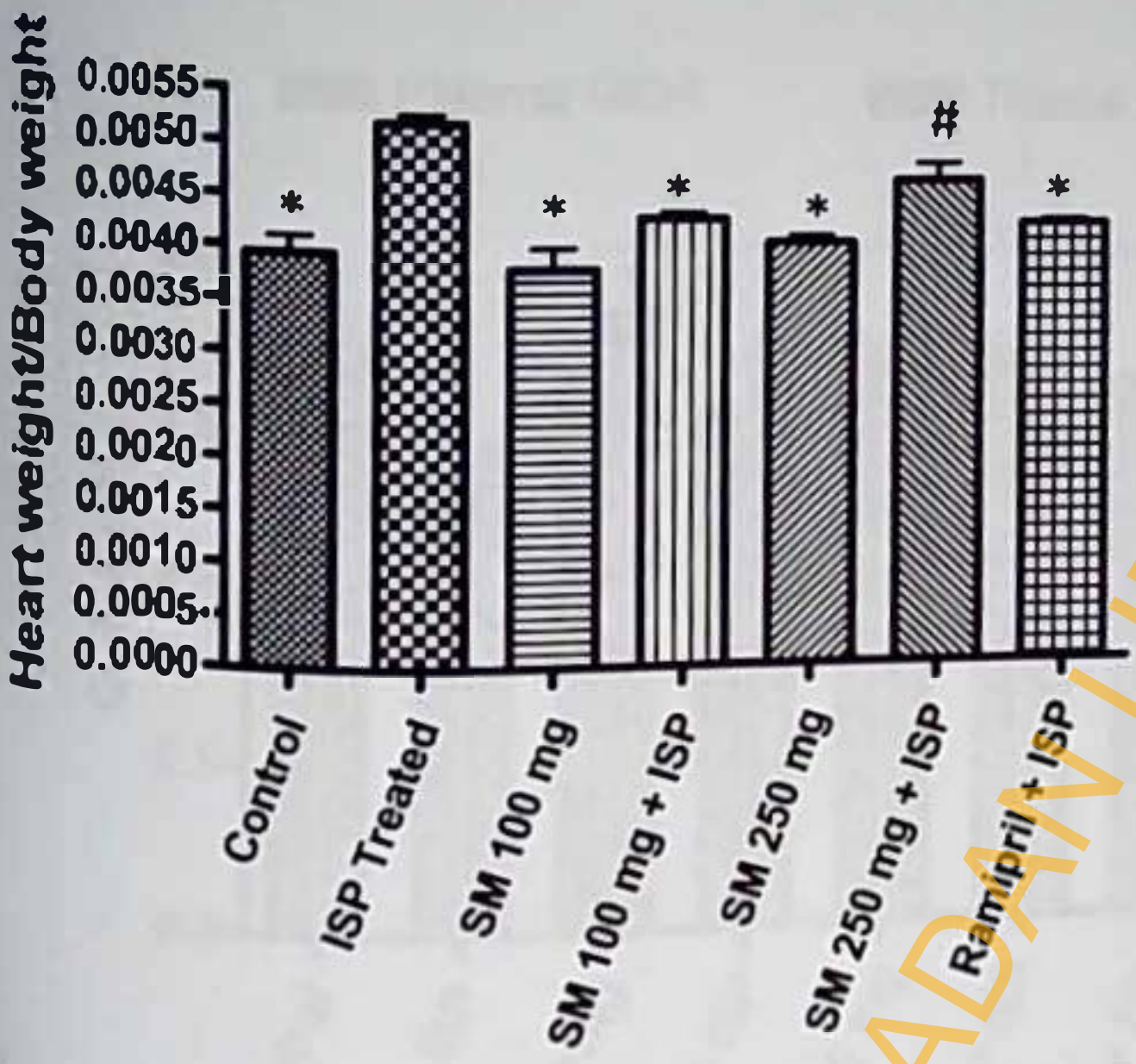


Figure 21: Heart weight/body weight ratio of experimental animals. Results are presented as mean ± SEM (n = 6). *Significantly different from ISP treated ($p<0.001$); #significantly different from ISP treated ($p<0.05$)

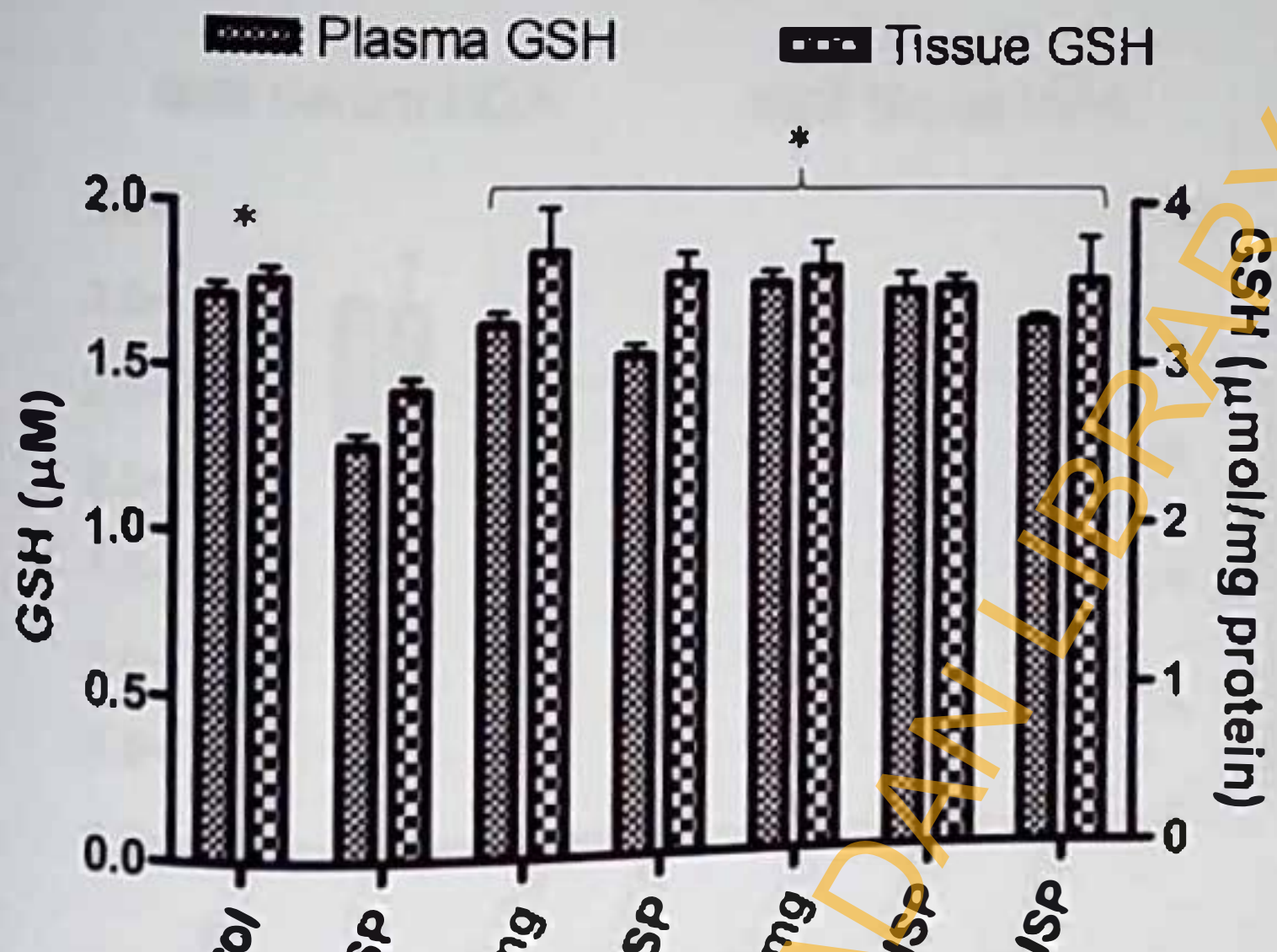


Figure 22: Plasma (μM) and tissue (μmol/mg protein) GSH levels. Results are presented as mean ± SEM (n = 5). * Significantly different from ISP treated group ($p < 0.05$).

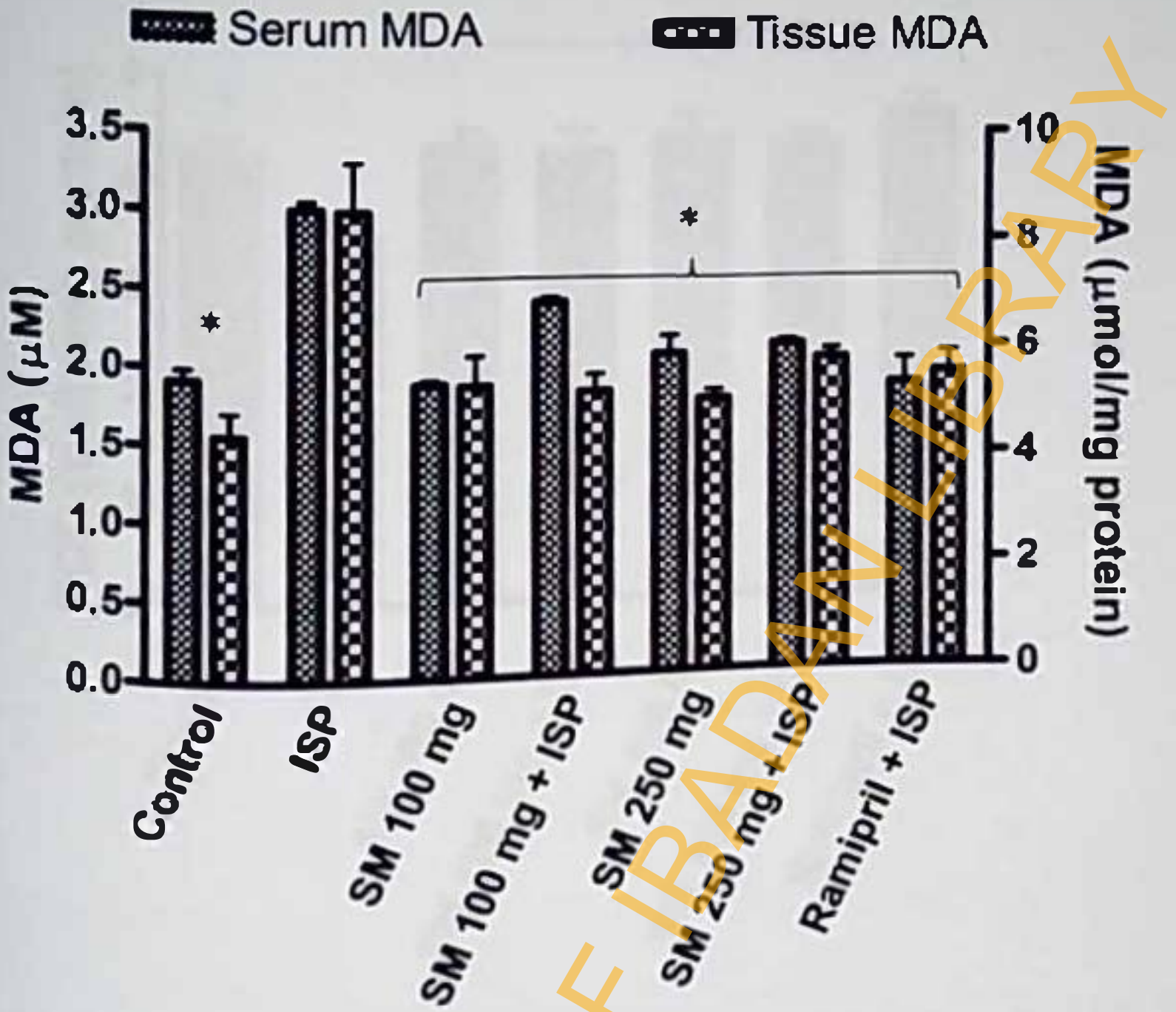


Figure 23: Serum (μM) and tissue (μmol/mg protein) MDA levels in serum of control and test groups. Results are presented as mean ± SEM (n = 6). * Significantly different from ISP treated ($p < 0.05$).

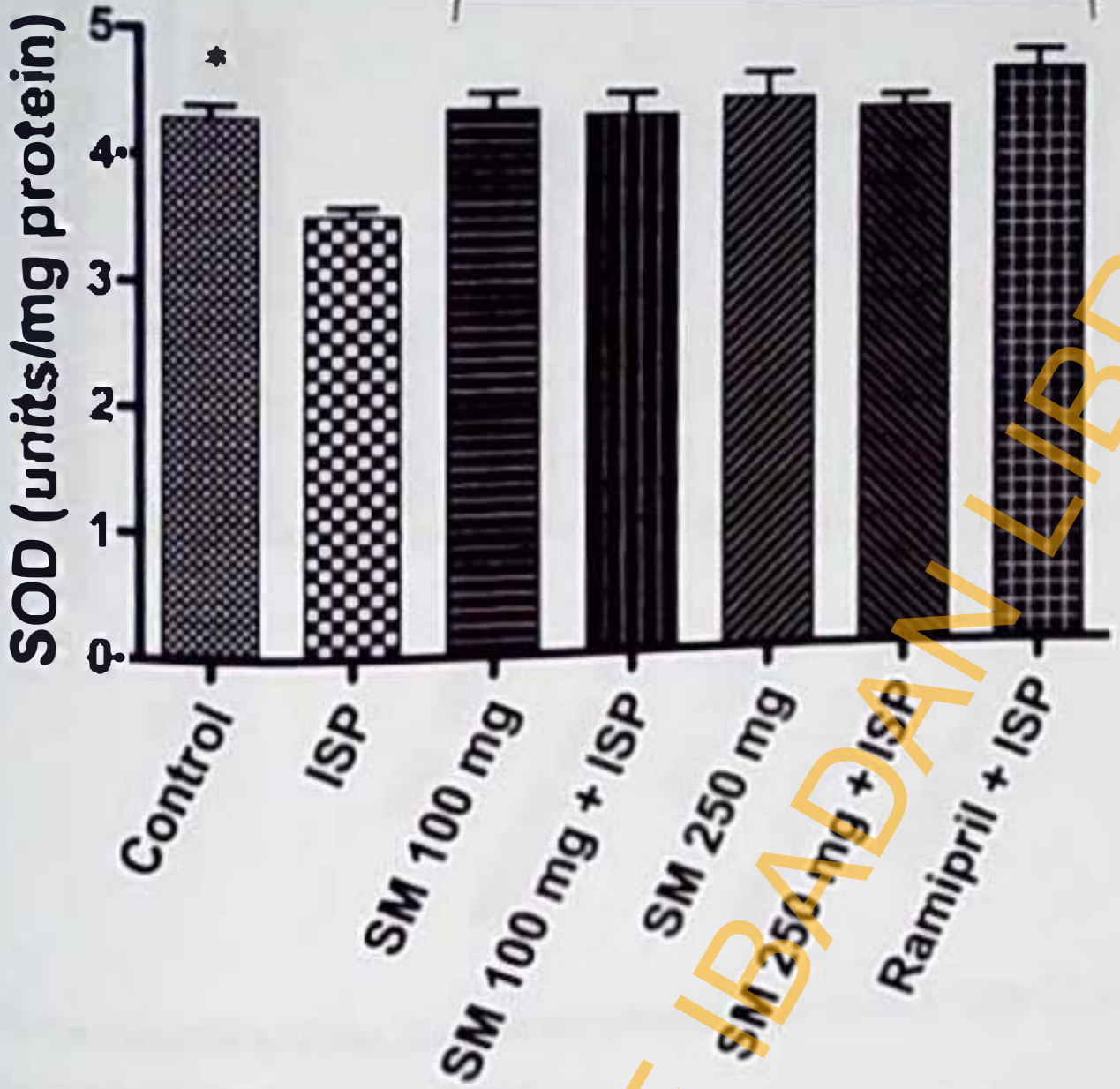


Figure 24: Tissue SOD activity. Results are presented as mean ± SEM (n = 5). * Significantly different from ISP treated group ($p < 0.05$)

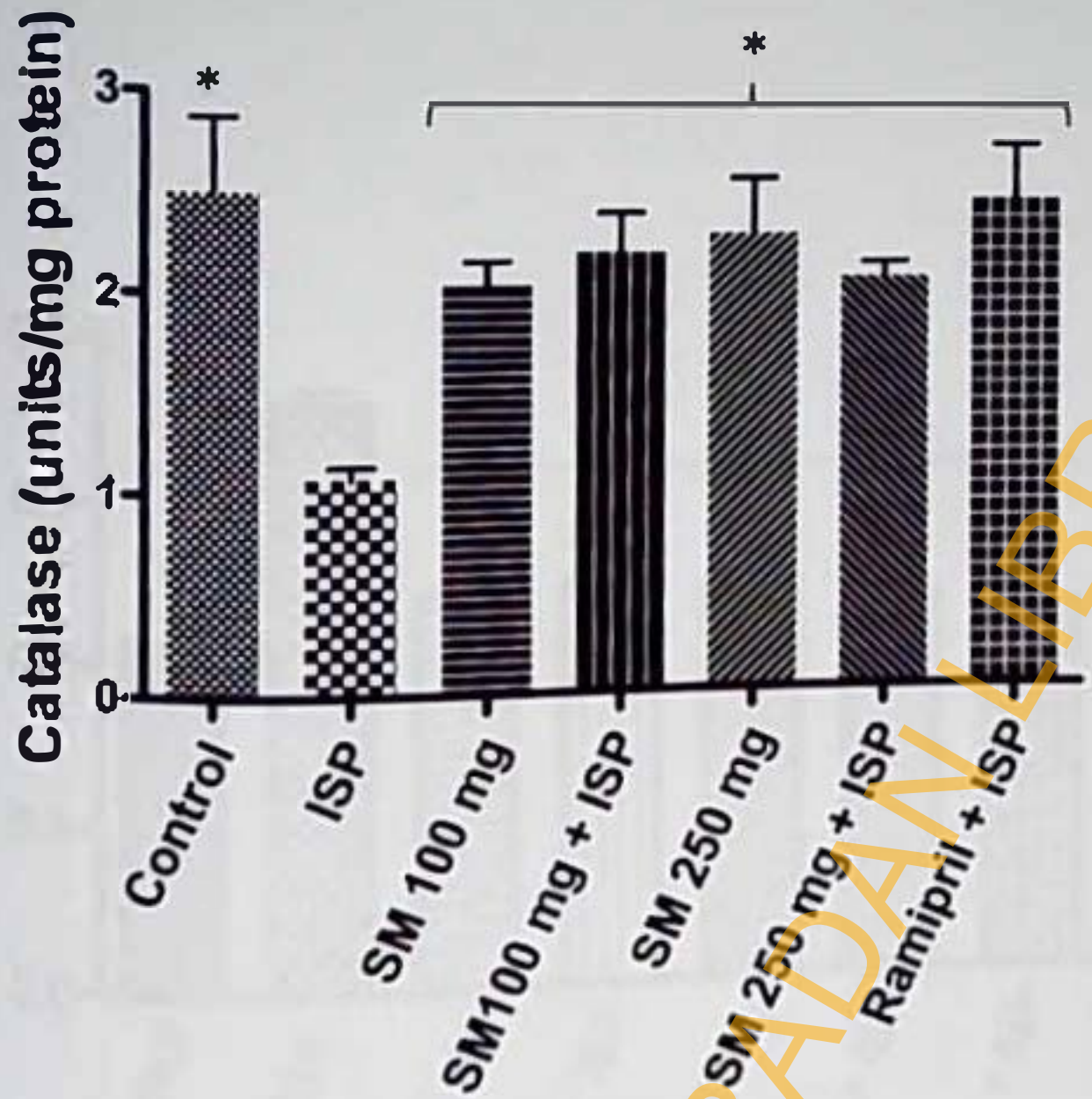


Figure 25: Tissue catalase activity. Results are presented as mean ± SEM (n = 5). * Significantly different from ISP treated group ($p < 0.05$)

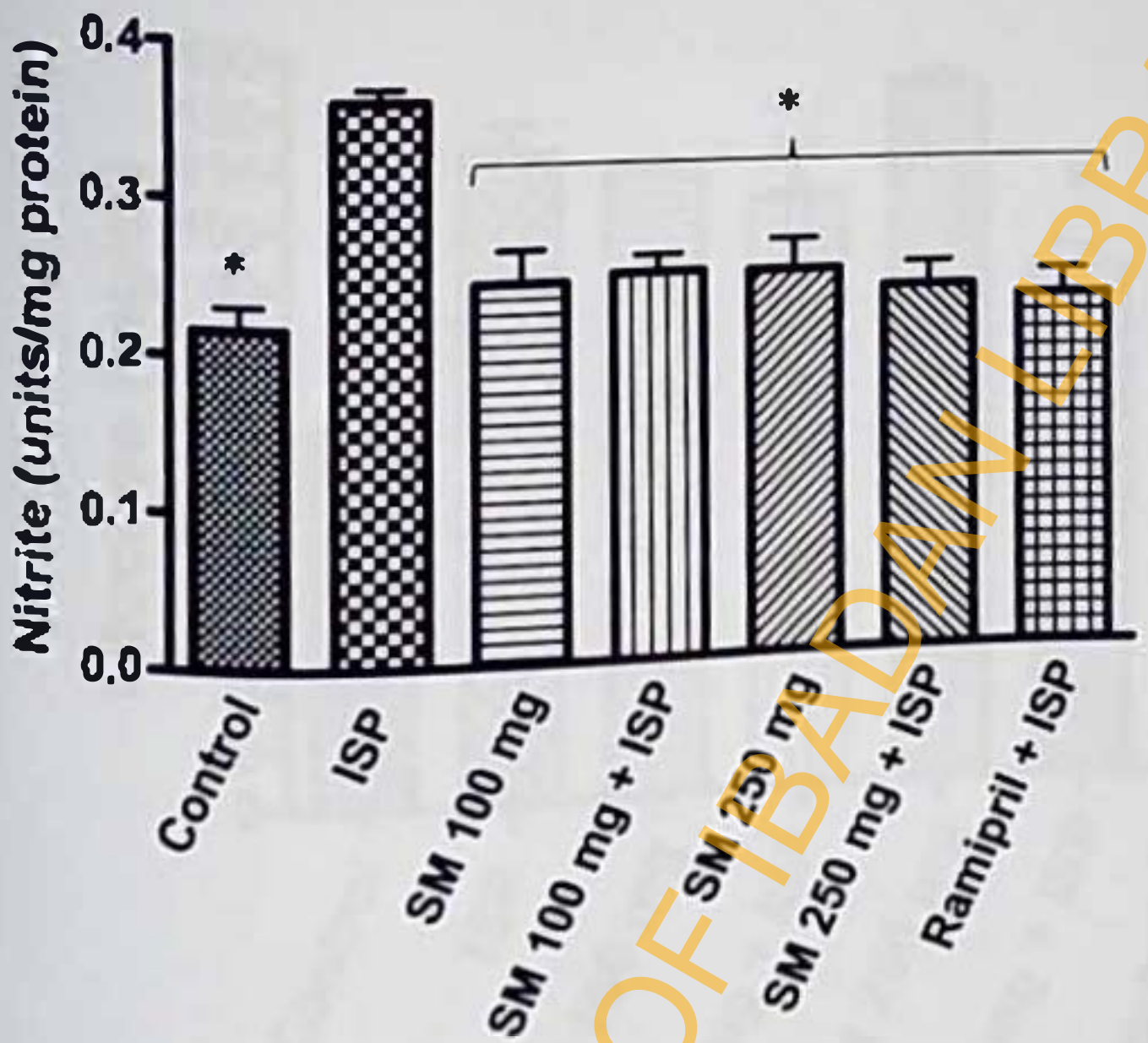


Figure 26: Tissue Nitrite levels. Results are presented as mean ± SEM (n = 5). * Significantly different from ISP treated group ($p < 0.05$)

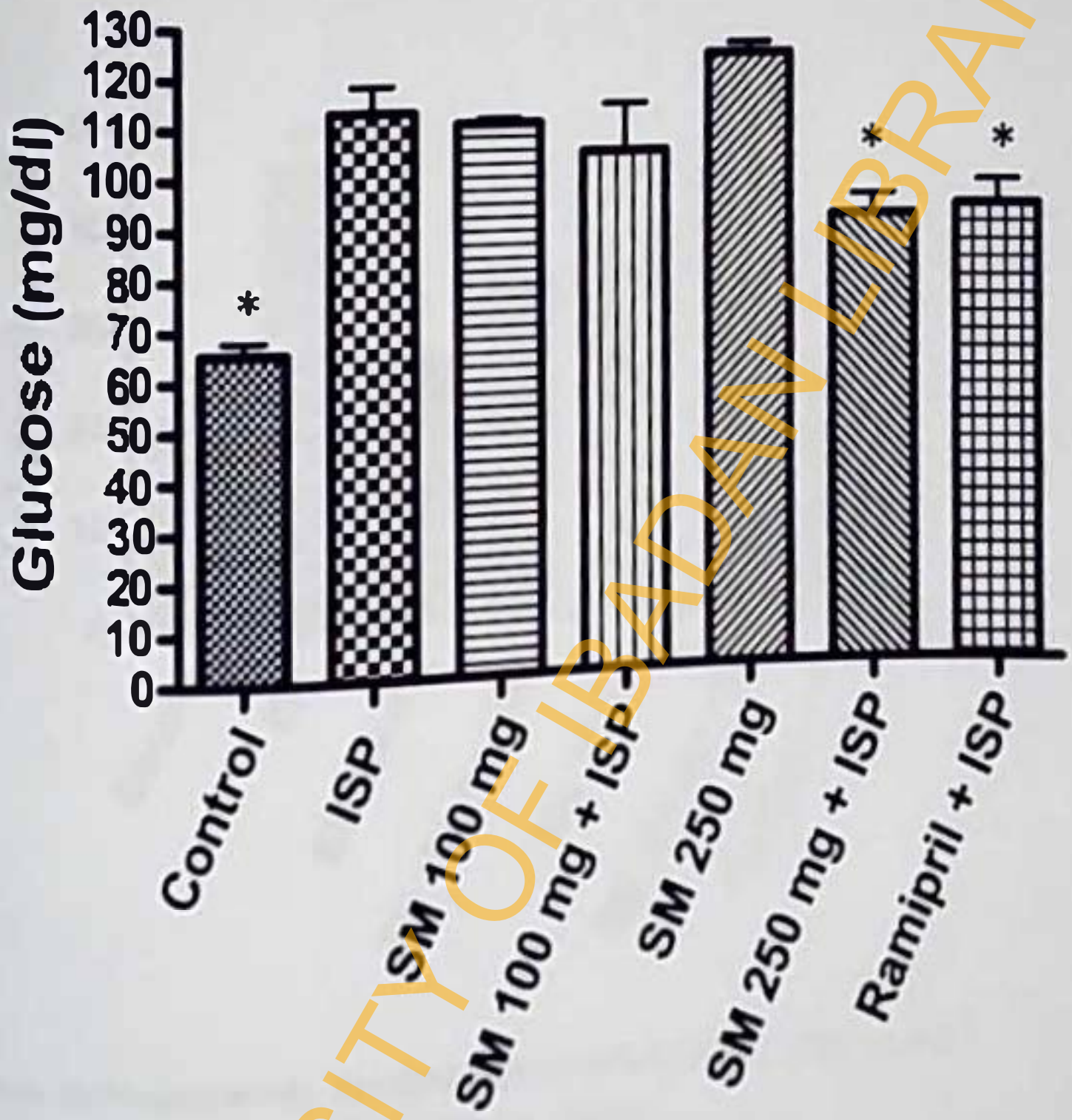


Figure 27: Blood glucose level in all groups. Results are presented as mean ± SEM (n = 6). *Significantly different from ISP treated group ($p < 0.05$).

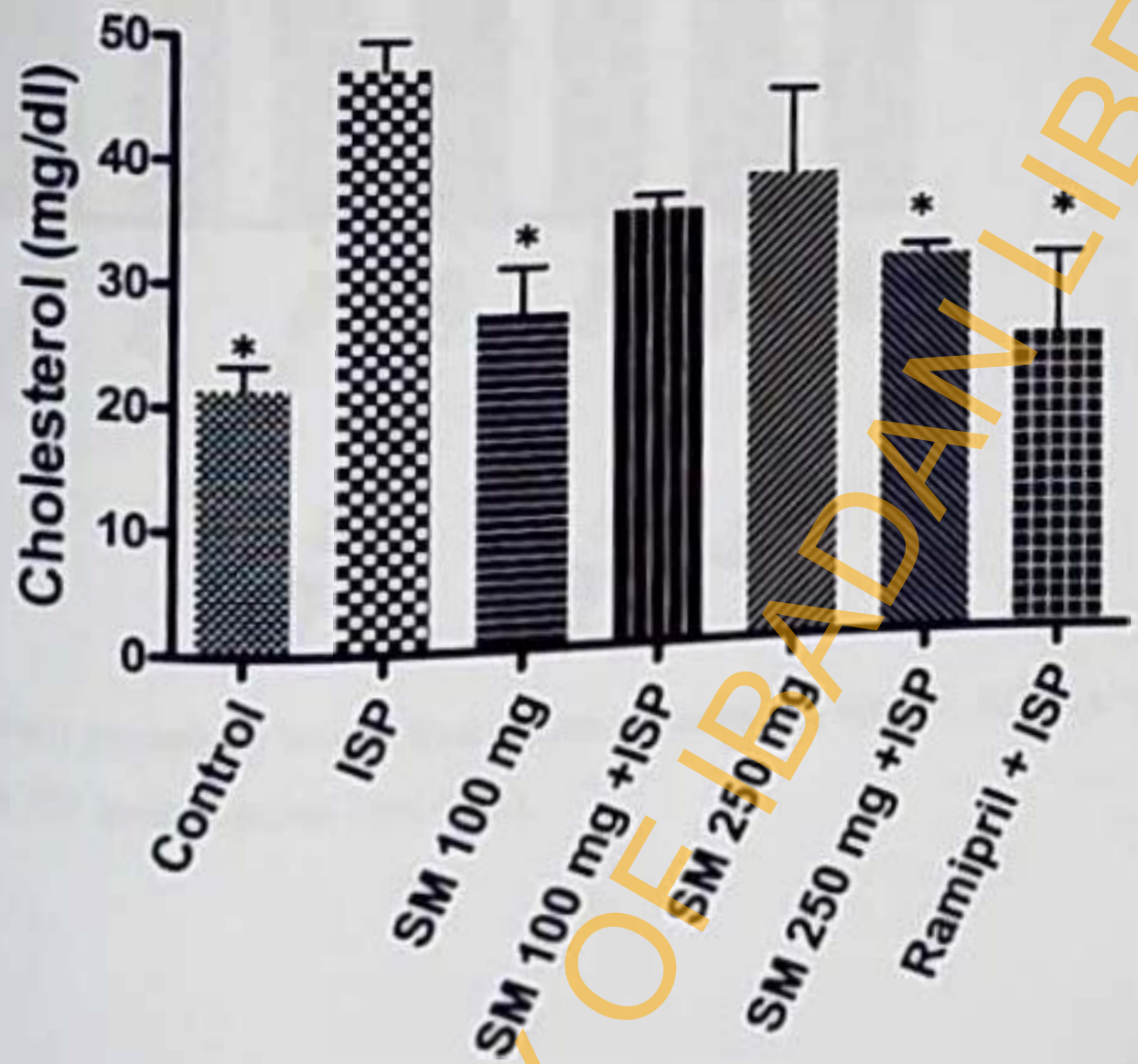


Figure 28: Serum cholesterol levels. Results are presented as mean ± SEM (n = 5).
* Significantly different from ISP treated group ($p < 0.05$)

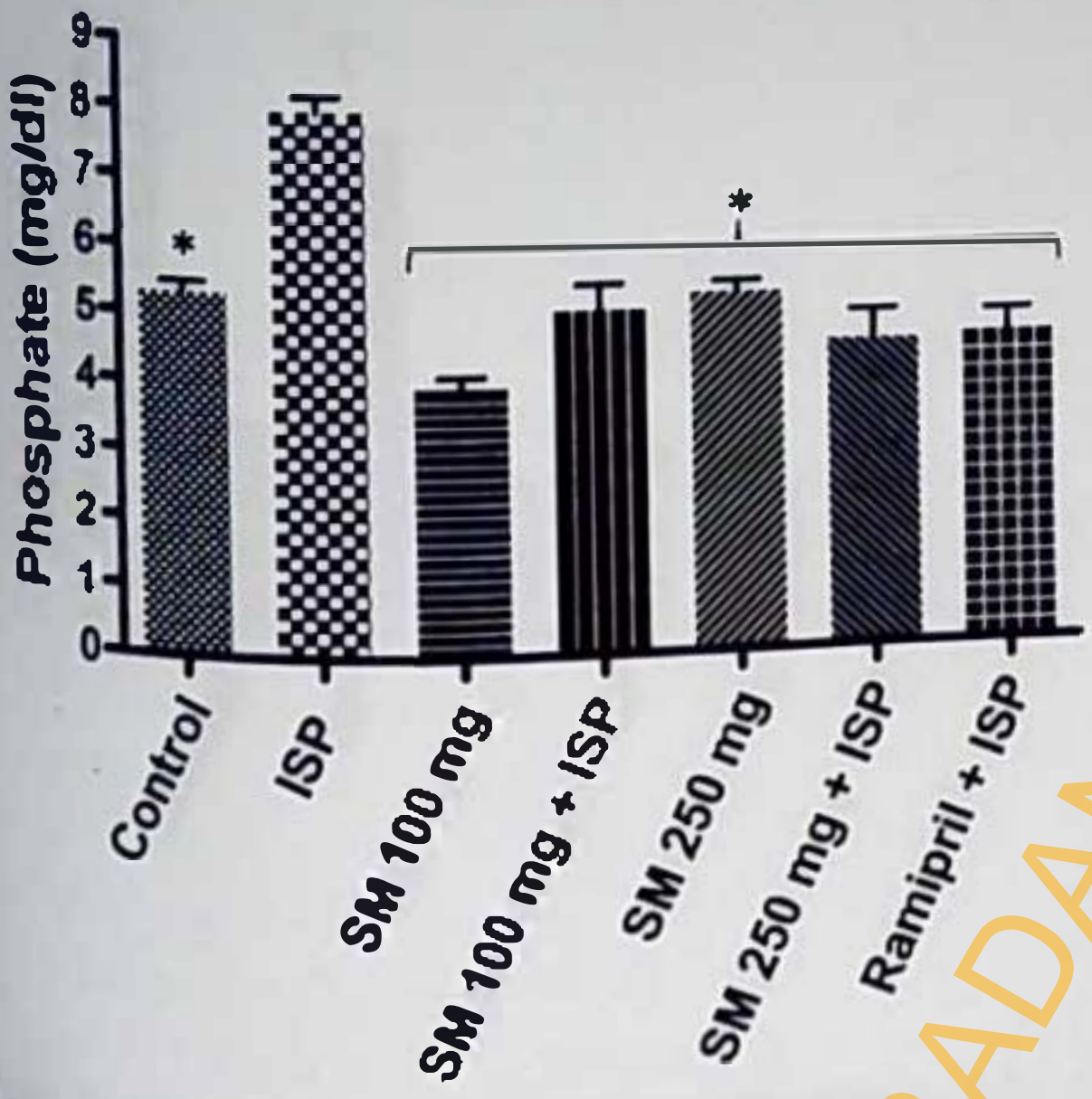


Figure 29: Serum phosphate levels. Results are presented as mean ± SEM (n = 5). * Significantly differed from ISP treated group ($p < 0.05$)

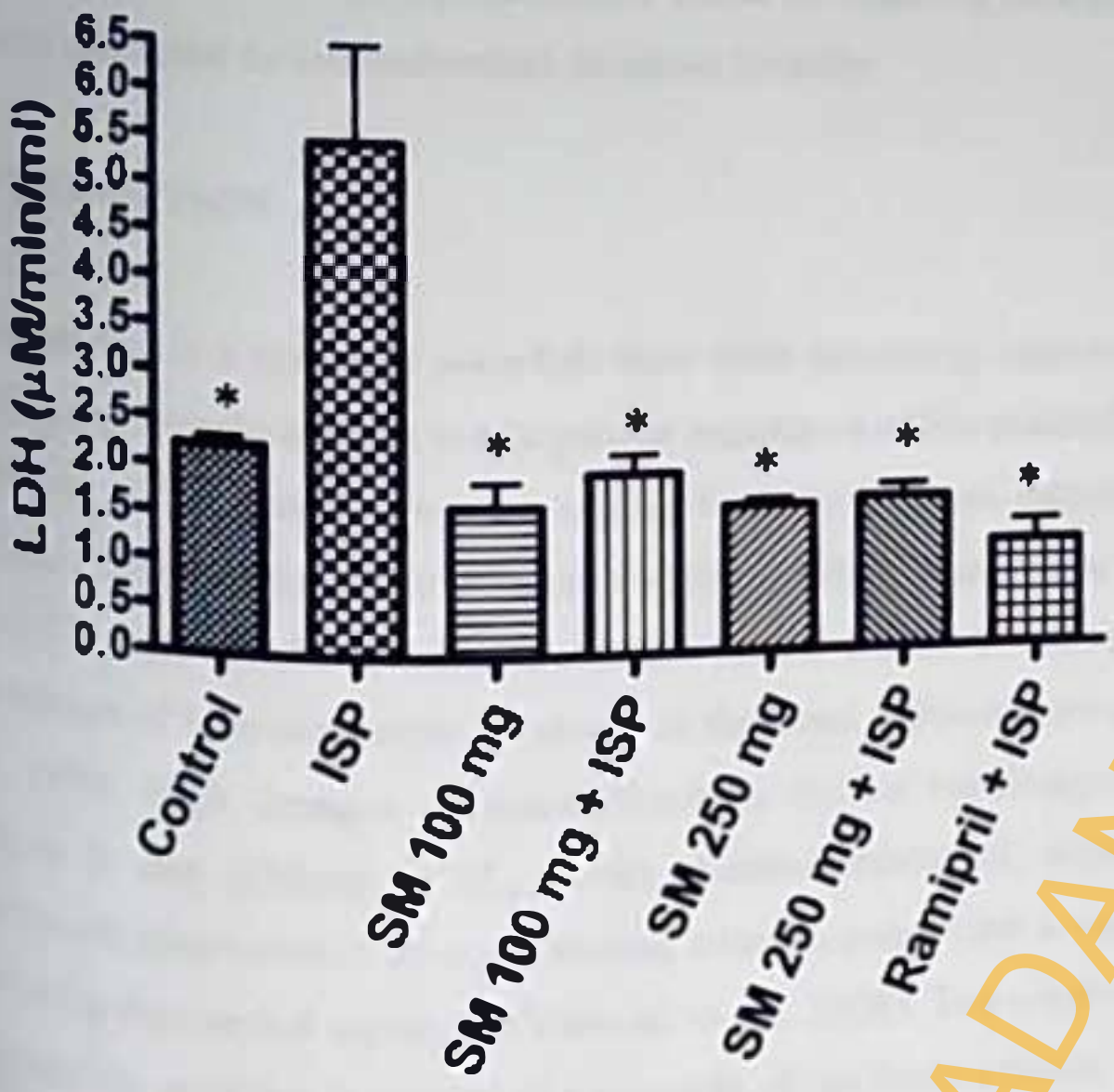


Figure 30: Serum LDH levels. Results are presented as mean ± SEM (n = 6). * Significantly different from ISP treated group ($p < 0.001$)

4.3.2. EXPERIMENT 6: Ameliorative effect of MES on histopathological alterations in rat hearts subjected to Isoproterenol-induced toxicity

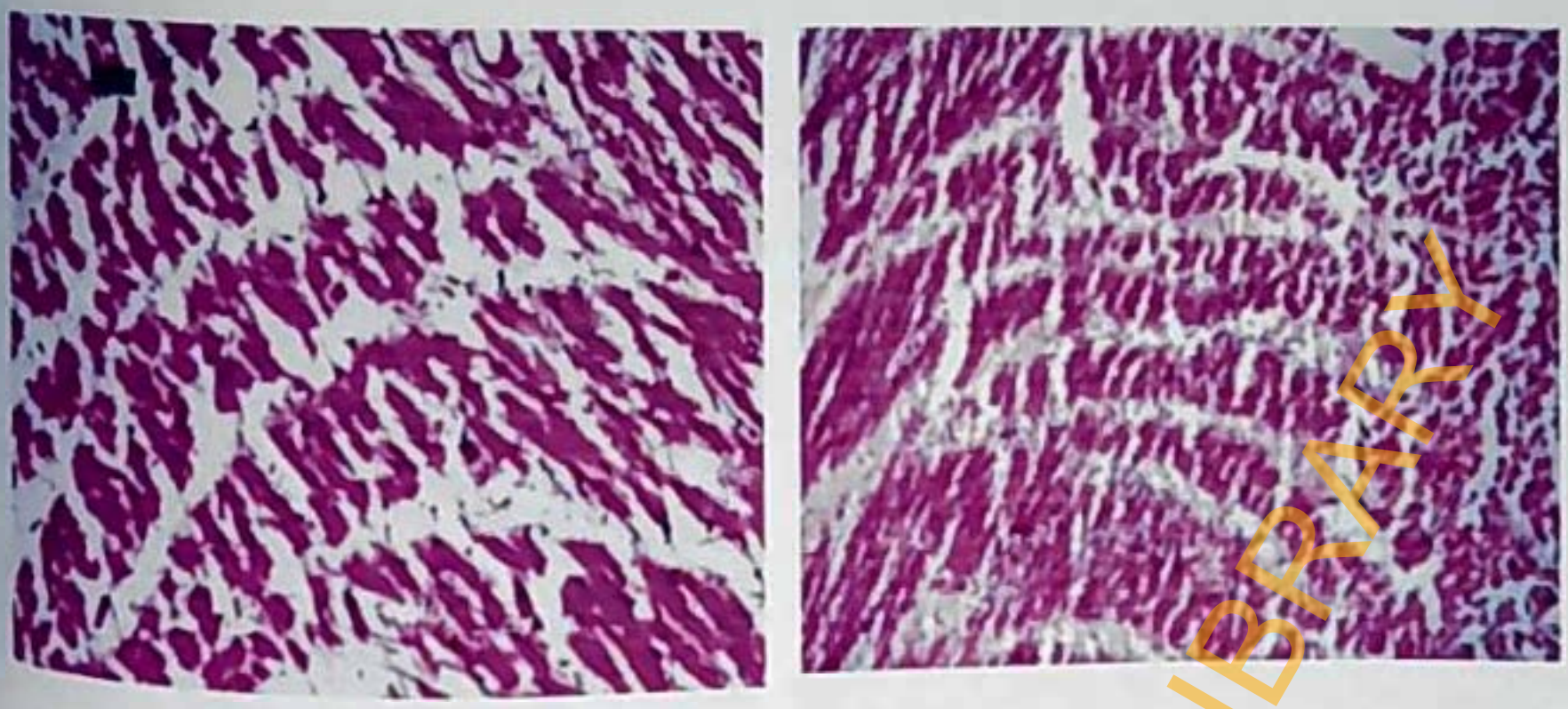
INTRODUCTION

Isoproterenol is a synthetic catecholamine with increasing attention owing to its application in cardiology. Catecholamines are important regulators of myocardial contractility and metabolism. However, it has been known for a long time that excess catecholamines are responsible for cellular damage, observed in clinical conditions such as transient myocardial ischemia, angina, acute Coronary insufficiency, and subendocardial infarct (Velavan *et al.*, 2008). The excessive stimulation of beta-adrenergic receptors in the heart induces myocardial hypertrophy (Busatto *et al.*, 1999). High dosages of catecholamines induce cardiomyocyte necrosis and interstitial fibrosis in rats (Grimm *et al.*, 1998). Administration of large amount of catecholamines, particularly isoproterenol to experimental animals constitutes a rapid and reproducible means of provoking myocardial ischemia (Velavan *et al.*, 2008). Isoproterenol causes severe stress in the myocardium resulting in infarct like necrosis of the heart muscle. It also increases the levels of serum and myocardial lipids, which in turn leads to coronary heart disease (Nair and Devi, 2006; Zhou *et al.*, 2008).

PROCEDURE

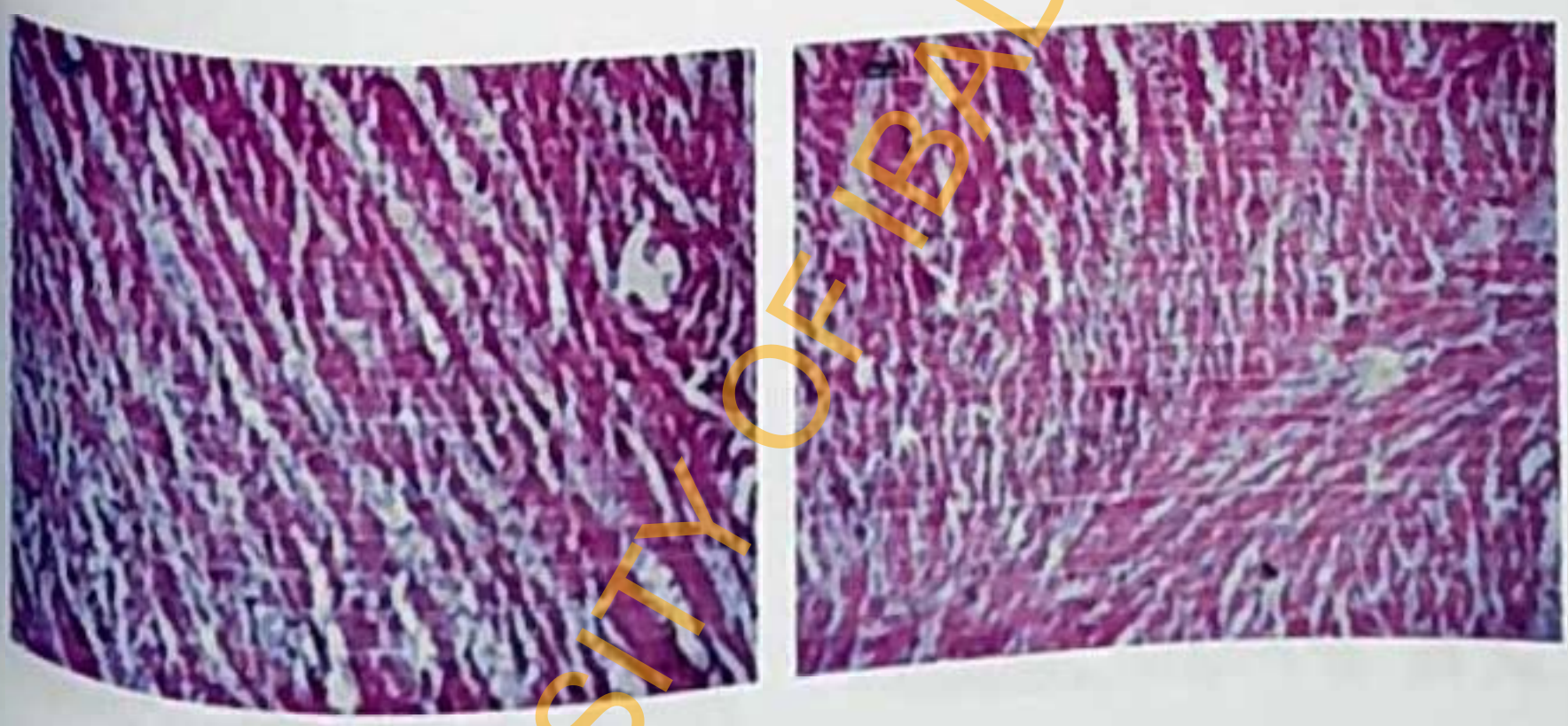
Animals were grouped as described in section 3.13.2. The dose of *Spondias mombin* used for the investigation was 250 mg/kg body weight. Myocardial tissue was perfused with saline and then 4 % paraformaldehyde. It was then fixed in 4 % paraformaldehyde for 24 h. The tissues were routinely processed and embedded in paraffin. Serial sections were cut and each section was stained with hematoxylin and eosin. The stained sections were examined under a microscope and photomicrographs were taken.

There was a massive disruption and fragmentation of heart myofibrils in ISP administered animals (Figure 31) but administration of SM (250 mg/kg) remarkably mitigated the ISP-induced damage (Figure 32). The myofibrillar structure of the heart in the group treated with 250 mg/kg extract alone seemed to be enhanced (Figure 33).



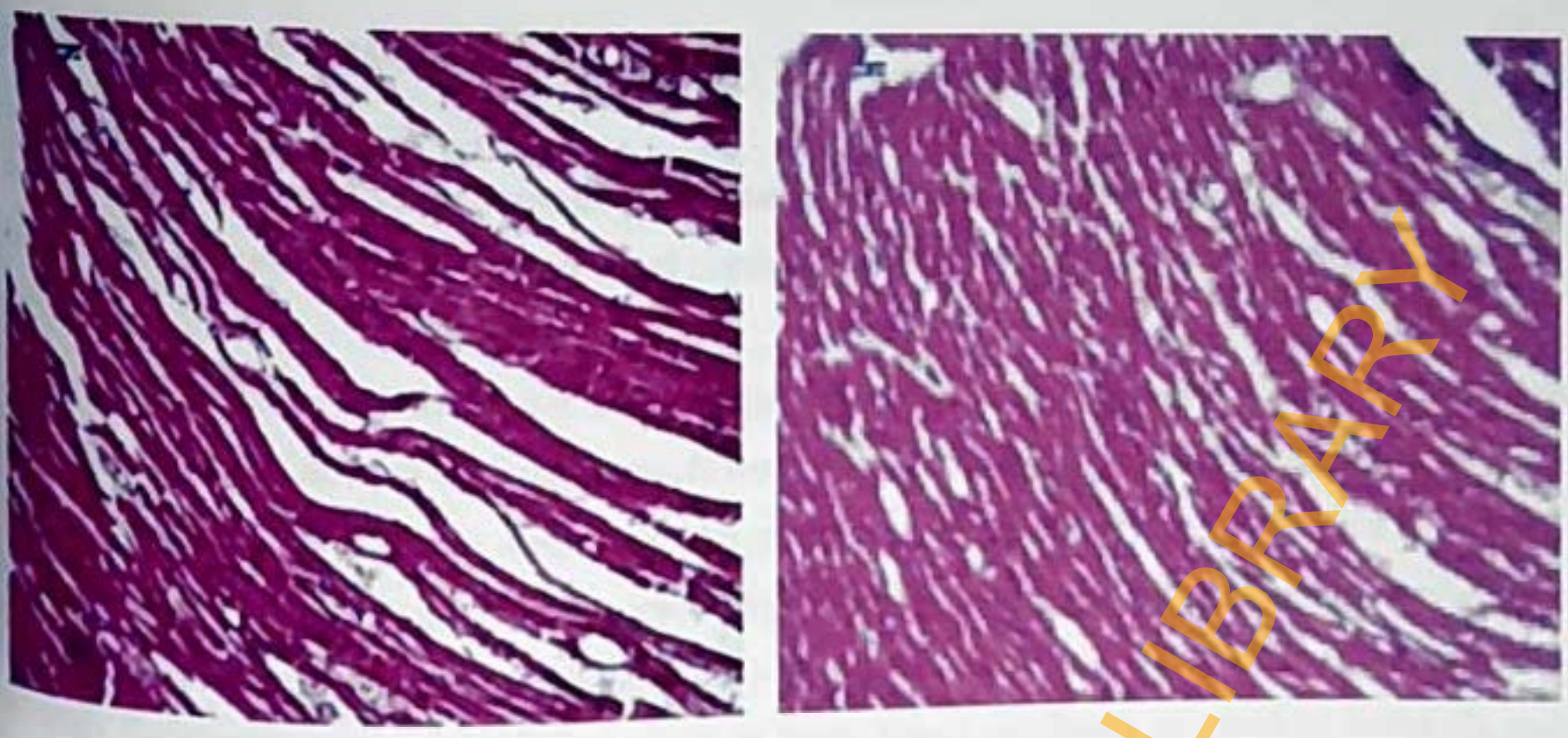
(A) (B)

Figure 31 (A and B): Representative sections from hearts of ISP-administered group (Mag. x10).



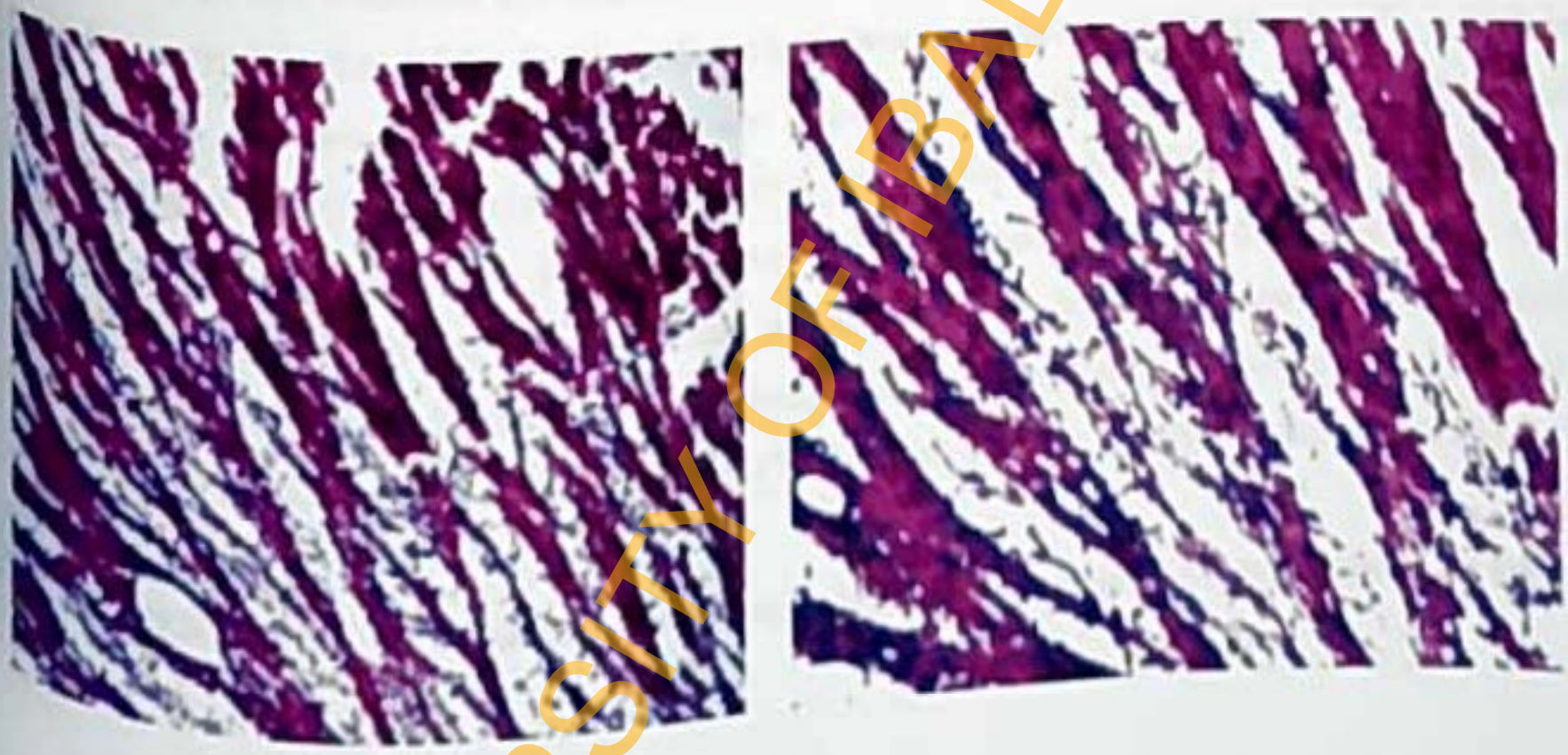
(A) (B)

Figure 32 (A and B): Representative sections of hearts of SM 250 + ISP group (Mag. x10).



(A) (B)

Figure 33 (A and B): Representative sections of hearts of SM 250 treated group (Mag. x10).



(A) (B)

Figure 34 (A and B): Representative sections from hearts of vehicle treated (control) group

4.4. Evaluation of the Neuroprotective property of *Spondias mombin* extract in rats using the middle cerebral artery induced focal cerebral ischemia model

Ischemic brain damage is the major cause of permanent disability in young adults. It has been suggested that up to 80% of all strokes result from ischemic damage in the middle cerebral artery area. The potential for clinical application of pharmacological agents has generated enormous interest in identifying the underlying intracellular signaling pathways and to develop therapeutic strategies that can benefit ischemic stroke injury in patients (Tsai *et al.*, 2007). Growing evidence supports the participation of oxidative stress in brain injury mediated by cerebral ischemia and stroke (Landemmer and Harrison, 2001). Reactive oxygen species (ROS) and lipid peroxidation (LPO) have been proposed to be important factors in reduction of cerebral blood flow and ischemia reperfusion injury. Oxidative stress has been regarded as a substantial underlying cause of brain damage and neuronal dysfunction after cerebral I/R (Chan 2001). The high demand for molecular oxygen, the high levels of polyunsaturated fatty acids in neural membrane phospholipids, and the high iron content are important factors rendering cells in the central nervous system (CNS) susceptible to oxidative stress (Sun *et al.*, 2008). The polyphenolics including flavonoids, which are found in many herbal extracts have been shown to be strong ROS scavengers, antioxidants and protectors of neurons from lethal damage *in vitro*. Phenolic constituents from medicinal plants have also been evaluated *in vivo* as neuroprotective agents in animal models of I/R induced oxidative stress (Yodium *et al.*, 2002; Takizawa *et al.*, 2003).

4.4.1. EXPERIMENT 7A: Assessment of neurological deficit and quantification of infarct size

INTRODUCTION

Occlusion of the middle cerebral artery at its origin interrupts blood flow to the vascular territory of this artery. This results in neurological deficit and brain infarction with severity depending on the duration of the ischemia and the period of reperfusion.

PROCEDURE

Focal cerebral ischemia was induced in male SD rats by occlusion of the middle cerebral artery (MCA) using a modification of the intraluminal, technique of Longa *et al.* (1989) as described in Section 3.14.1. In sham-operated animals, all the procedure except for the insertion of the nylon filament was carried out. Animals in the vehicle group received 0.2 % CMC while the treated groups were administered 100 mg/kg *Spondias mombin* extract suspended in 0.2 % CMC. On recovery from anaesthesia, rats were examined for neurological deficit on a ten – point scale as shown in Table 3.

For the quantification of the infarct size, rats were anaesthetized with ether and the brains were taken out. Each brain was cut into seven 2mm thick slices and incubated with 1% TTC (dissolved in 0.1M phosphate buffered saline) at 37°C for 30 min. The slices were scanned and analyzed by using computerized image analysis system (Biovis Image Plus). The infarct area of all brain slices of each rat slice was multiplied by slice thickness to give the infarct volume.

RESULTS

Neurological deficit

The neurological deficit caused by 1 h of cerebral ischemia followed by 24 h of reperfusion was significantly reduced (43 %) by pretreatment with 100 mg/kg SM extract prior to MCAO (Figure 35).

Infarct size and volume

Pretreatment with SM extract remarkably reduced cerebral infarct size and infarct volume (Figures 36, 37 and 41) of rats subjected to MCAO for 1 h followed by 24 h of reperfusion by 56%.

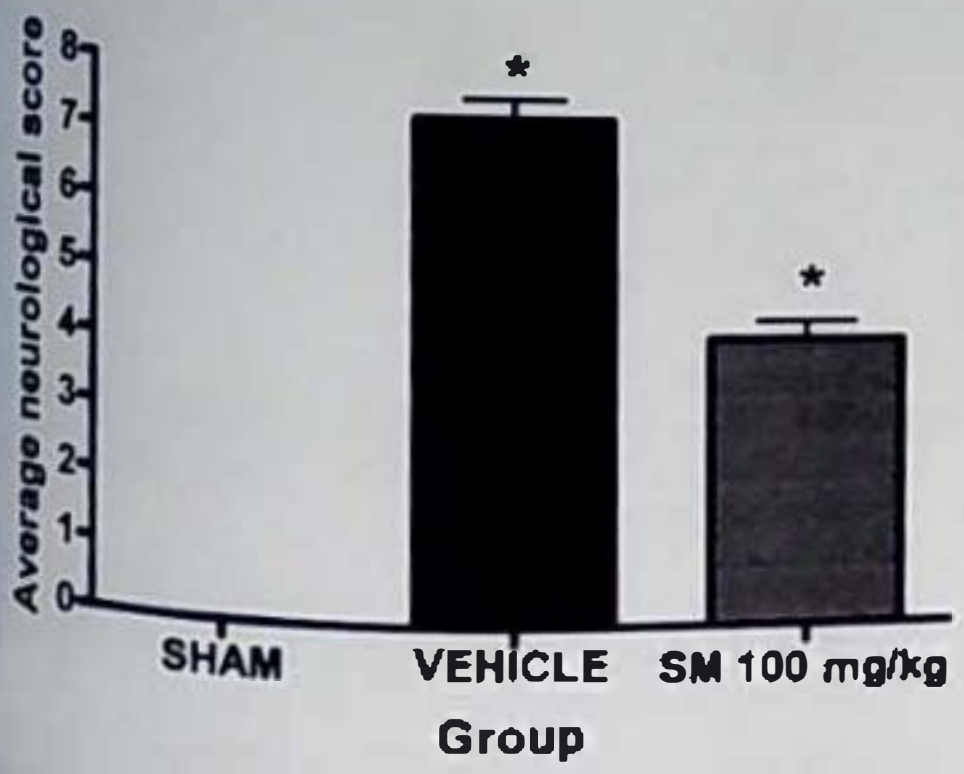


Figure 35: Neurological scores for all groups. Results are presented as mean ± SEM (n = 7). *Significantly different ($p < 0.001$)

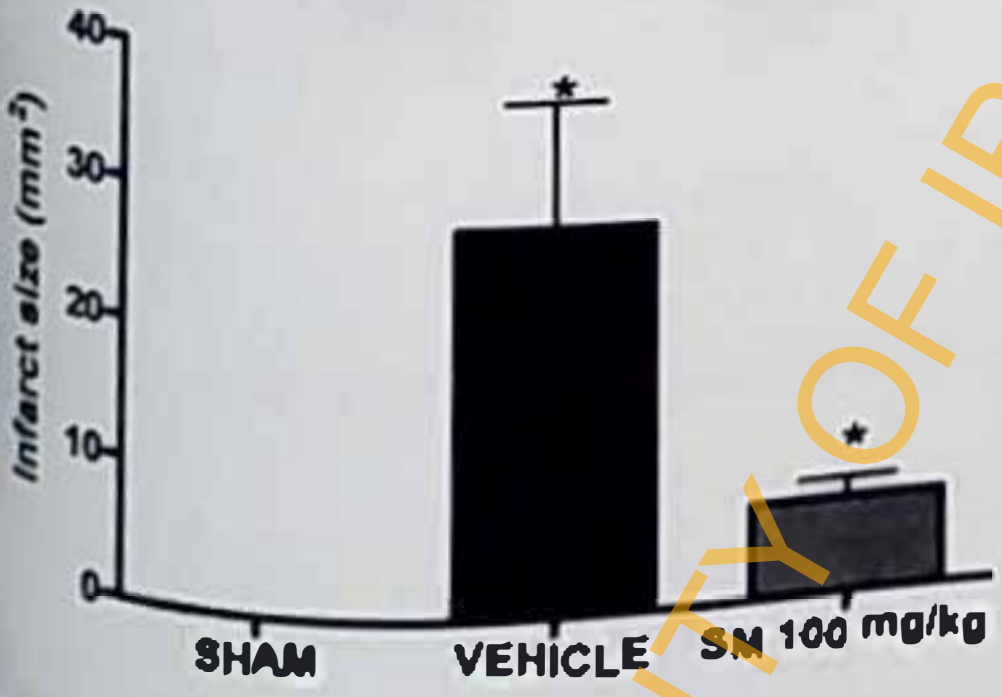


Figure 36: Infarct size in all groups. Results are presented as mean ± SEM (n = 5). *Significantly different ($p < 0.05$).

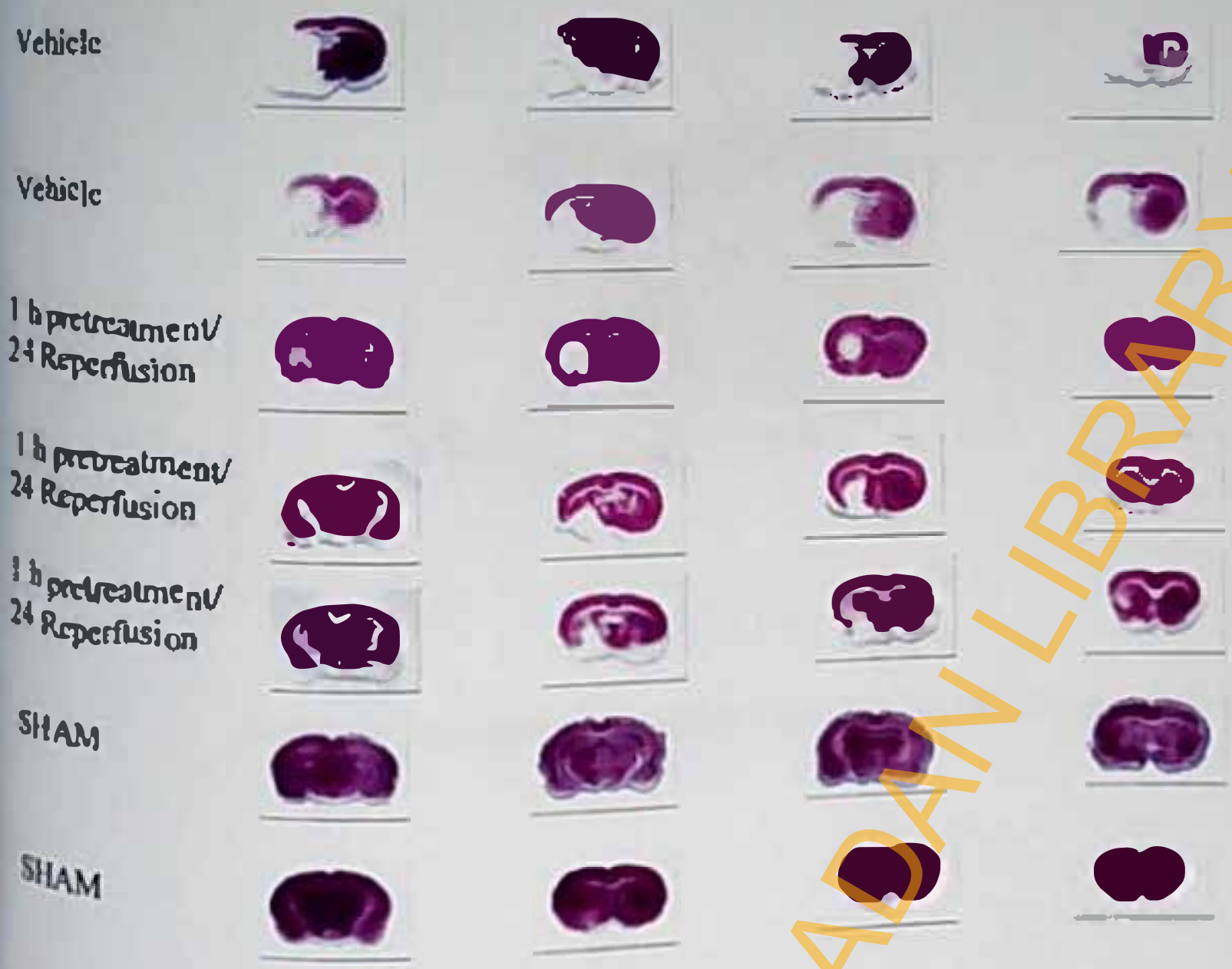


Figure 37: Representative sections of brains from all groups

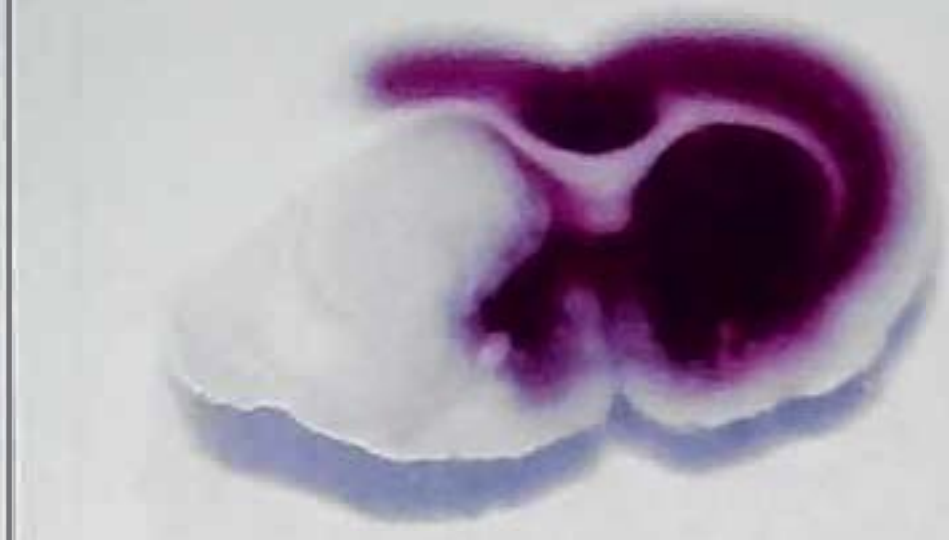


Figure 38: A section of rat brain subjected to 1h MCAO and /24 h reperfusion

Figure 39: A section of SM 100 mg/kg pre-treated subjected to 1 hMCAO and 24 h reperfusion

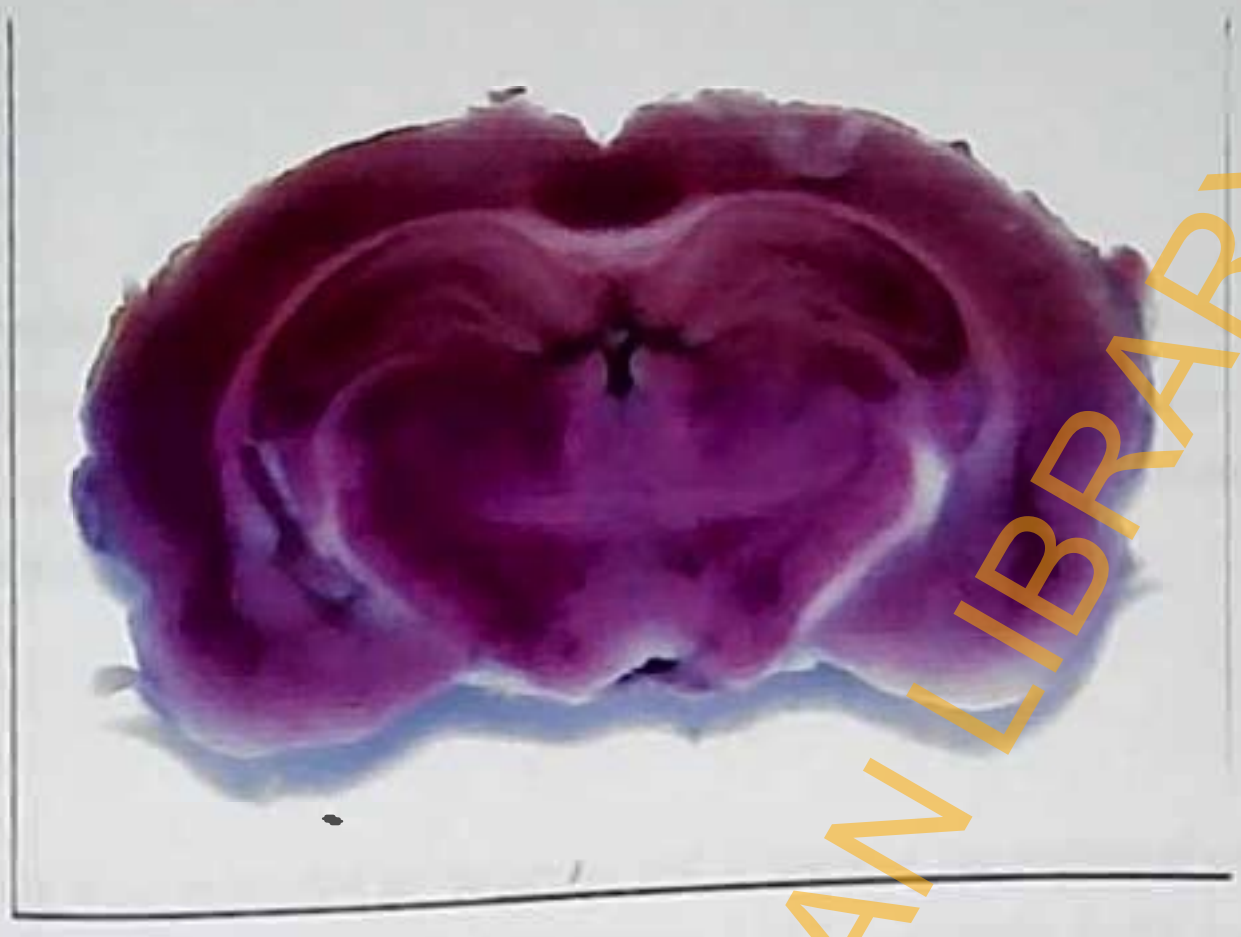


Figure 40: A section of rat brain from the sham treated group

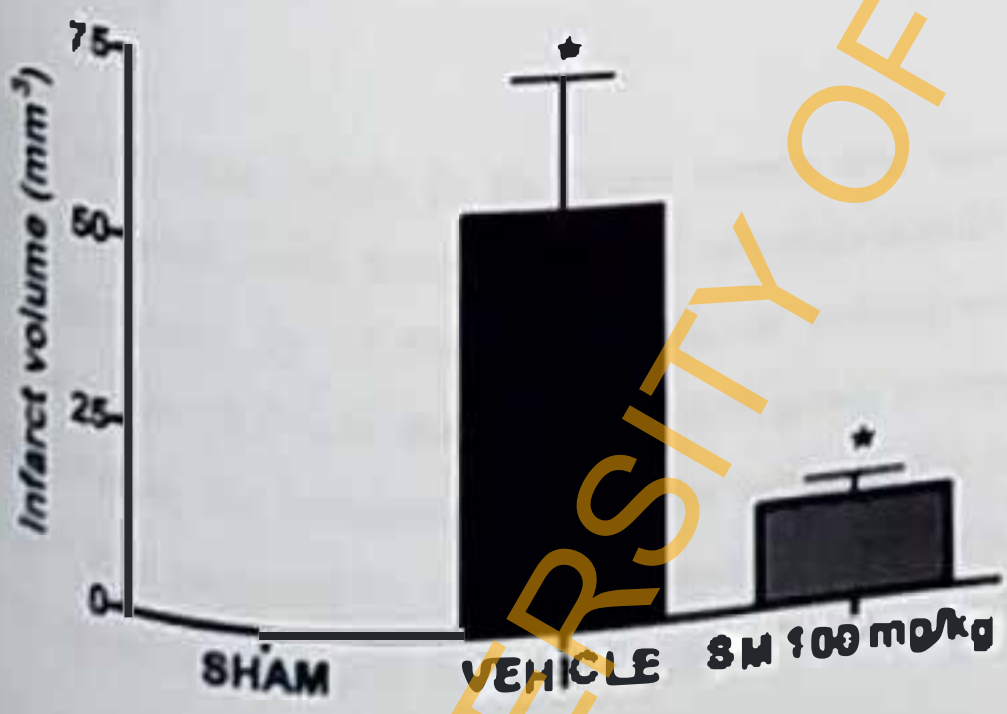


Figure 41: Infarct volume of all groups. Results are presented as mean ± SEM (n = 5). significantly different ($p < 0.05$). Newological deficit, infarct size and volume were significantly reduced by the administration of 100 mg/kg SM 1 h prior to MCAO.

4.4.2. EXPERIMENT 7B: Evaluation of GSH and MDA levels in cortical and striatal regions

INTRODUCTION

Oxidative stress is implicated in the pathogenesis of ischemic brain injury. Levels of endogenous antioxidants and activities of antioxidant enzymes have been found to decrease in ischemic reperfusion brain injury. In particular, GSH and MDA levels are routinely used as markers of cerebral damage and for the evaluation of potential neuroprotectants.

PROCEDURE

GSH and MDA levels were estimated in the cortex and striatum of the ipsilateral portion of the brains of experimental animals as described in section 3.13.2.1. Animals in the vehicle group received 0.2 % CMC while the treated groups were administered 100 mg/kg *Spondias mombin* extract suspended in 0.2 % CMC. Sham-operated animals underwent all the procedure except the insertion of the nylon filament.

RESULTS

GSH and MDA levels in the brain cortex and striatum of the animals were decreased and elevated respectively following 1 h of ischemia and 24 h of reperfusion. In the cortex, GSH level was decreased by 37.5% in the vehicle administered group compared to sham group but increased by 28.6% in the extract-treated group compared to the vehicle-administered group. In the striatum, the corresponding figures were 47.6% and 31.3% respectively. MDA level in the cortex was increased by 34.8% as a result of MCAO but decreased by 26.1% by the extract. The corresponding figures in the striatum were 75% and 25% respectively.

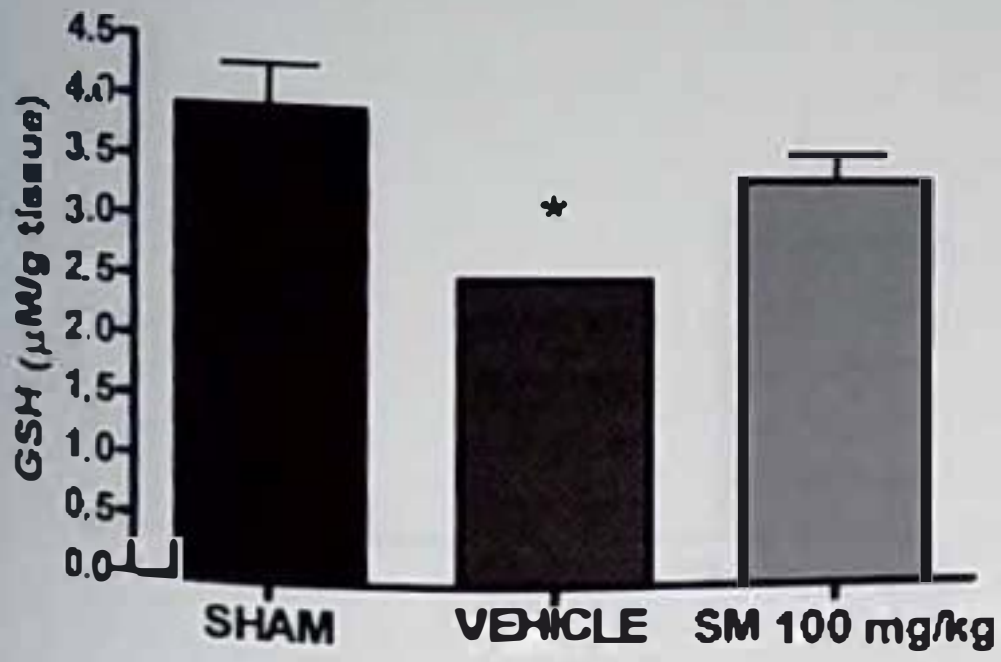


Figure 42: GSH level in the brain cortex of all groups. Results are presented as mean ± SEM (n = 4). *Significantly different from Sham and treated groups

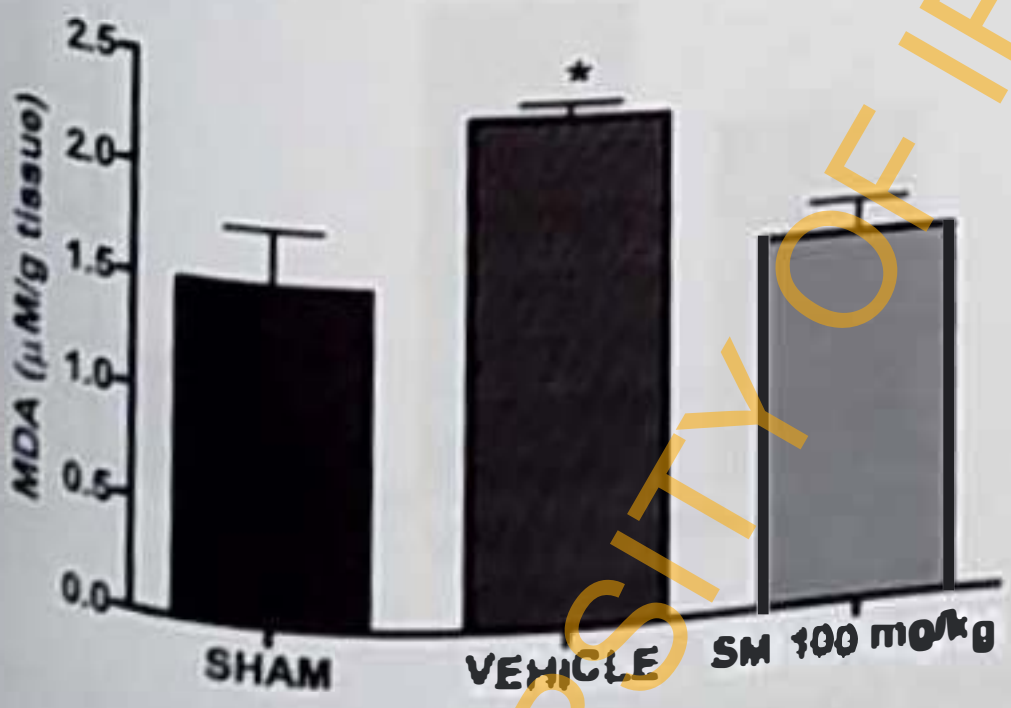


Figure 43: MDA level in the brain cortex of all groups. Results are presented as mean ± SEM (n = 4). *Significantly different from Sham and treated groups

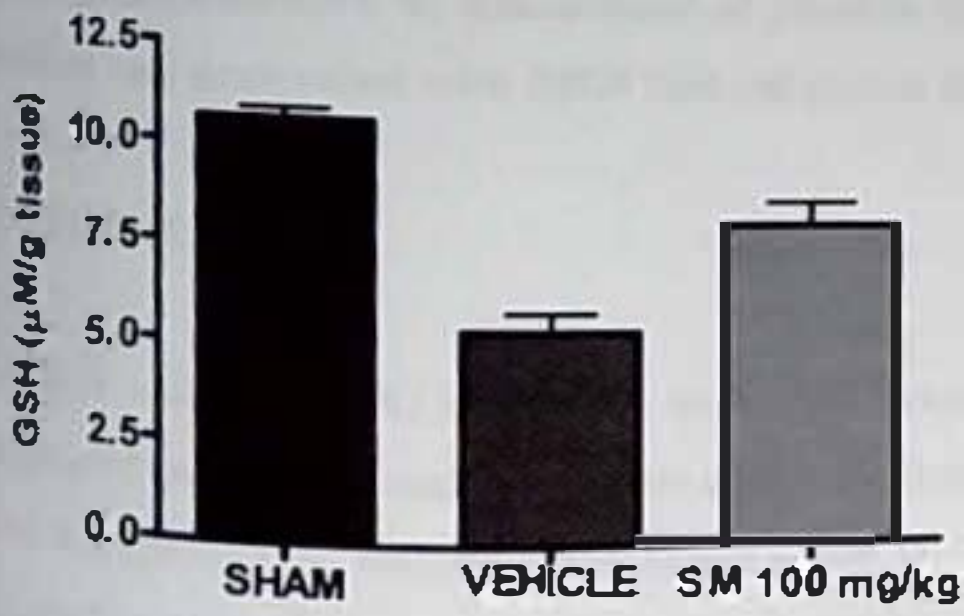


Figure 44: GSH level in the brain striatum of all groups. Results are presented as mean ± SEM (n = 4). All groups are significantly different (p <0.05).

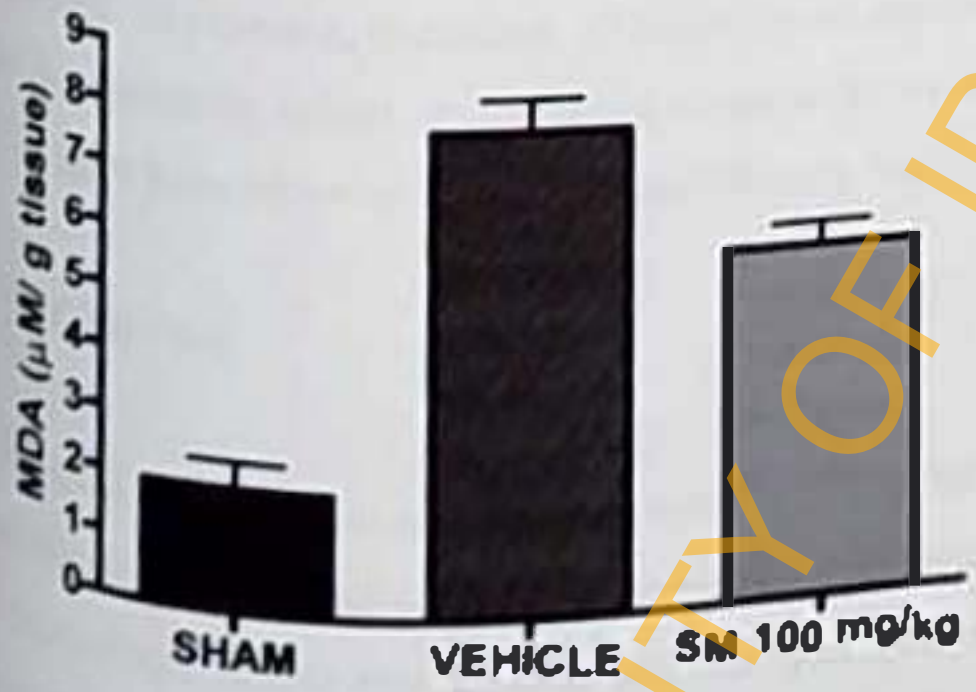


Figure 45: MDA level in the brain striatum of all groups. Results are presented as mean ± SEM (n = 4). All groups are significantly different (p <0.05).

4.4.3. EXPERIMENT 8: Assessment of protein expression of gp91, p22, eNOS, nNOS and SOD in rats pretreated with MES and subjected to MCAO

INTRODUCTION

NADPH oxidase (NOX) is a major source of reactive oxygen species (ROS) which has been implicated in ischemic and other types of brain injury. NADPH oxidase is a pro-oxidant enzyme that is expressed in various brain regions and its level is regulated by ischemia. NOX is a multisubunit complex whose functional subunits include $gp91^{phox}$ and $p22^{phox}$ (Chen, 2009; Lo *et al*., 2007). It has been reported that $gp91^{phox}$ KO mice showed significantly improved neurological scores compared to controls (Lo *et al*., 2007). Overexpression of the $p22^{phox}$ subunit in cerebral ischemia is indicative of neuroprotection. NO, produced by NOS, is an important messenger molecule involved in many physiological and pathological processes (Mayer and Hasmens, 1998). There are different isoforms of NOS. During ischemia reperfusion injury, NO (produced mainly by cNOS) mediates effects that would be protective following cerebral ischemia. In contrast, excessive NO (produced initially by nNOS, and later by iNOS) mediates the neurotoxicity effect of cerebral ischemia (Li *et al*., 2010). Transgenic mice overexpressing SOD have been reported to exhibit significantly less brain infarct and edema (Chen, 2009).

PROCEDURE

The sham operated rats and rats subjected to 1 h of MCAO followed by 24 h of reperfusion, were sacrificed by overdose of anesthetic ether. The ipsilateral portion of brain tissue subjected to ischemia/reperfusion was quickly excised and homogenized in ten volumes of ice cold lysate buffer (200 mmol/l HEPES (pH 7.5), 250mmol/l sucrose, 1 mmol/l dithiothreitol,1.5 mmol/Mgcl2,10mmol/l KCl, 1mmol EDTA, 1mmol EGTA, 1mmol Phenyl methyl sulfonyl chloride, 10μg/ml leupeptin, 5μg/ml pepstatin, 2μg/ml aprotinin) using a teflon homogenizer. Homogenates were processed as described in Section 3.15.5. The immunoreactive bands were visualized by enhanced chemiluminiscence (ECL) detection. The band intensity was measured using upon densitometry analysis software of Alphaeaser ™ 2200.

The studies on protein expression showed that expression of gp91 was decreased in the MES treated group compared with the vehicle treated group ($P<0.05$) while the expression of the p22 subunit was enhanced in SM treated animals compared with the vehicle treated group ($p<0.05$). The expressions of eNOS and SOD were also enhanced while that of nNOS was decreased in MES treated animals compared with the vehicletreated group ($p<0.05$) (Figure 46-50).

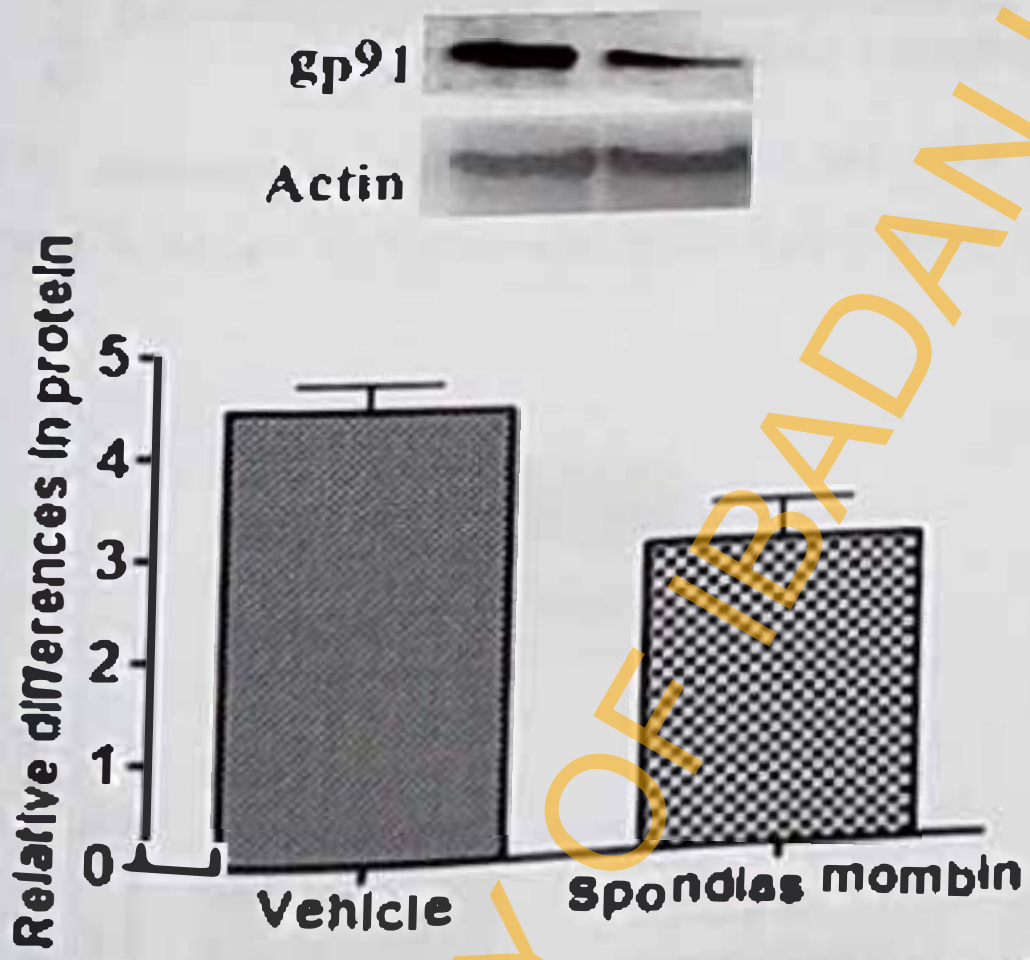


Figure 46: Expression of the gp91 subunit in vehicle administered and MES treated group. Results are presented as mean ± SEM (n = 3). Values are significantly different ($p<0.05$).

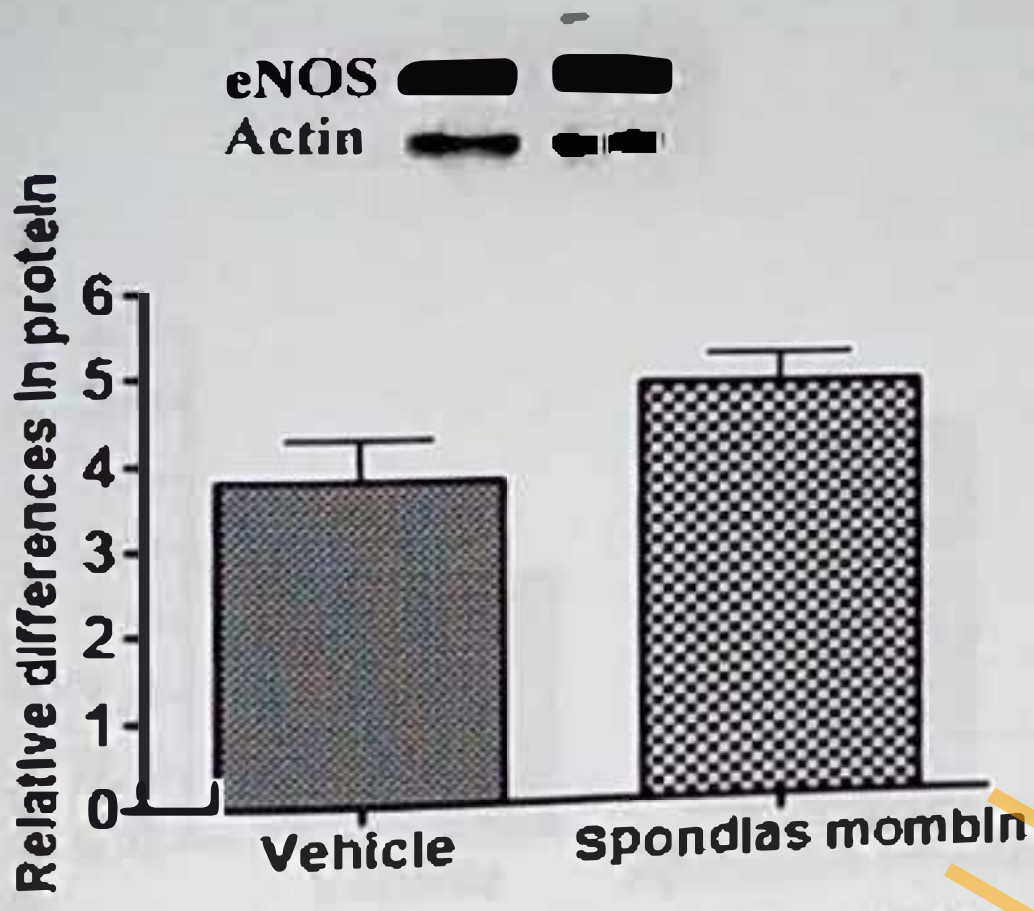


Figure 47: eNOS expression in vehicle administered and MES treated groups. Results are presented as mean ± SEM (n = 3). Values are significantly different ($p<0.05$).

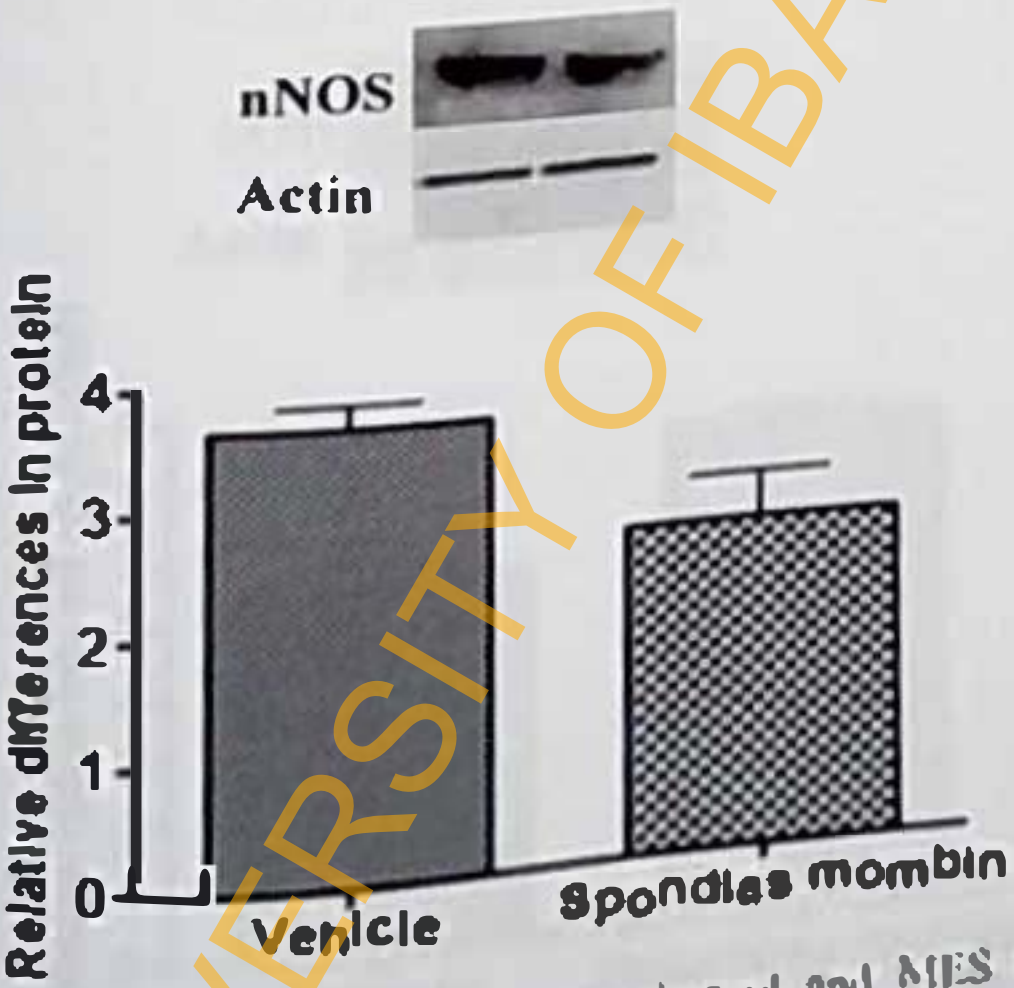


Figure 48: nNOS expression in vehicle administered and MES treated groups. Results are presented as mean ± SEM (n = 3). Values are significantly different ($p<0.05$).

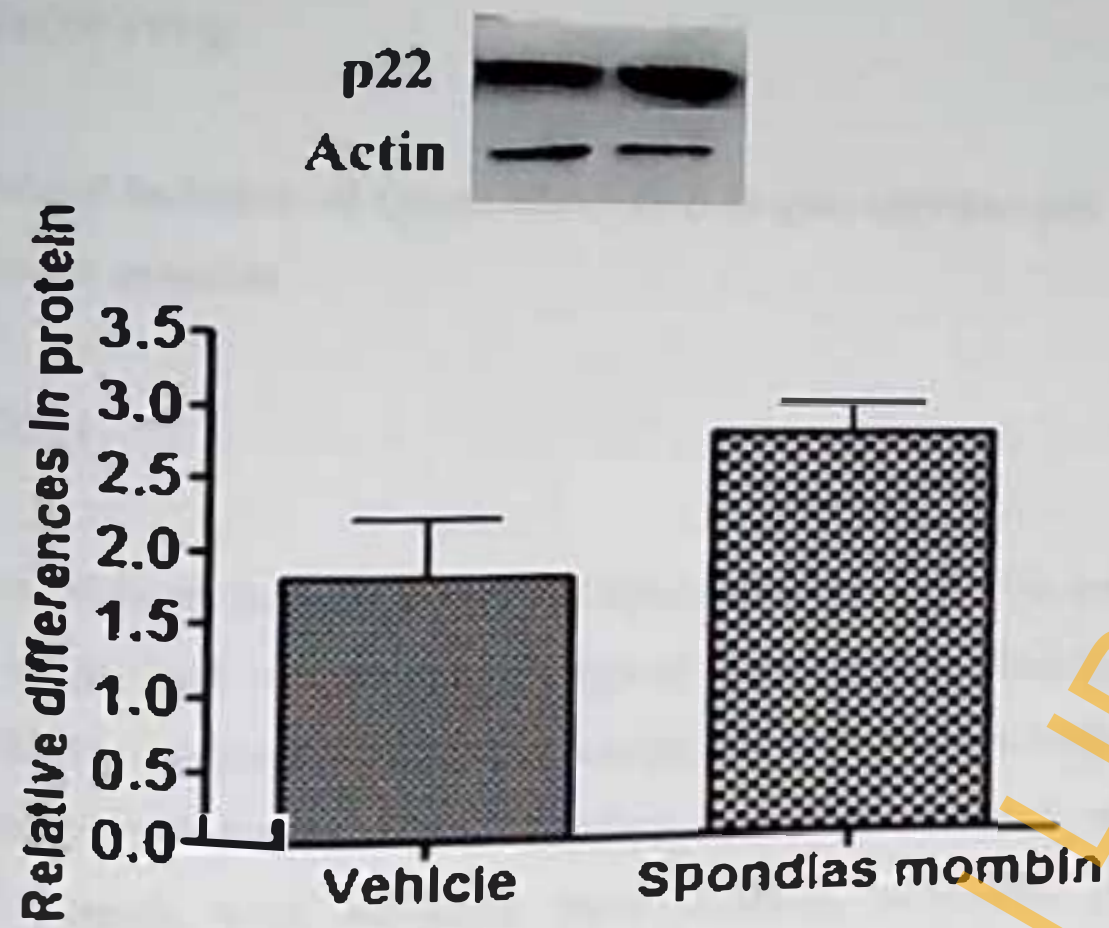


Figure 49: Expression of the p22 subunit in vehicle administered and MES treated groups. Results are presented as mean ± SEM (n = 3). Values are significantly different (p<0.05).

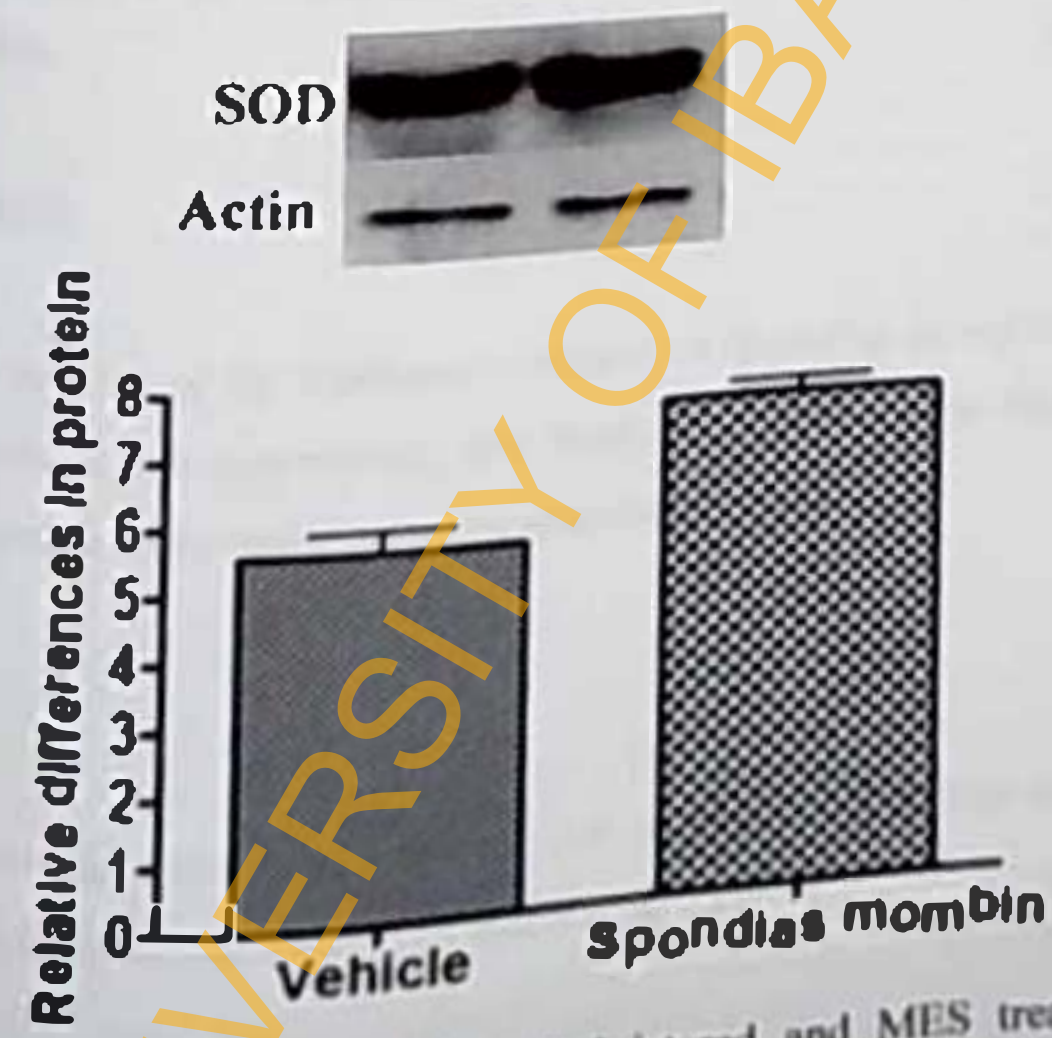


Figure 50: SOD expression in vehicle administered and MES treated groups. Results are presented as mean ± SEM (n = 3). Values are significantly different (p<0.05).

INVESTIGATION FIVE

4.5. Activity guided isolation of Quercetin-3-O-β-D-glucopyranoside and Undec-1-ene from extract of *Spondias mombin*

INTRODUCTION

Bioactive natural products have an enormous economic importance as specialty chemicals. They can be used as drugs, lead compounds, biological or pharmacological tools, feedstock products (raw materials for the production of drugs), excipients and nutraceuticals. When compared with libraries of synthetic substances, natural products offer the prospects of discovering a greater number of compounds, with sterically more complex structures. Bioguided isolation of pharmacologically active plant components is a valuable strategy for finding new lead compounds (Pieters and Vlietinck, 2005)

4.5.1. EXPERIMENT 9: Antioxidant activity of fractions from methanolic extract of *Spondias mombin*

INTRODUCTION

The antioxidant activity of the methanolic extract of *Spondias mombin* has been confirmed from our earlier studies. In this experiment, five fractions obtained from SM have been evaluated for in vitro antioxidant activity using selected antioxidant tests.

PROCEDURE

The various antioxidant tests were carried out as described in Section 3.16. H_2O_2 scavenging activity was evaluated by the method of Ruch *et al.* (1989).

The results of the free radical scavenging activity for *Spondias mombin* and its fractions (Figure 51-56) showed that ethyl acetate and n – butanol fractions have the highest activities in most of the assays while the dichloromethane fraction showed the least activity. The lipid peroxidation inhibitory activity of all the fractions in rat brain was generally high with the dichloromethane fraction showing a particularly remarkable activity.

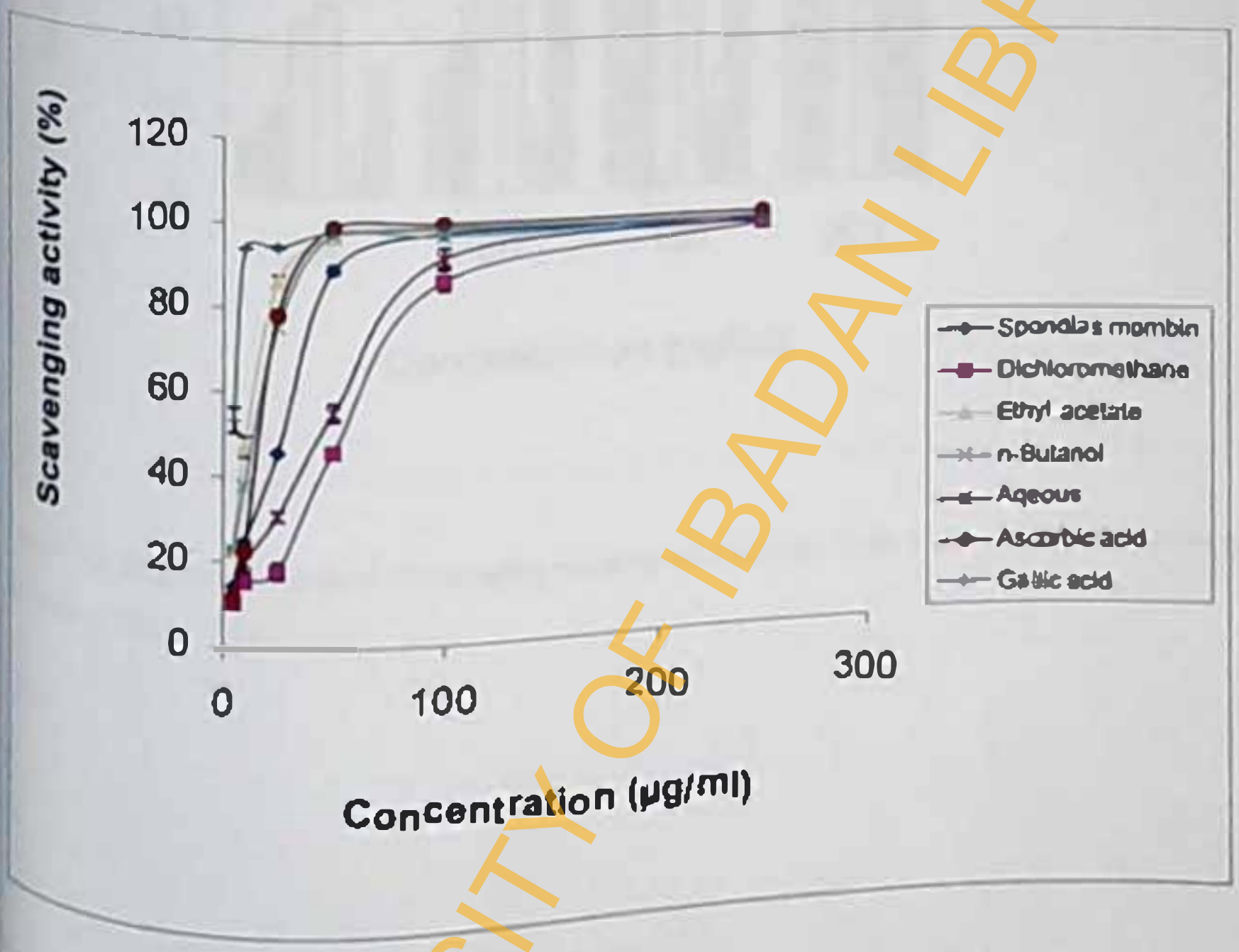


Figure 51: DPPH free radical scavenging capacity of fractions from MES. Results are presented as mean ± SD (n = 3).

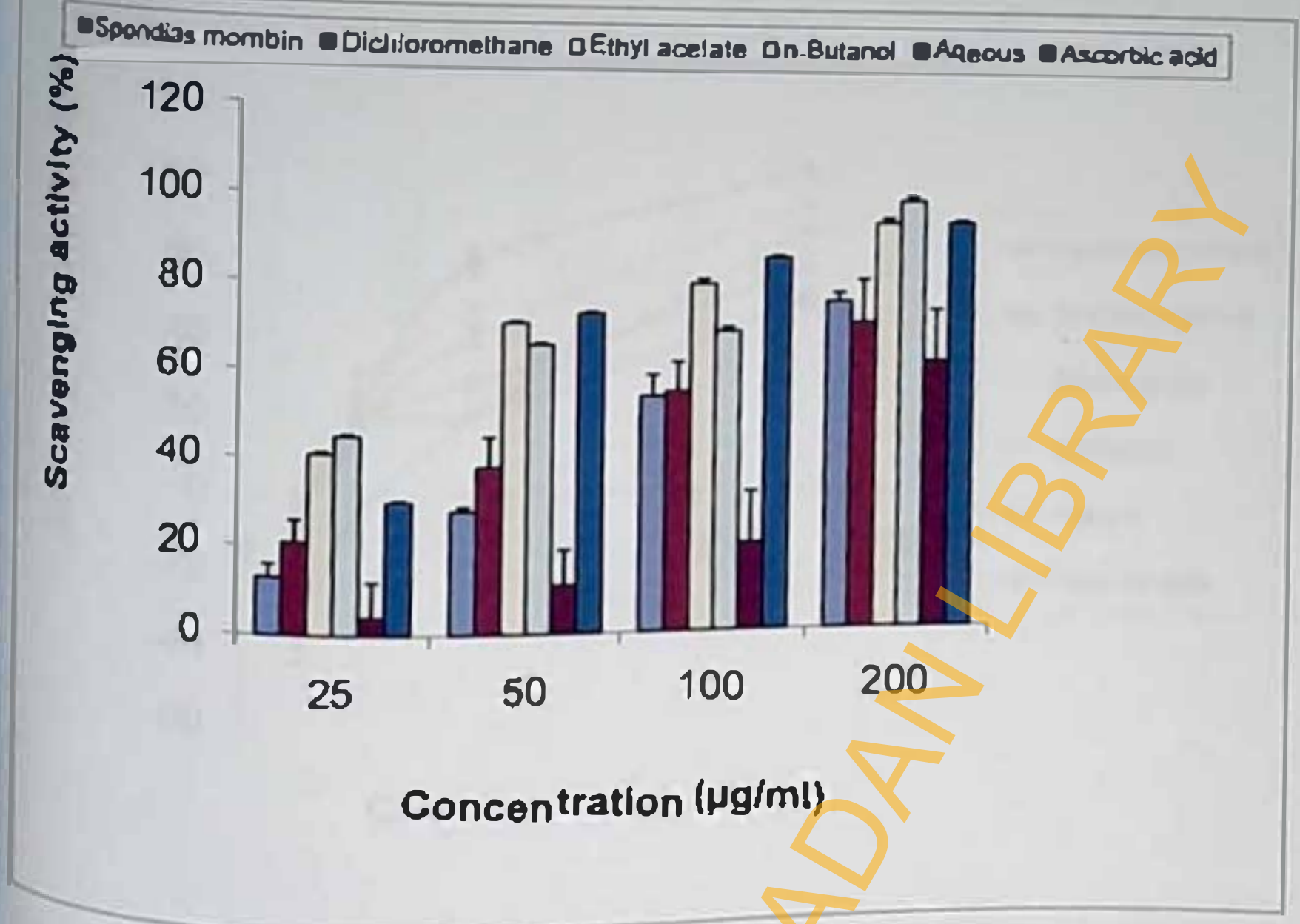


Figure 52: Superoxide radical scavenging capacity of fractions from MES. Results are presented as mean ± SD (n = 3).

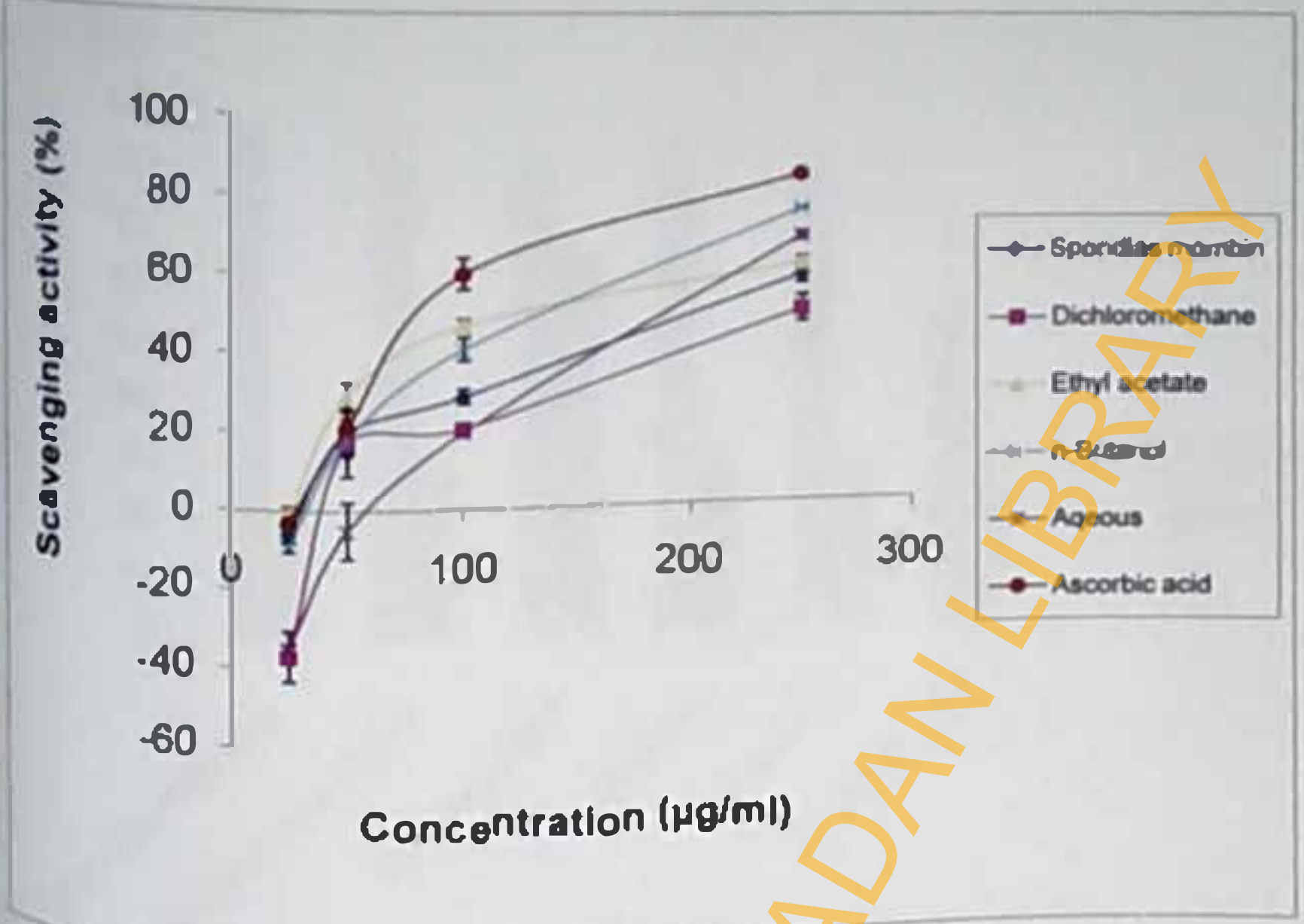


Figure 53: Nitric oxide radical scavenging capacity of fractions from MES. Results are presented as mean ± SD (n = 3).

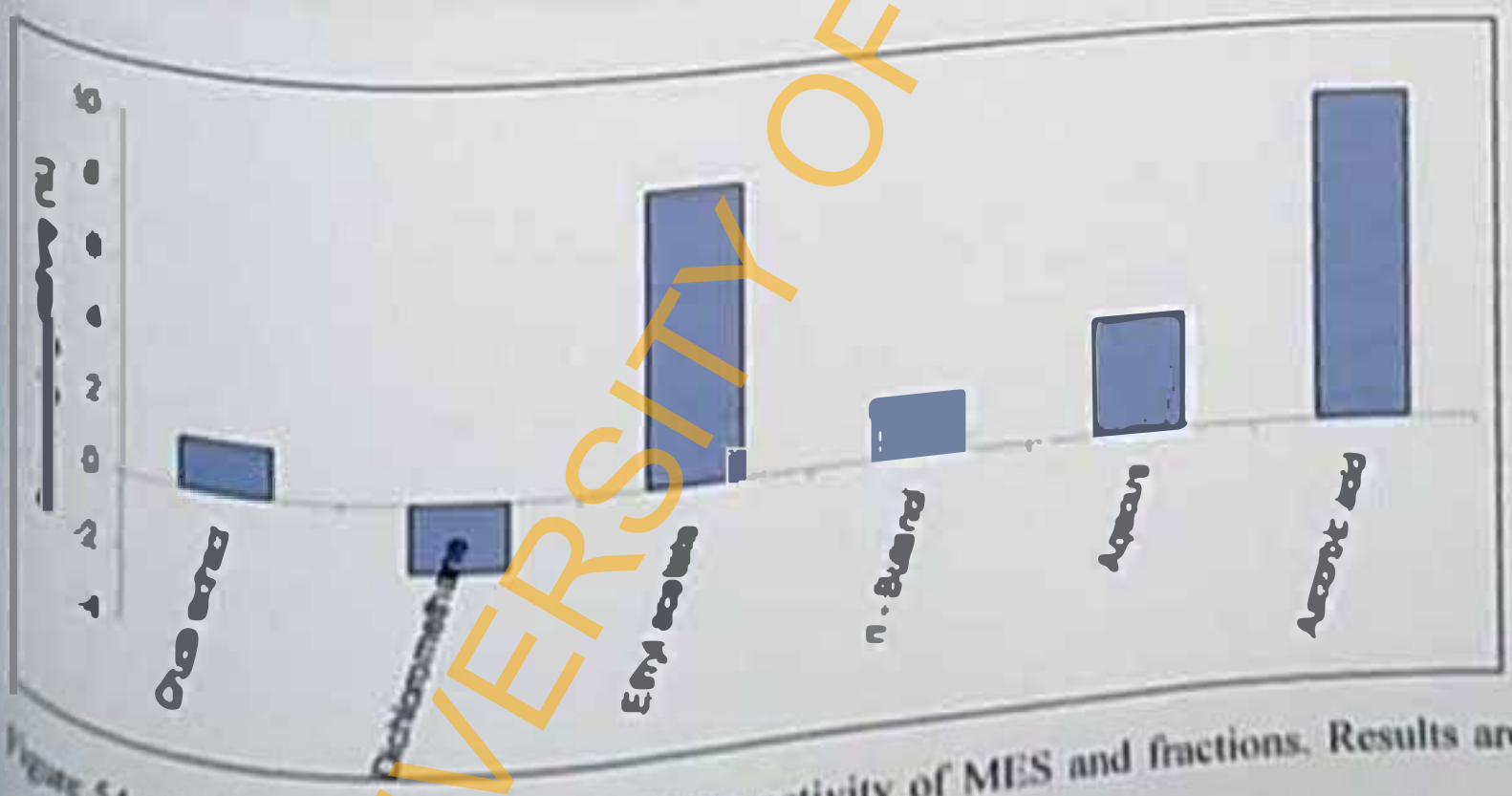


Figure 54: Hydrogen peroxide scavenging activity of MES and fractions. Results are presented as mean ± SD (n = 3).

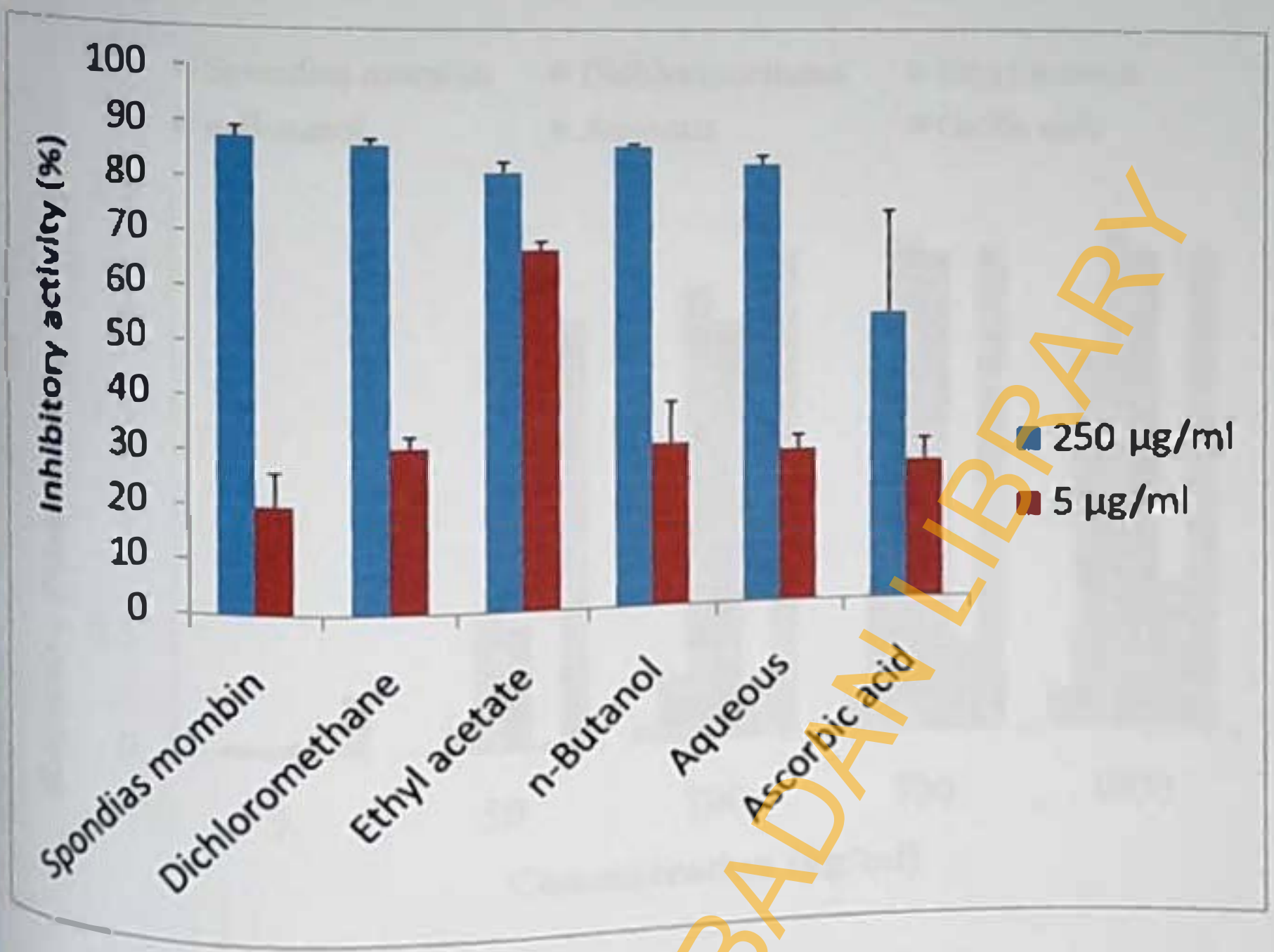


Figure 55: Lipid peroxidation inhibitory activity of MES in rat brain. Results are presented as mean ± SD (n = 3).

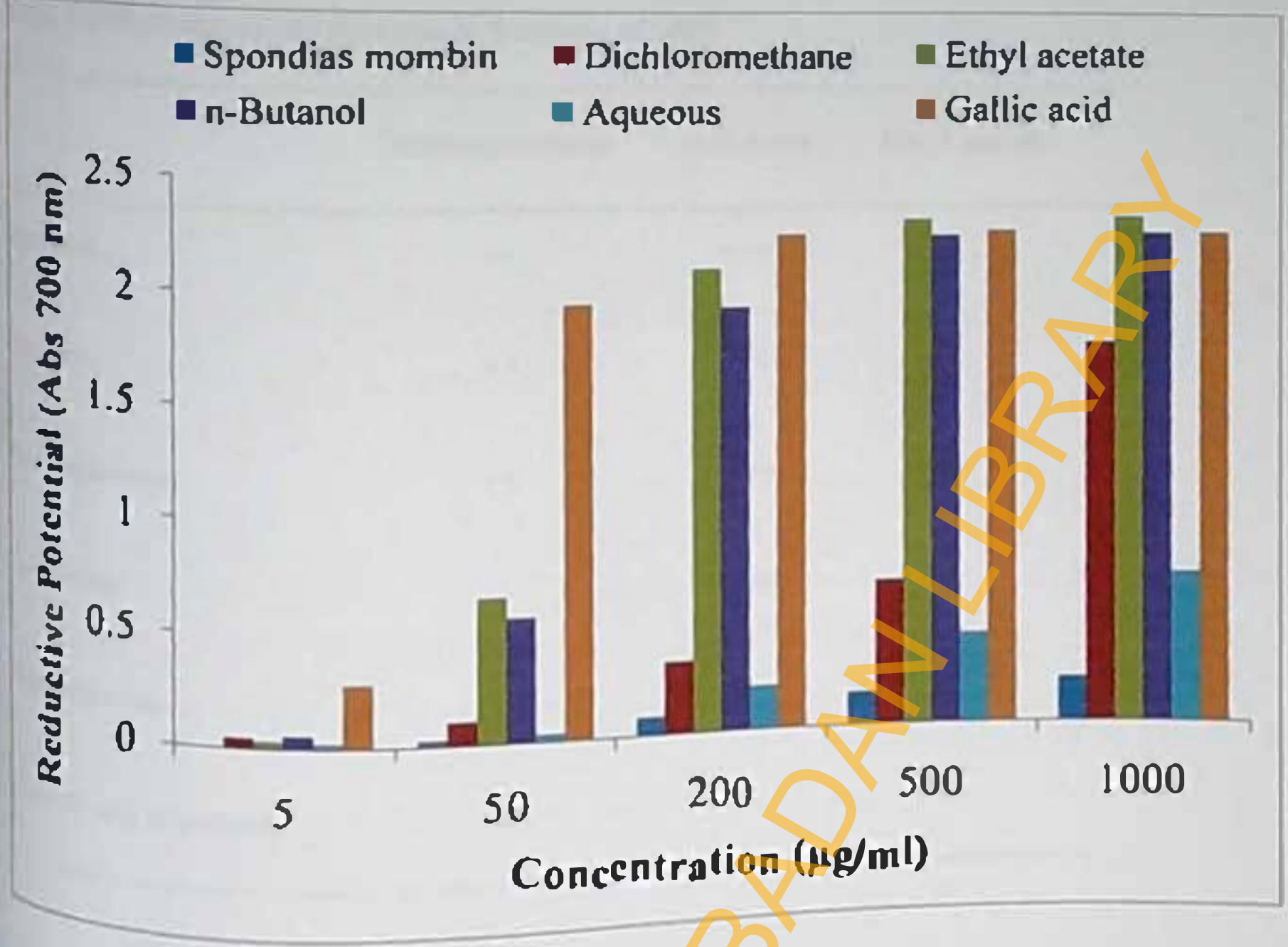


Figure 56: Reductive potential of fractions from MES. Results are presented as mean ± SD (n= 3).

Table 11: Phytochemicals detected in fractions of MES

	Dichloromethane	n-Butanol	Ethyl acetate
Saponins	++	+++	++
Tannins	++	+++	+++
Anthraquinones	++	++++	+++
Flavonoids	–	+++	++
Phlobatannins	–	–	–
Steroids and terpenoids	++	+	+

4.5.2. EXPERIMENT 10: Antiproliferative activity of fractions from methanolic extract of *Spondias mombin*

INTRODUCTION

Fractions of *Spondias mombin* were screened for antiproliferative activity using four cell lines and a standard.

PROCEDURE

A colorimetric sulphorhodamine B (SRB) assay was used for measurement of cell proliferation (Noughton *et al.*, 2007). The cytotoxic effect of the extract and fractions (50 µg/ml) was assessed as the percentage of inhibition of cell growth, where untreated cells were taken as 100 % viable. Four cell lines were used: KB (Oral cancer), C – 33A (Cervical cancer), MCF – 7 (Breast cancer) and A – 549 (Lung cancer). NIH3T3 (Mouse fibroblast) was used as control cell line.

RESULTS

For the antiproliferative activity, only the dichloromethane fraction showed >50% growth inhibition against KB and C-33A cancer cell lines. It also had the highest percentage growth inhibition for the A549 cell line. Generally, the crude extract and the various fractions did not exhibit strong anticancer activities.

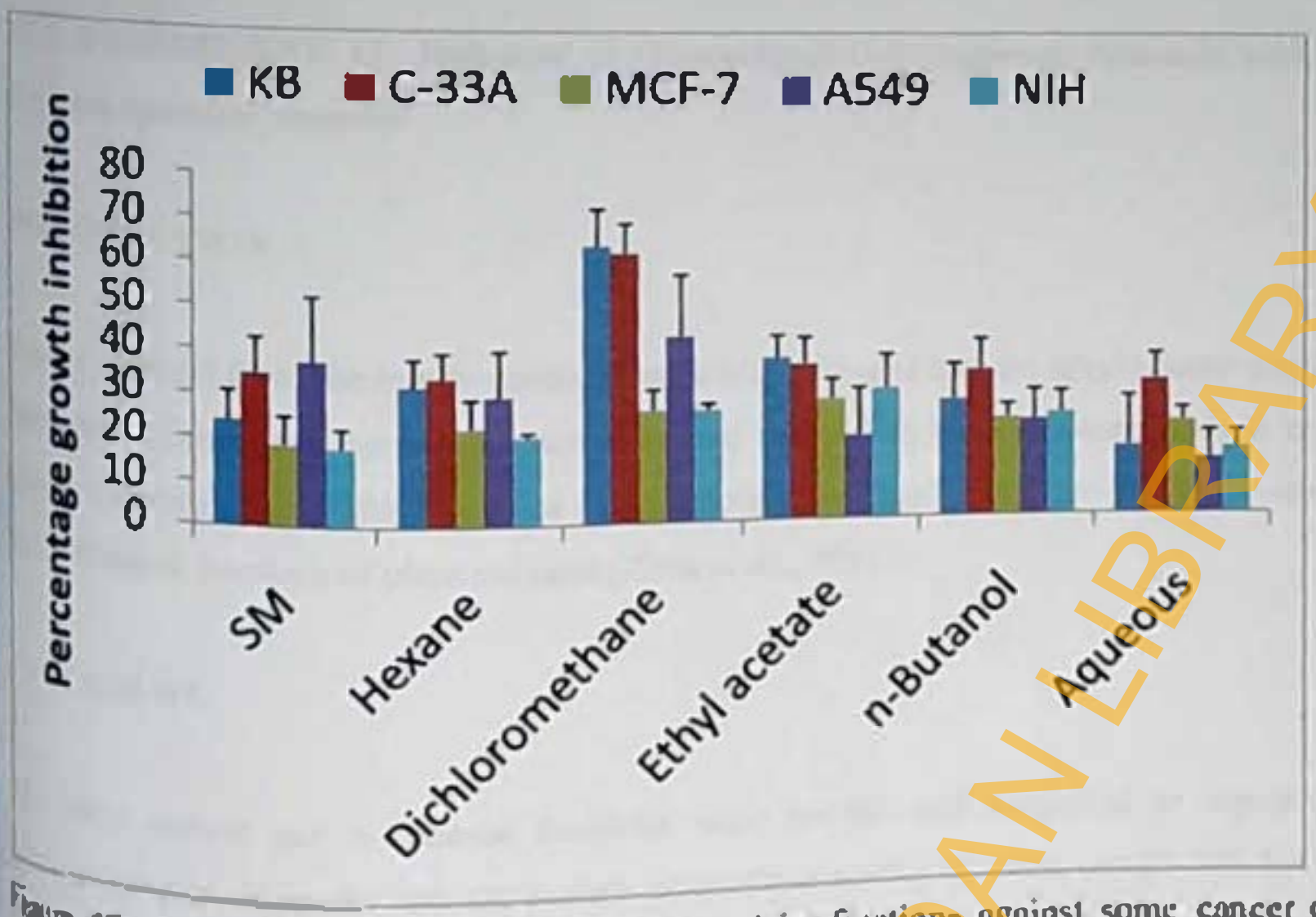


Figure 57: Antiproliferative activity of MES and its fractions against some cancer cell lines. Results are presented as mean ± SD (n = 3).

4.5.3. EXPERIMENT 11: Isolation of Quercetin-3-O-β-D-glucopyranoside and Undec-1-ene from *Spondias mombin*

INTRODUCTION

Results obtained from the in vitro antioxidant studies showed that the ethyl acetate and n-Butanol fractions demonstrated the highest activities and seemed to be equipotent. This is in line with previous investigations which showed that phenolics are usually concentrated in the ethyl acetate and n-Butanol fractions of plant extracts (Zhou *et al*., 2011).

PROCEDURE

The ethyl acetate and n-Butanol fractions were pooled and subjected to repeated column chromatography using the solvent system chloroform:methanol:water (65:25:20). Isolated pure compounds were characterized as described in Section 3.18. The only stationary phase used was silica gel.

RESULTS

Full characterization combining UV, NMR and MS (Tables 12 and 13) revealed the compounds to be a flavonol glycoside, tentatively identified as quercetin-3-O-β-D-glucopyranoside (Figure 57) and a fatty chain, undec-1-ene (Figure 59).

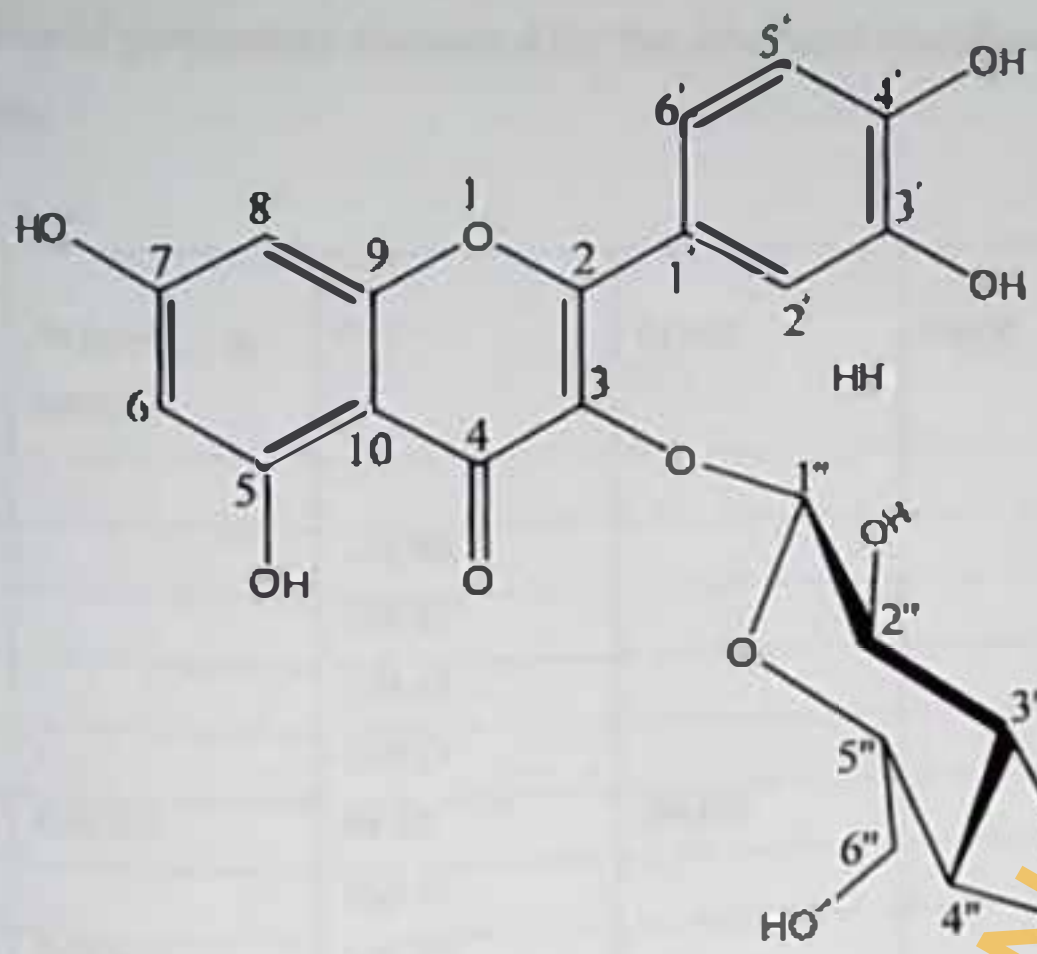


Figure 58: Structure of quercetin-3-O-β-D-glucopyranoside. Glucose moiety is attached to carbon-3, it is established by HMBC correlation. Coupling constant (of anomeric proton) is 7.53 and doublet so it is β glycoside.

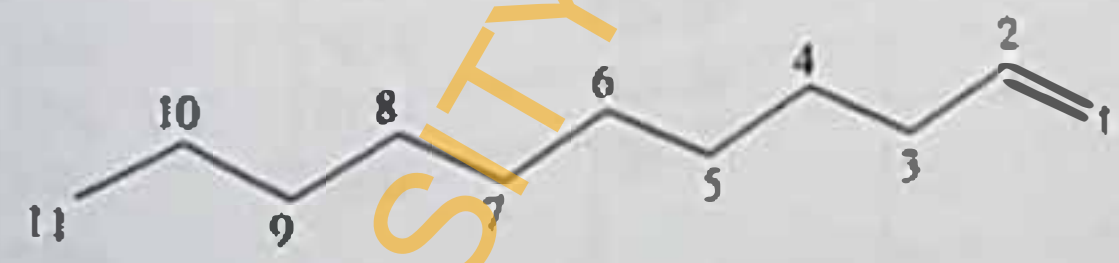


Figure 59: Structure of undec-1-ene

Table 12. Values of parameters evaluated for the structural elucidation of quercetin-3O-β-D-glucopyranoside

S/No	^{1}H (mult., J in hertz)	^{13}C	COSY	HSQC	HMBC
1	-				
2	-	158.90			
3	-	135.88			
4	-	179.65			
5	-	163.11			
6	6.407(s)	94.85	116-118		C8,C10,C9,C7
7	-	166.17			
8	6.204(s)	100.02			C5,C6,C7,C10
9	-	158.55			
10	-	105.74			
1'	-	123.66			
2'	7.849(s)	117.90	112-116		C1',C4',C6'
3'		145.94			
4'		150.08			C1',C3',C4'
5'	6.871(d,8.79)	116.23	115-116		C1',C2',C4'
6'	7.586(d,8.64)	123.05			C3'
1''	5.166(d,7.53)	103.95	111-112		
2''	3.24m	73.31	112-113		
3''	3.38m	73.96	113-114		
4''	3.26m	70.14	114-115		
5''	3.74m	77.30	115-116		
6''	4.10m	62.04	115-116		
	4.35m				

Table 13: Values of parameters evaluated for the structural elucidation of undec-1-ene

S.No.	^{1}H(multiplication, coupling constant)	^{13}C	HMBC
1	5.024 4.950(dd,j=9.38,9.38)	114.29	H1 –C2,C3
2	5.820(m)	139.49	H2- C1,C3,C4
3	2.050(q)	34.60	H3-C1,C2,C4
4	1.382(m)	29.86	H4-C2,C6
5	0.893(m)	29.75	H5-C7,C3
6	0.893(m)	29.60	H6-C8,C3
7	0.893(m)	29.40	H7-C9,C5
8	0.893(m)	29.20	H8-C10,C6
9	0.893(m)	32.17	H9-C11,C7
10	1.592(m)	22.93	H10-C11,C8,C9
11	0.845(m)	14.33	H11-C10,C9

CHAPTER FIVE

DISCUSSION AND CONCLUSION

DISCUSSION

Focus on the medicinal properties of natural products especially those of plant origin have been on the increase in recent times. In particular, research on the therapeutic and chemoprophylactic efficacy of individual and combined phytochemicals against chronic and debilitating diseases afflicting man has been on the increase. The distinctive advantages of plant-based remedies over synthetic drugs such as reduced adverse or side effects, affordability and availability have fuelled the upsurge in the research. Another important factor is the positive results confirming the bioactivities of phytoconstituents which are emanating from laboratories all over the world. Only very few of the known plant species have been investigated. This necessitates increasing and sustained search for bioactive components that might still be locked up among the thousands of plants not yet evaluated for their medicinal properties.

1). Antioxidant and free radical scavenging activities of study plants and correlations among assay methods

It is well recognized in plant chemistry that the mode of preparation and administration of herbal remedies are often crucial variables in determining efficacy for pharmacological evaluations (Mensor et al., 2001). In the traditional use of these plants, decoctions or infusions of the relevant parts are usually made with either water or alcohol as the solvent. The nature of solvent may influence the medicinal or other effects exhibited by plants because solvents extract antioxidant components to different degrees.

Antioxidant activity in higher plants has often been associated with phenolic compounds (Meskew et al., 1998). In addition to their roles in plants, phenolic compounds in our diet may provide health benefits associated with reduced risk of chronic diseases (Liu, 2004). Flavonoids

are the largest group of phenolics. They have been identified in fruits, vegetables, and other plant parts and linked to reducing the risk of major degenerative diseases. More than 4,000 distinct flavonoids have been identified (Liu, 2004). The antioxidant activity of plant extracts has been reported to correlate with their phenolic content (Hidalgo *et al.*, 1994; Jayaprakasha and Jaganmohan, 2000). Data from the present work indicate that this correlation is dependent on the nature of the antioxidant assay employed. The results of this work clearly illustrate that different methodologies can provide completely different responses with respect to the antioxidant capacity of a pure compound or a mixture of compounds.

Significant correlations were observed between some of the assay methods. DPPH free radical scavenging activity had an excellent correlation with TPC and RP (Fig. 17). These three methods have a similar underlying mechanism of reaction. The DPPH assay evaluates antioxidant activity by testing the ability of compounds to act as free radical scavengers or hydrogen donors (Prakash, 2001). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They also have a metal chelating potential (Lewis *et al.*, 1998). The RP assay also has to do with the redox properties of substances being investigated. Each assay or group of assays with a similar underlying mechanism may be specific for a particular group of antioxidant substances, and where this group occurs in a substantial amount, such tests will yield high values. Exceptions can occur where these groups are bound or masked leading to their nondetection by the specified assay(s). There are different types of antioxidants in plants. There are elements like selenium, vitamins such as ascorbic acid, and phytochemicals such as carotenoids, phenolics, organosulfur compounds, and nitrogen-containing compounds. The nature and position of functional groups in some antioxidant compounds, e.g., the hydroxyl groups of flavonoids, influence their reactivity and consequently their activity.

It was reported by Odabasoglu *et al.*, (2005) that there was no correlation between antioxidant activity and TPC of extracts of some lichen species, a contradiction to previous reports (Hidalgo *et al.*, 1994; Jayaprakasha and Jaganmohan, 2000). The present investigation also contradicts the submission of Odabasoglu *et al.* (2005). There were strong correlations between TPC on the one hand and DPPH, RP, and TPTA assays on the other ($r = 0.76$, 0.81, and 0.55, respectively). A

strong correlation between reducing power and total antioxidant activity was however reported Odabasoglu *et al.* (2005). In line with this, the present investigation also revealed significant correlations between reducing power on one hand and DPPH and TPC on the other. It was suggested by them that individual phenolics may have distinct antioxidant activities, and there may be antagonistic or synergistic interactions between phenolics and other compounds like carbohydrates and proteins.

A good correlation between antiradical activity (DPPH) and TPC was reported by Miliauskas *et al.* (2004). Findings from the present work agree with this. The results of the present work also confirm their findings that there was low correlation between TFC and DPPH assay and between TPC and TFC. The values for the correlation coefficients between TFC and DPPH assay and between TPC and TFC in our own study ($r = 0.21$ and 0.43, respectively) were similar to those obtained by Miliauskas *et al.* (2004) (0.32 and 0.43, respectively). The results of the present work showed only a low correlation between TFC and TPC (Table 4) and also between TFC and radical scavenging assays. For example, the correlation coefficient between the DPPH assay and TFC was 0.21, and that of hydroxyl radical scavenging capacity and TFC was 0.03 (Table 7). These results are also in agreement with the findings of Miliauskas *et al.* (2004).

The present investigation further revealed that DOR and NO showed no strong correlation with any of the other assays that were carried out. It will therefore be logical to include these two assays when antioxidant activities of herbal extracts are being evaluated. Although previous investigators used very few assays or few plants for the purpose of investigating correlations, in the present study, seven assays and ten plants have been employed to ensure more accurate results.

It has been observed that only flavonoids of a certain structure and, in particular, the hydroxyl position in the molecule determine antioxidant properties. These properties, in general, depend on the ability to donate hydrogen or electron to a free radical. Miliauskas *et al.* (2004) found, in the same study, some correlation between TPC and flavonols. In support of the above observations, Chol *et al.* (2002a) reported that the interaction of a potential antioxidant with a free radicle depends on its structural conformation and that this structural requirement is correlated

with the presence of hydroxyl groups on the flavonoids. Cos *et al.* (2002) reported that allopurinol showed remarkable activity in inhibiting xanthine oxidase and scavenging superoxide radical, whereas taxifoline showed relatively weak activity. The difference in activities was attributed to variation in the location of the hydroxyl groups and double bonds.

Choi *et al.* (2002b) found that the scavenging activity of flavonoids on peroxynitrite was governed by the position of the hydroxyl group. *o*-Hydroxyl structures increased the scavenging activity on peroxynitrite. Structural comparison of the flavonols in their study and their scavenging activities clearly shows that the C-3 hydroxyl group plays a pivotal role in the observed scavenging activity. These authors inferred that the higher scavenging potency of galangin compared with galangin 3-*O*-methyl ether may suggest that C-3 methoxylation reduced the scavenging effect of flavonols.

Phytochemicals are complex in nature. Therefore, the antioxidant activities of plants extracts cannot be evaluated by only a single method. The antioxidant defense system of the body is composed of different antioxidant components. The antioxidant capacities of these antioxidant components depend upon which free radicals or oxidants are produced in the body. The various methods used in evaluating the antioxidant activity of samples can give varying results depending on the specificity of the free radical being used as a reactant. The results of the present work highlight the diversity and complexity of phytochemicals present in plant extracts and the specificity of different assay protocols for specific antioxidant species based on the mechanism of reaction.

The DPPH assay appears to be a reliable method of measuring total antioxidant capacity of substances or systems. Its values correlated well with about three other methods used in this study (Table 7). As noted by Prakash, it is rapid, simple, and inexpensive, and its value applies to the overall antioxidant capacity of the sample and is not specific to any particular antioxidant component.

Using the frequency of high antioxidant capacity values as the basis, results obtained in this work revealed that extracts from *P. guajava*, *S. mombin*, and *G. cupulare* demonstrated consistent high

activities in the various assays, followed by *C. alata* and *O. gratissimum*. It should be noted that some of the remaining plants have higher activities in some assays than the plants listed above. For example, the NO radical scavenging activity of *A. boonei* (44.88 ± 0.55%) was higher than that of *P. guajava* (21.68 ± 1.51%) and *O. gratissimum* (30.576 ± 1.61%) ($P < 0.05$) buttressing the fact about the complexity of phytochemicals and their mechanism of action.

Overall, the results obtained indicated that Nigerian indigenous medicinal plants could be a source of natural chemoprophylactic antioxidants against reactive oxygen species and as such could be relevant in the treatment of cardiovascular disease, cancer, arthritis and other pathologies in which free radical mechanisms have been implicated. In view of the potential beneficial properties of the studied plants, our results led to further investigations of the antioxidant, antilipoproliferative, cardioprotective and neuroprotective property of *Spondias mombin*.

12. Antiischemic and cardioprotective properties of *Spondias mombin*

The Langendorff studies revealed that *Spondias mombin* leaf extract at 0.005 g/L improves the contractility of the heart which has been weakened by ischemia. *Spondias mombin* showed positive inotropic and anti-ischemic properties. A comparison of the per se effect of the buffer and that of *Spondias mombin* showed that the extract did not exhibit adverse effect on the isolated heart. The variety and scope of cardiovascular drugs have increased tremendously in the last few decades, and new drugs are being approved annually. While treatment with these synthetic drugs are usually effective, there are often side effects. For example, alpha blockers like prazosin and terazosine may cause orthostatic hypotension, nausea and palpitations. Angiotensin converting enzyme inhibitors like captopril and enalapril are less effective in African-Americans than in Whites and may cause a hacking unpredictable cough. Some antiarrhythmic drugs like amiodarone are extremely toxic. Anticoagulants, antiplatelets and thrombolytics may cause excessive bleeding while Beta blockers may cause nightmare and precipitate asthmatic attack (Cohen *et al.*, 1992). *Spondias mombin* methanolic leaf extract appear to show a combination of effectiveness and safety.

For the in vivo cardioprotective studies, isoproterenol was used as the cardiotoxicant. Isoproterenol, a synthetic β-adrenoceptor agonist, has been found to induce myocardial infarction in rat as a result of disturbance in physiological balance between production of free radicals and antioxidative defense system. It is well known to generate free radicals and stimulate lipid peroxidation, which is a causative factor for irreversible damage to the myocardium. It causes severe stress in the myocardium resulting in infarct like necrosis of the heart muscle. It also increases the levels of serum and myocardial lipids, which in turn leads to coronary heart disease (Nair and Devi, 2006; Zhou *et al.*, 2008).

Figure 26 reveals that administration of the SM extract alone had no adverse effect on the heart weight/body ratio and also suggests that it may possess anti-inflammatory potential since it was able to significantly reduce the Heart weight/Body weight ratio which was elevated by ISP administration. Abad et al., (1996) had reported that extract from the bark of SM showed weak anti-inflammatory activity.

The antioxidant and lipid peroxidation inhibitory activities of SM are also clearly demonstrated in the results obtained in this study. The endogenous antioxidant GSH and Malondialdehyde which is a major product of lipid peroxidation are biomarkers of oxidative damage in living systems. Changes in the serum or tissue level of GSH is a reflection of changes in the enzymes associated with the metabolism of GSH such glutathione peroxidase, glutathione reductase and glutathione-S-transferase. In both serum and tissue, decreased GSH levels caused by ISP were remarkably augmented by the administration of SM while elevated MDA and nitrite levels caused by ISP administration were significantly attenuated, in both instances, to a level comparable with the effect shown by ramipril (Figures 27-30, 33). The in vivo antioxidative property of SM was further demonstrated by the significant increases in the activities of the antioxidant enzymes SOD and catalase in the SM and ramipril-treated groups compared with the ISP intoxicated group ($p<0.05$) (Figures 31 and 32).

The damage caused to the cardiomyocytes as a result of the administration of ISP already demonstrated by deleterious alterations in the serum and tissue antioxidative indices was further highlighted by the massive leakage of LDH into the serum (Figure 37) in the ISP intoxicated

group compared with the remaining groups ($p<0.001$). The leakage of LDH was significantly reduced in the SM and Ramipril treated groups ($p<0.001$). Since leakage of LDH into the blood occurs when the plasma membrane is damaged, SM could be said to restore membrane integrity compromised by ISP intoxication.

Elevated serum phosphate concentration has been linked with cardiovascular and renal diseases. Specifically, phosphate excess has been implicated in the substantial cardiovascular morbidity and mortality observed among people who receive chronic dialysis. Hyperphosphatemia has been independently linked with calcification of the coronary arteries and aorta as well as cardiovascular and all-cause mortality in the setting of end stage renal disease (ESRD) (Goodman *et al.*, 2000; Raggi *et al.*, 2002; Kestenbaum *et al.*, 2005). ISP intoxication led to a significant increase in the phosphate level which was decreased by treatment with MES (Figure 36).

Blood glucose level was not lowered in groups treated with MES alone compared with the ISP intoxicated group ($p>0.05$). However, the blood glucose level in the group administered ISP and treated with 250 mg/dl of MES was significantly lower compared to the ISP intoxicated group ($p<0.05$) (Figure 34). The antidiabetic activity of SM has been reported (Fred-Jaiyesimi and Kio, 2009). However, the evaluation of the antidiabetic activity was based on acute administration of SM in which exposure to SM was < 24 h in animals with induced diabetes. There appears to be no report on the hypoglycaemic activity of SM in normoglycaemic animals. In the present study, SM was administered to the experimental animals for 30 days. SM could be hypoglycaemic following acute administration but hyperglycaemic following chronic use. Also, the hypoglycaemic activity of SM may only be expressed in a hyperglycaemic state. However, SM was able to reduce the cholesterol level ($p<0.05$) which was elevated as a result of the administration of ISP (Figure 35). This suggests that SM possibly possesses hypocholesterolemic potential. Further studies would be needed to establish the hypolipidemic property of the plant. The histological studies show that SM was able to prevent the disorganization and fragmentation of cardiac myofibrils (Figures 38-41) which is in consonance with the results of the LDH assay.

In the present study, SM showed similar effects to that of Ramipril, an angiotensin converting enzyme (ACE) inhibitor, in most of the parameters evaluated. Experimental investigations and large clinical trials have shown that ACE inhibitors prevent deleterious events related to ischemia-reperfusion injury and atherosclerosis (Juggi et al, 1993; Heusch *et al.*, 1997; Remme, 1997). In particular, Ramipril has been demonstrated to be beneficial in a wide range of patients who are at high risk of cardiovascular events and has been indicated for congestive heart failure, left ventricular dysfunction and the prevention of myocardial infarction, stroke and cardiovascular death by the FDA (Annapurna and Kumar, 2000). Ramipril has a higher lipophilicity and therefore penetrates tissues better than other drugs in its class. ACE inhibitors prevent the degradation of bradykinins, possess vasodilatory activity and have oxygen free radical scavenging property which has been postulated to contribute significantly to the reduction of myocardial infarction (Annapurna et al., 2000). The excellent antioxidative activity shown by SM in this study and its anti-ischemic activity which is suggestive of a vasodilatory activity suggests some similarities to ramipril. Phenolics and flavonoids in foods and extracts from plants have been reported to possess anti ACE activity (Actis-Goretta et al., 2006; Park and Jhon, 2010). Phenolics are present in SM (Ayoka *et al.*, 2006; Igwe *et al.*, 2010).

It has been observed that taken alone, individual antioxidants studied in clinical trials do not appear to have consistent preventive effects as the isolated compound may not behave the same way as the compound in extracts or fractions (Yusuf *et al.*, 2000; Liu, 2004). Bioactivity may be a result of synergistic interactions among diverse phytochemicals. For these reasons, bioactivities shown by extracts from medicinal plants as obtained in this study should not be misinterpreted. Results of bioactivity studies on isolated compounds should be juxtaposed with those of extracts or fractions to ascertain which pharmacological formulations will be most beneficial.

This investigation appears to be the first major study exploring the cardioprotective property of SM. In this study, SM demonstrated *ex vivo* anti-ischemic property and its protective effect against myocardial damage. The mechanisms of SM cardioprotection seems to involve improvement of the contractility of the heart through the strengthening of the cardiac myofibrils, inhibition of the breakdown and disorganization of the myofibrils, preservation of the integrity of

cardiomyocyte membrane and reduction of oxidative stress. Moreover, the lowering of blood cholesterol levels in SM treated animals suggests that inhibition of atherosclerotic plaque formation may also be a contributory mechanism. Further investigations especially at the molecular level are needed to unravel the precise mechanisms and bioactive principles responsible for the cardioprotective property of SM and to ascertain whether cardioprotection by SM is attributable to an individual compound or a group of phytochemicals.

5.3 Neuroprotective property of *Spondias mombin* in middle cerebral artery occlusion induced brain infarction

The NeuroProtective property of MES was evaluated in the MCAO-induced focal cerebral ischemia model. Ischemia of cerebral tissue and cellular death underlie all forms of stroke, including focal ischemia. In the ischemic brain, cells die by means of two major processes: necrosis and apoptosis. Cells in the ischemic core die with the necrotic process and, depending on the location within the penumbra, cells die by means of either method (Smith, 2004). Salvage of the cells and tissue in the penumbra is a target for stroke therapy. A single dose of 100 mg/kg of SM extract was chosen based on results from pilot studies and previous works. *Spondias mombin* showed excellent neuroprotection in the model employed remarkably reducing infarct size and neurological deficit. This justifies the folkloric use of the plant for the treatment of cerebral disorders in some parts of the world. Since excessive apoptosis is a feature of cerebral ischemia, one of the neuroprotective mechanism of *Spondias mombin* may be suppression of apoptotic cell death in the penumbra.

The redox imbalance triggered by the oxidative burst following reperfusion often manifests in elevated MDA levels and reduced GSH levels. The negative alterations in the levels of these biomarkers of oxidative stress in both cortical and striatal regions of the brain following ischemia were ameliorated by *Spondias mombin*. This indicated that another key mechanism of MES-induced neuroprotection is through the normalization of the redox status culminating in the reduction of oxidative stress in the brain.

Nitric oxide (NO) generated by endothelial NO synthase (eNOS) plays a crucial role in vascular function and homeostasis. NO possesses vasodilatory, anti-inflammatory, antithrombotic and antiproliferative properties. Several modalities that upregulate eNOS expression and/or activity have been shown to enhance protection from ischemic stroke (Endres *et al.*, 2004). Depending on the cellular source and the stage of evolution of the ischemic process, the role of NO might be protective or destructive. This dual role of NO in brain ischemia underlines the necessity for selective therapeutic approaches to inhibit nNOS and iNOS and augment eNOS. This is because inducible nitric oxide synthase (iNOS) have been found to play an important role in the inflammatory process after cerebral ischemia which contributes to increase cerebral edema or infarct volume (Thiyagarajan and Sharma, 2004) and nNOS have also been reported to contribute to neuronal damage after ischemic or excitotoxic insult (Bayir *et al.*, 2005). The selective upregulation of eNOS expression and downregulation of the expression of iNOS might be another mechanism of neuroprotection by MES against cerebral ischemia.

SOD expression was also upregulated in MES-treated animals. Superoxide along with hydroxyl radical produce modification in primary, secondary and tertiary structure and aggregation and/or fragmentation of cellular proteins including SOD and peroxidase. Dysfunction or downregulation of SOD may result in loss of its protective function which manifested as increased infarction in many studies (Thiyagarajan and Sharma, 2004). Superoxide anion produced as a result of IR injury reacts with nitric oxide to form peroxinitrite which can produce severe damage to neurons by oxidizing the sulfhydryl groups in cytosolic proteins and producing peroxidation of lipids. Thus the enhanced expression of SOD by MES is another indication of its neuroprotection. This is confirmed by reports that homozygous SOD1 transgenic mice (fivefold increase in SOD activity) demonstrated a 35% decrease in infarct volume compared with control mice in a permanent focal ischemia model. A reduction ranging from 25 to 50% in neuronal cell death following transient MCA occlusion has also been reported in SOD1-overexpressing mice. The reductions were linked to decreased DNA damage through the blockage of the early release of cytochrome c from the mitochondria. Accordingly, mice lacking SOD1 demonstrated an increase in both infarct size and oedema following transient focal cerebral ischemia (Taylor and Sun, 2004).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is well known as a major source for superoxide radical generation in leukocytes (Chen, 2009). NADPH oxidase is a multisubunit complex composed of membrane-associated $gp91^{phox}$ and $p22^{phox}$ subunits and cytosolic subunits, including $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$. As NOX is activated, cytosolic subunits, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$, translocate into membranes and fuse with the catalytic subunit, $gp91^{phox}$. The activated enzymic complex transports electrons to oxygen, thus producing the superoxide anion (O_2^-), a precursor of reactive oxygen species (Bedard and Krause, 2007). The activation of the $gp91^{phox}$ subunit enhances cerebral damage while the activation of the $p22^{phox}$ subunit is neuroprotective. Chen (2009) reported that $gp91^{phox}$ expression increased after ischemia, and was further aggravated by genetic copper/zinc-superoxide dismutase (SOD1) deletion, but ameliorated in SOD1-overexpressing mice. This suggests that NOX plays a role in oxidative stress and inflammation, thus contributing to ischemic brain injury. MES-treated rats showed increased expression of $p22^{phox}$ compared to vehicle treated animals while the expression of $gp91^{phox}$ was suppressed. From these results, the mechanisms involved in neuroprotection by MES seem to include attenuation of oxidative stress, increased expression of enzymes and other factors that bolster neuronal preservation and the decreased expression of those that promote or exacerbate neuronal damage

Results from the bioactivity studies taken together suggest that augmentation of the endogenous defense system and attenuation of lipid peroxidation via different routes are principal mechanisms by which MES exhibited its medicinal properties.

5A Characterization of Quercetin-3-O-β-D-glucopyranoside and undec-1-ene from n-butanol and ethyl acetate fractions of MES

The strong antioxidant profile of SM in our previous studies was the basis for its selection for antiprotective and neuroprotective investigation. The high antioxidant indices for the n-butanol and ethyl acetate fractions informed their selection for chromatographic analyses which led to the isolation of some compounds out of which two were characterized. Previous investigations have reported the isolation of some lead compounds from *Spondias mombin*. These include hydrolysable tannins and caffeoyl esters with antiviral properties and alkenyl phenols

with molluscicidal and insecticidal properties (Pieters and Vlietinck, 2005). Although phenolics and many other compounds have been reported to be isolated from SM leaves (Ayoka *et al.*, 2006; Fred-Jaiyesimi et al., 2009), to the best of our knowledge there is no previous record of the isolation of the flavonol glycoside Quercetin-3-O-β-D-glucopyranoside and the fatty chain undec-1-ene from the leaves of the plant. The strong antioxidative property of Quercetin3-O-β-D-glucopyranoside has been reported (Hai-Lan et al., 2007, Liu et al., 2010). The relative ease of the isolation of this compound and the strong antioxidant and other bioactive properties that have been alluded to it in previous works suggest that it may be abundant in MES as well as be the bioactive compound in the extract. Undec-1-ene may also contribute to the antioxidant and other bioactive property of MES. The relative ease of the isolation of the compound may also be indicative of its abundance in the plant. Along with other essential oils, undec-1-ene was found to inhibit LPS-induced NO and PGE(2) production in RAW 264.7 cells. These inhibitory effects of the essential oils were accompanied by dose-dependent decreases in the iNOS and COX-2 mRNA expression (Kim *et al.*, 2008).

Antiproliferative activity

For the antiproliferative activity, only the dichloromethane fraction showed >50% growth inhibition against KB and C-33A cancer cell lines. It also had the highest percentage growth inhibition for the A549 cell line. Generally, the crude extract and the various fractions did not exhibit strong antiproliferative activities. The strong antiproliferative activity of the dichloromethane fraction suggests that other fractions from MES may contain other bioactive phytochemicals. As such, focus must not be centered on only the phenolic rich n-butanol and ethyl acetate fractions.

CONCLUSION

The present investigation corroborated reports from previous studies on the cardiotoxicity of isoproterenol and substantiated the role of oxidative stress and ischemia in the pathogenesis of myocardial and cerebral infarctions. Results from this study indicated that methanolic extract from the leaf of *Spondias mombin* possessed antioxidant, antiproliferative, cardioprotective and neuroprotective properties. The medicinal properties demonstrated by MES are principally mediated via its ameliorative effect on oxidative stress. These properties are due to the bioactive compounds present in the plant. Quercetin-3-O-β-D-glucopyranoside and undec-1-ene characterized from MES may be two of the bioactive compounds in MES responsible for the beneficial medicinal effects. The present investigation sets the tone for further evaluation of *Spondias mombin* for the management of cardiovascular diseases and stroke. Further pharmacological and mechanistic studies are needed.

CONTRIBUTION TO KNOWLEDGE

1. The phenolic content, total flavonoid content and the antioxidant profiles of ten indigenous Nigerian medicinal plants were presented in this study. Correlations among the different assay methods were analyzed.

2. The n-butanol and ethyl acetate fractions of MES showed strong antioxidant activity while the dichloromethane fraction exhibited significant antilipoproliferative activity against KB and C-33A cell lines.

3. MES at 0.005 g/l demonstrated *ex vivo* anti-ischemic property and *in vivo* cardioprotective property at 100- and 250 mg/kg.

4. MES at a dose of 100 mg/kg protected against middle cerebral artery induced focal cerebral ischemia in rats.

5. MES suppressed the expression of $gp91^{phox}$ and nNOS but enhanced the expression of $p22^{phox}$, eNOS and SOD in rat brain subjected to 1 h of MCAO and 24 h of reperfusion.

6. Quercetin-3-O-β-D-glucopyranoside (a flavonol glycoside) and undec-1-ene (a fatty chain) were characterized from MES.

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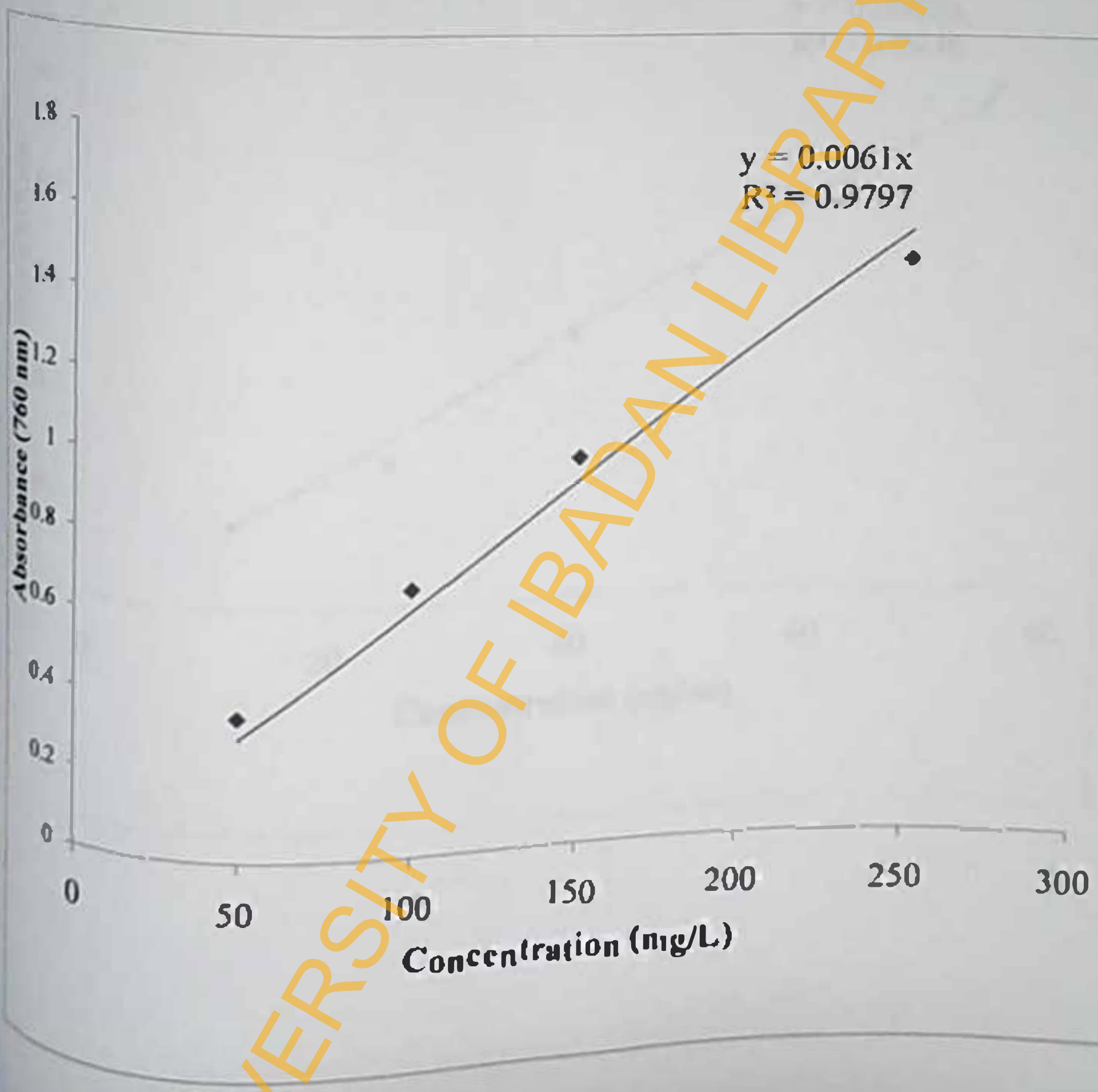
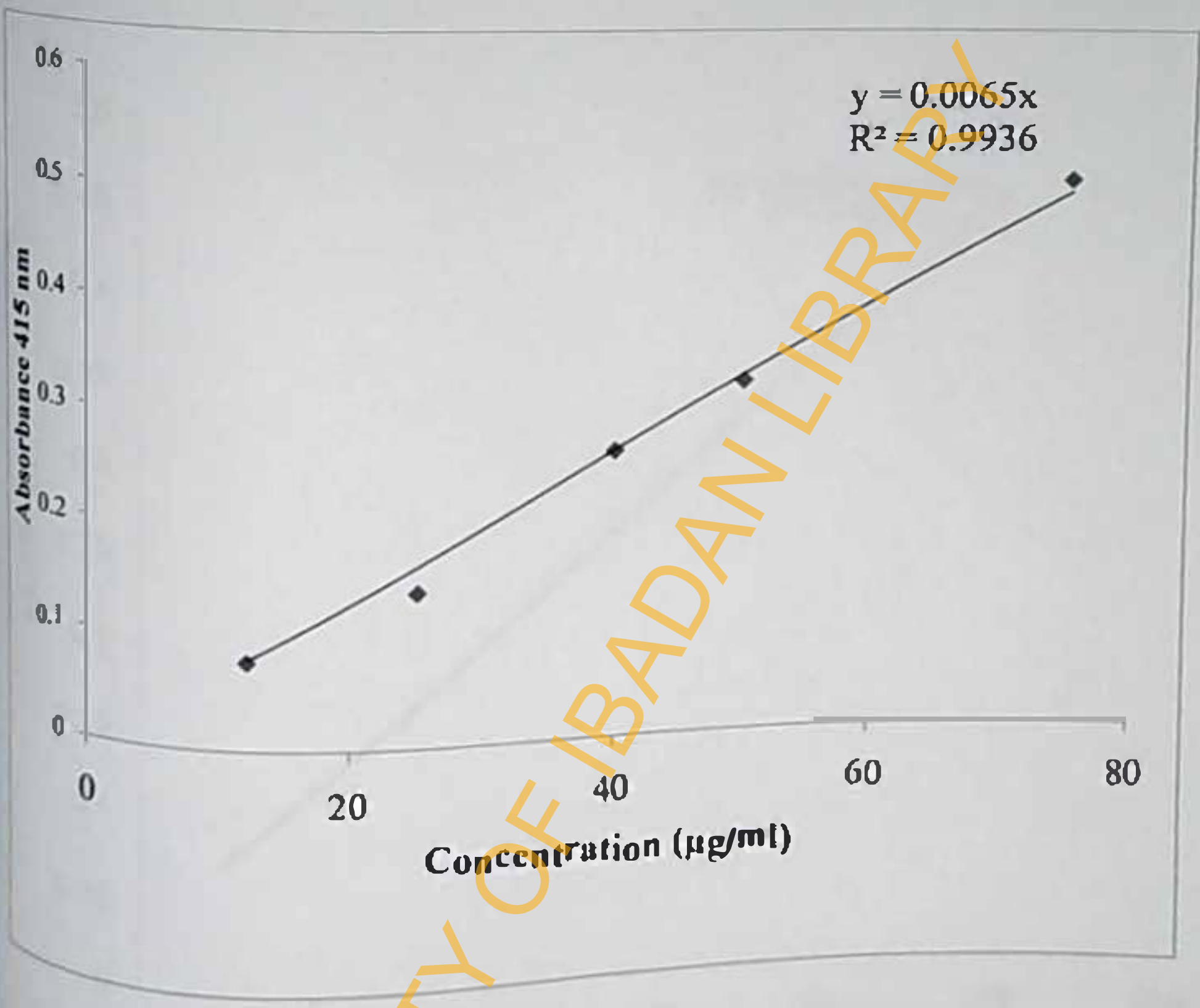
APPENDIX

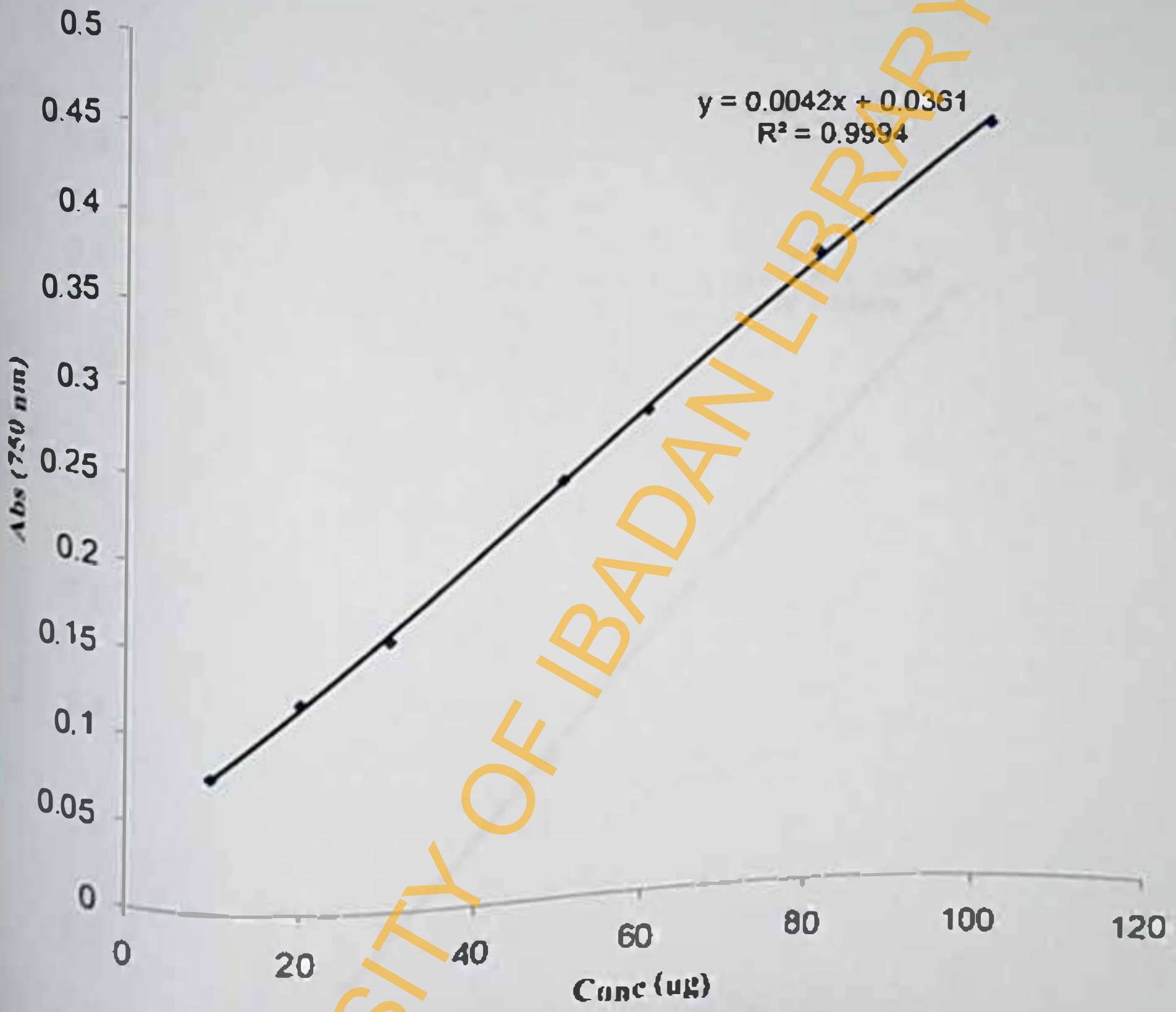
Appendix I: Gallic acid standard curve



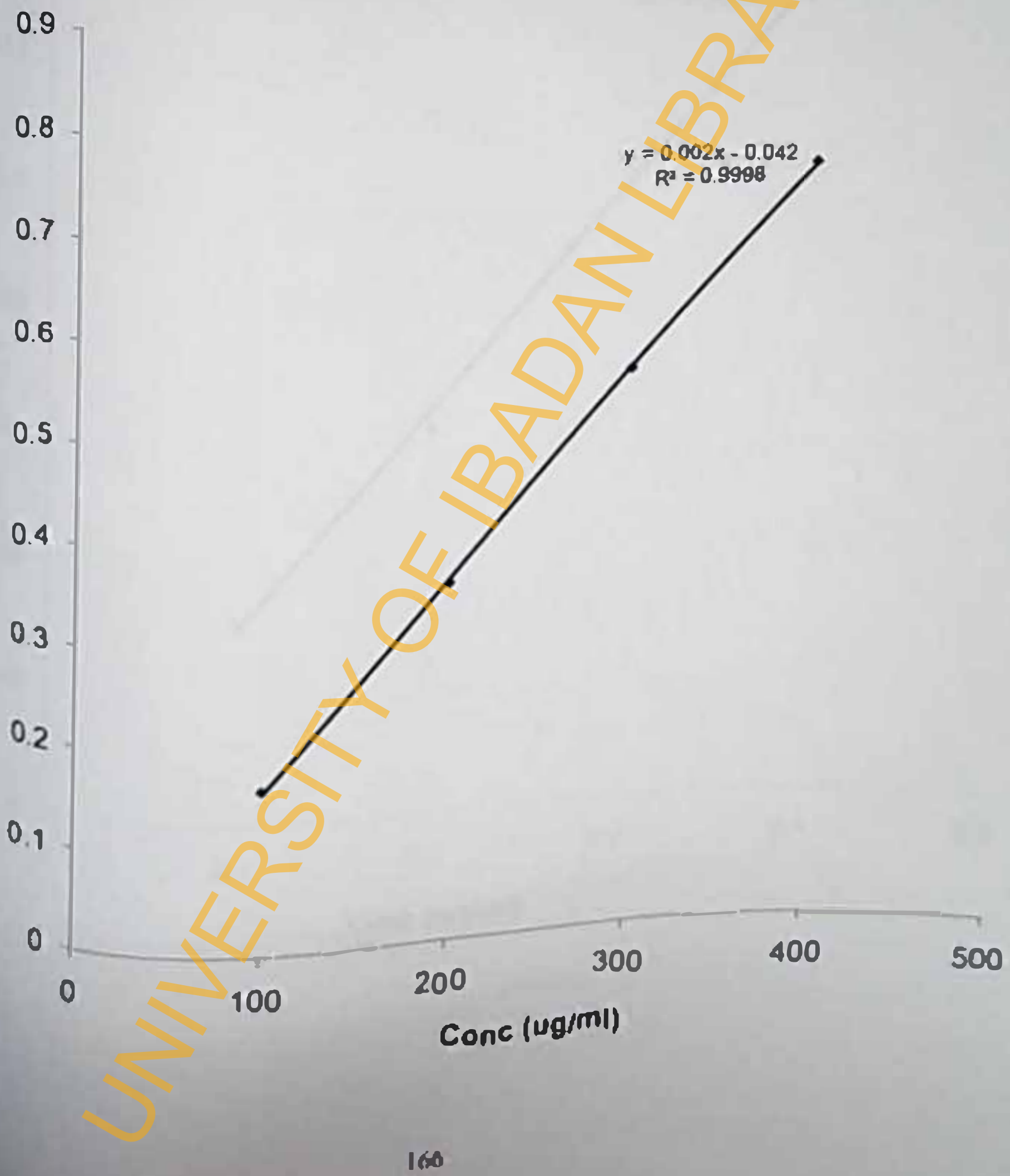
Appendix I: Quecertin standard curve



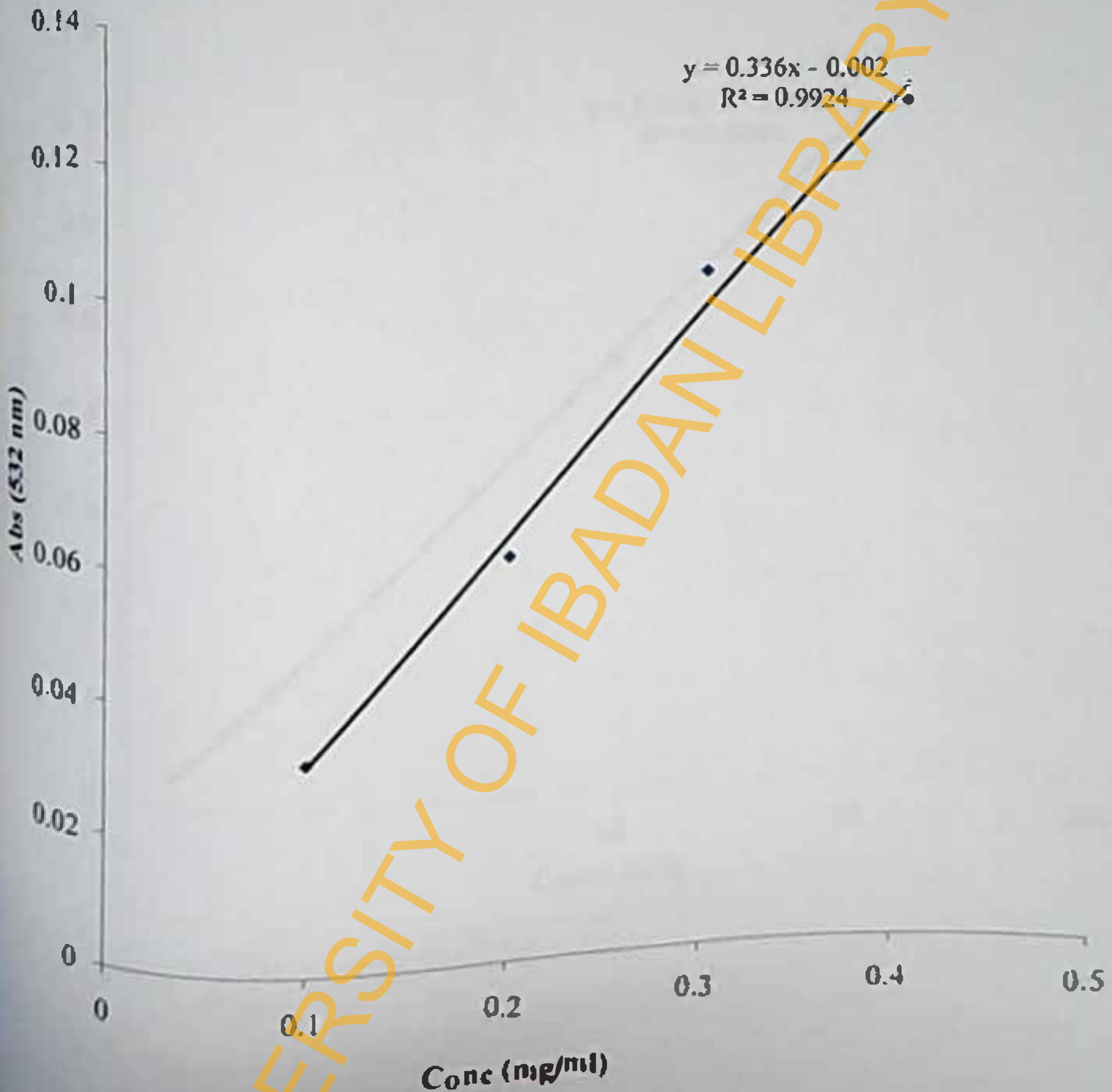
Appendix III: Protein standard curve



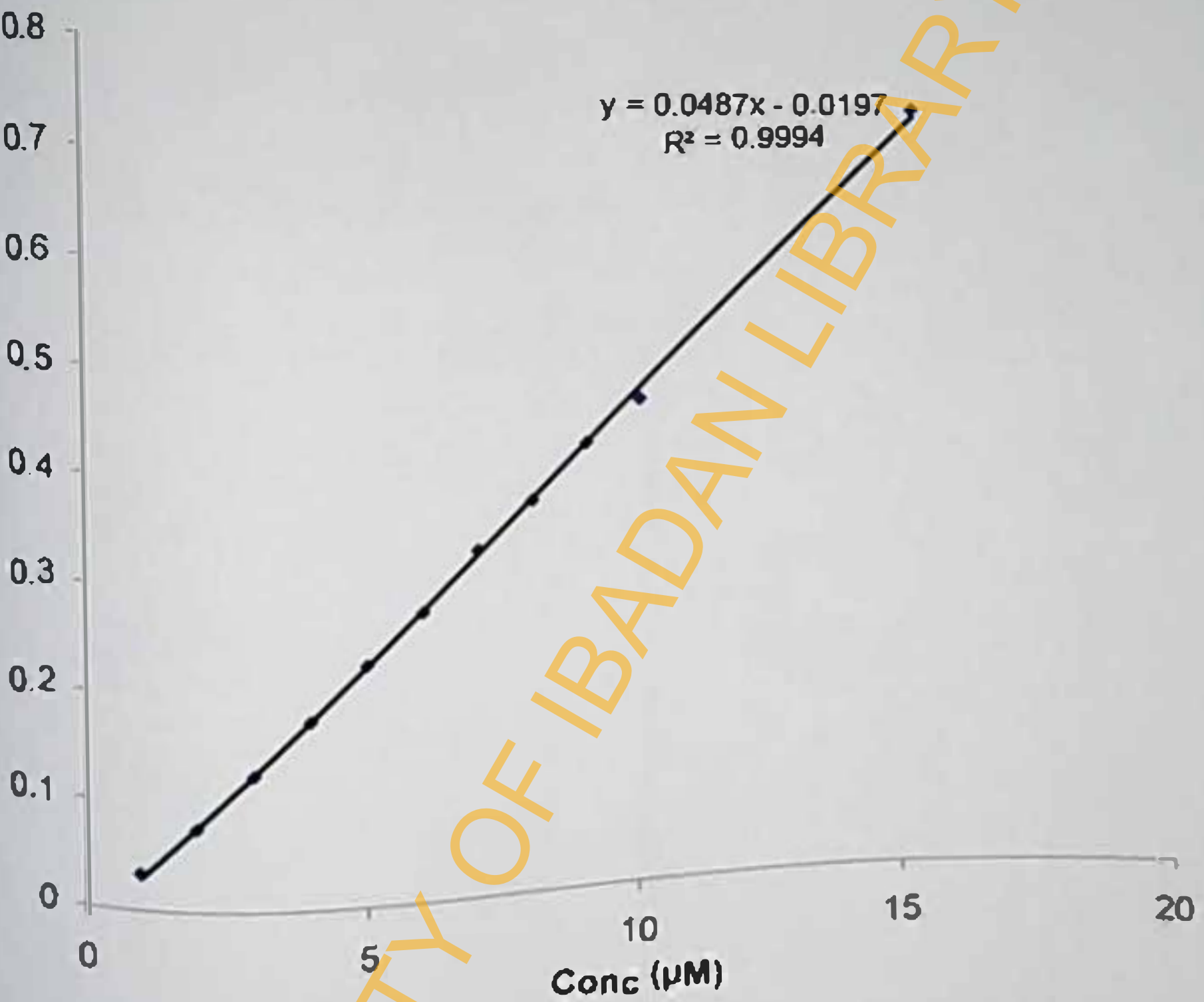
Appendix IV: GSH standard curve



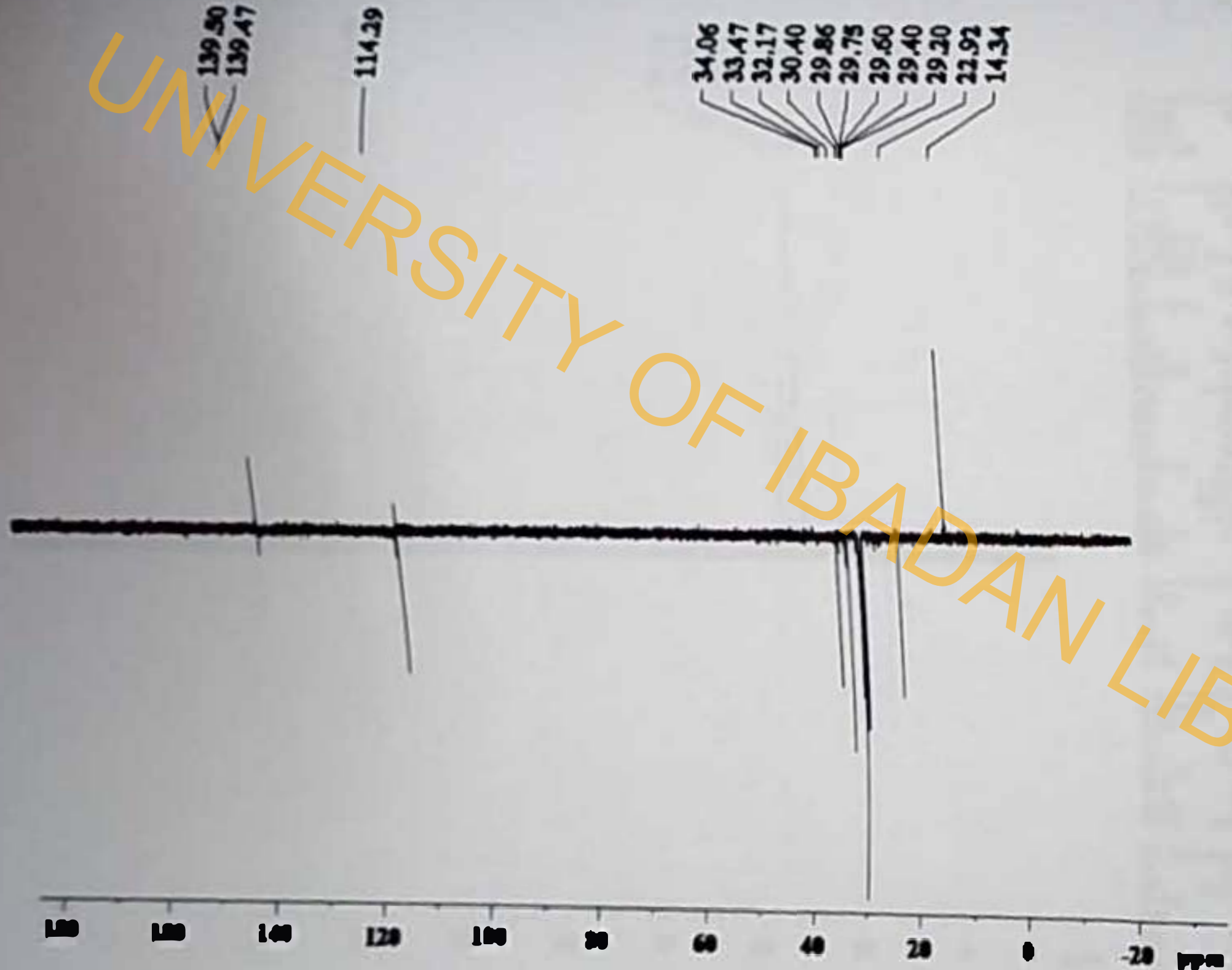
Appendix V: MDA standard curve



Appendix VI: Nitrite standard curve



Appendix VII-XXII: Structural elucidation charts



CLEAN-02

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139.47
114.39
34.06
33.47
32.17
30.40
29.86
29.75
29.60
29.40
29.20
22.92
14.34

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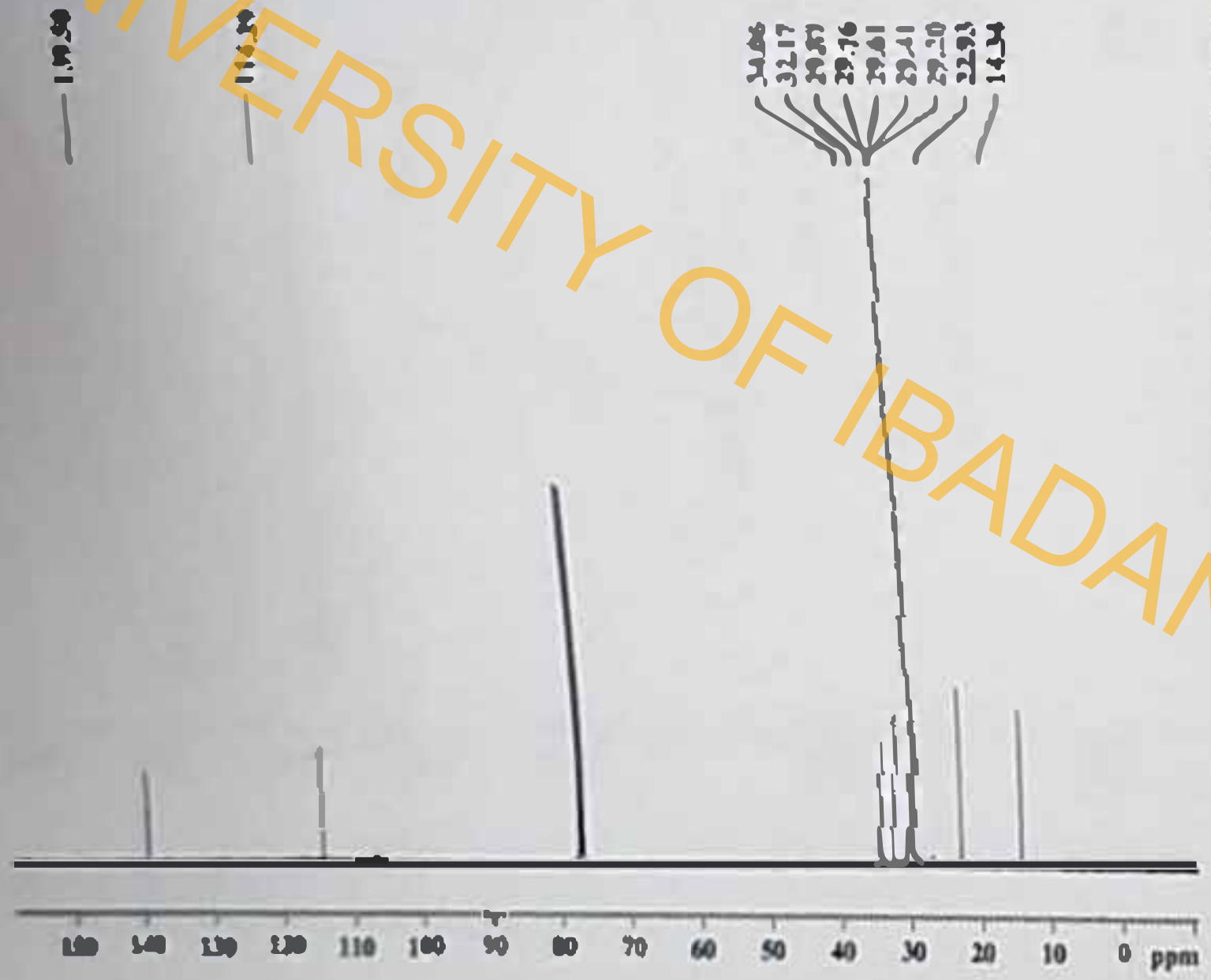

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TD0 1

======== CHANNEL
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======== CHANNEL
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PL2 2.
PL12 2
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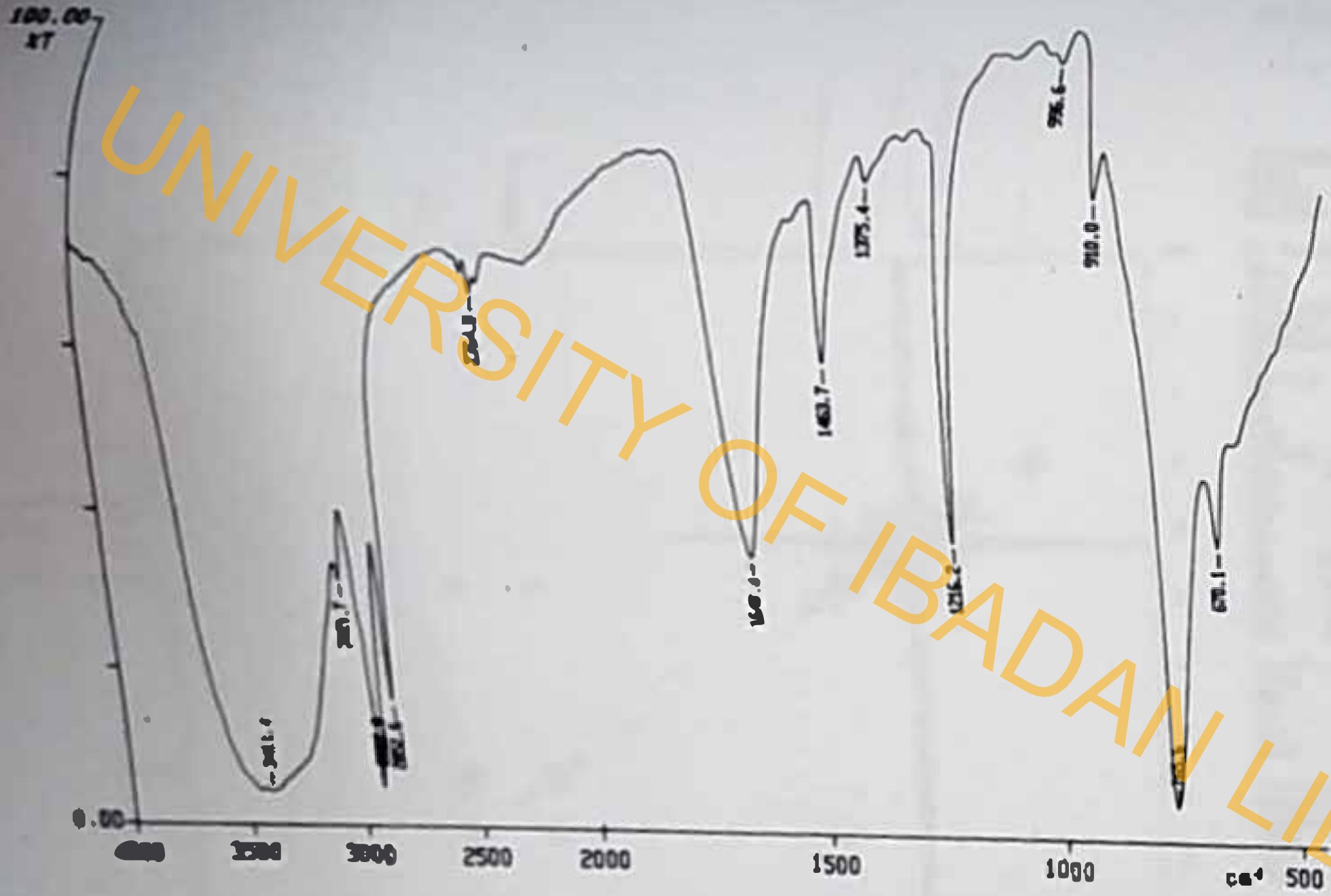

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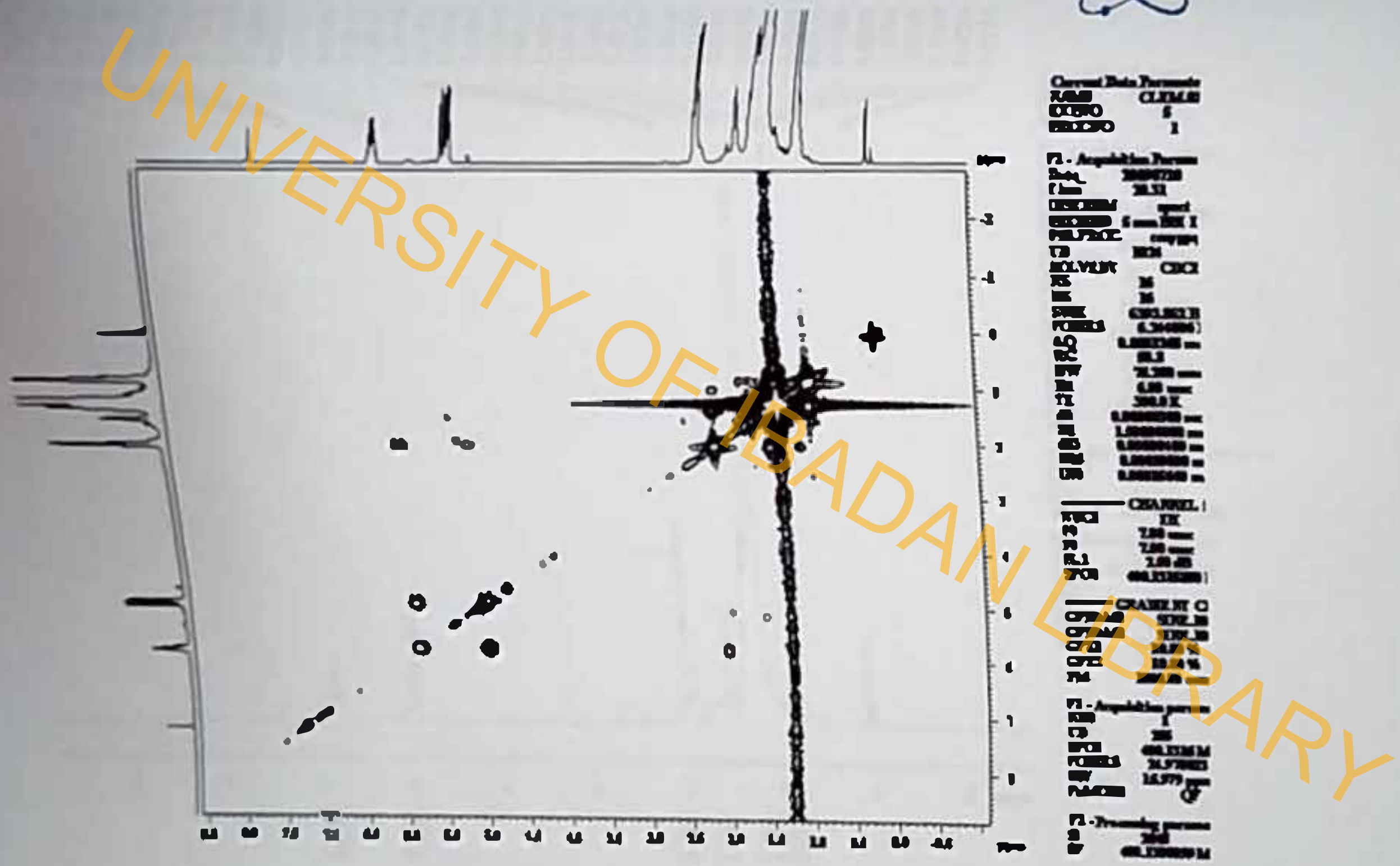

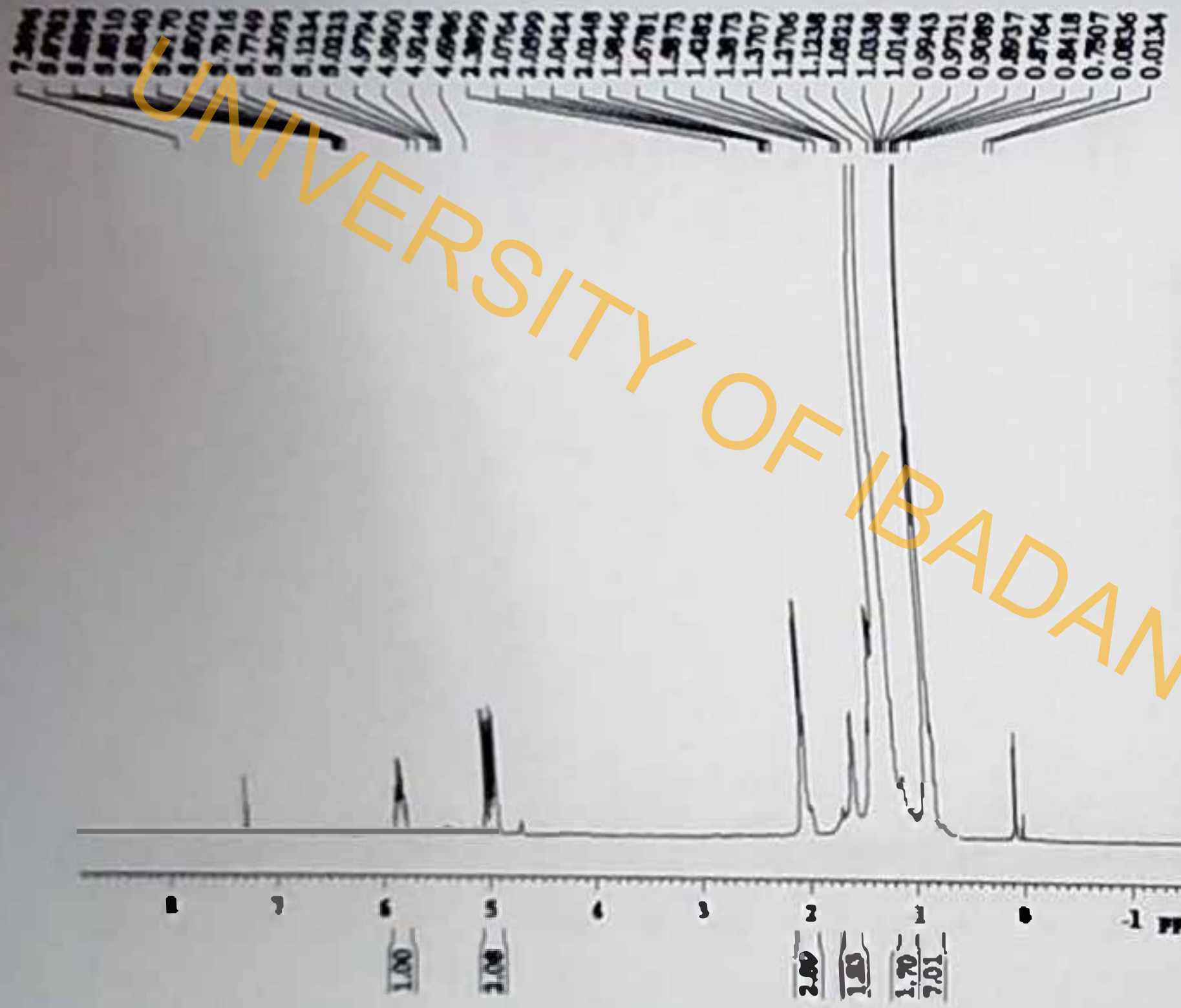

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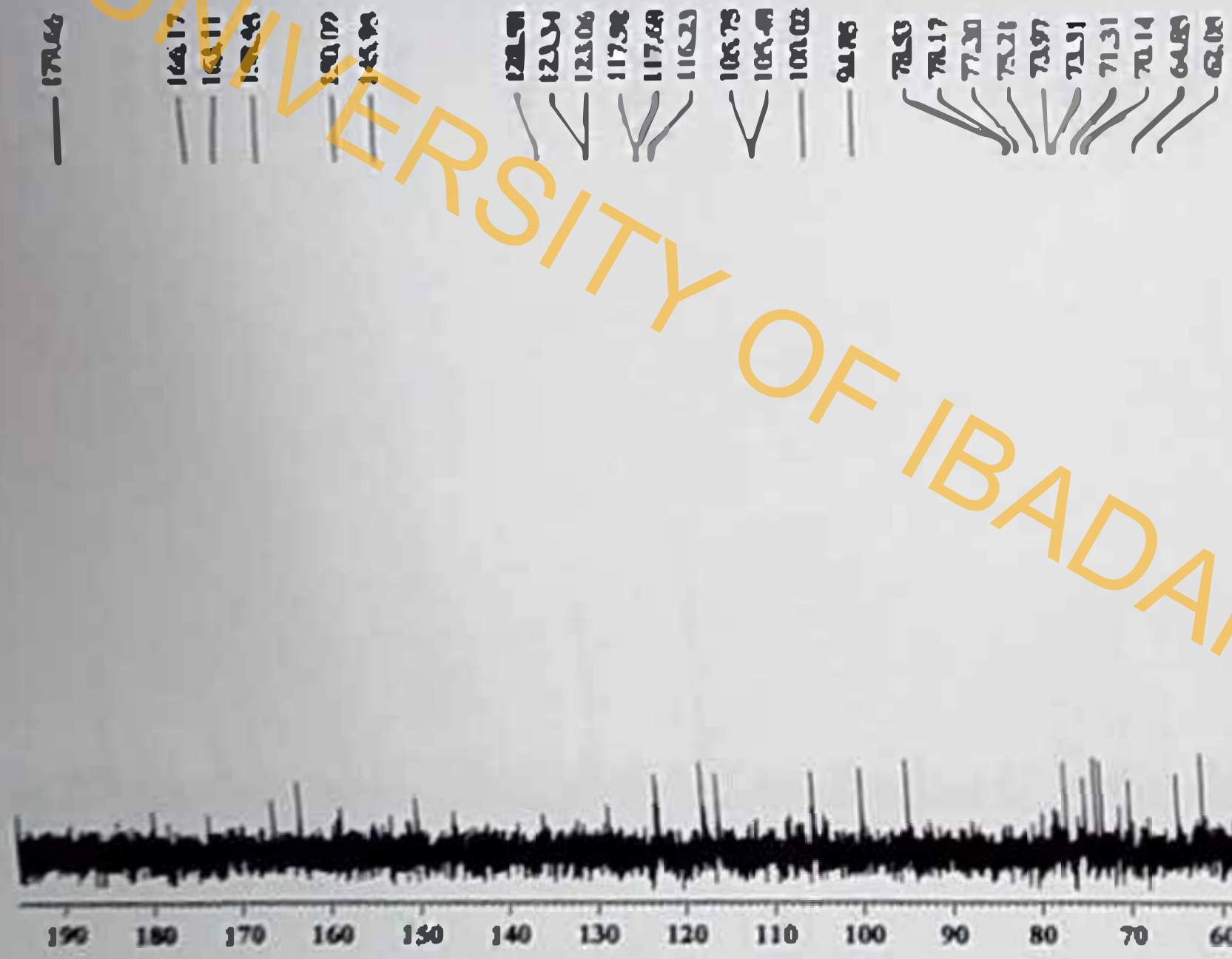

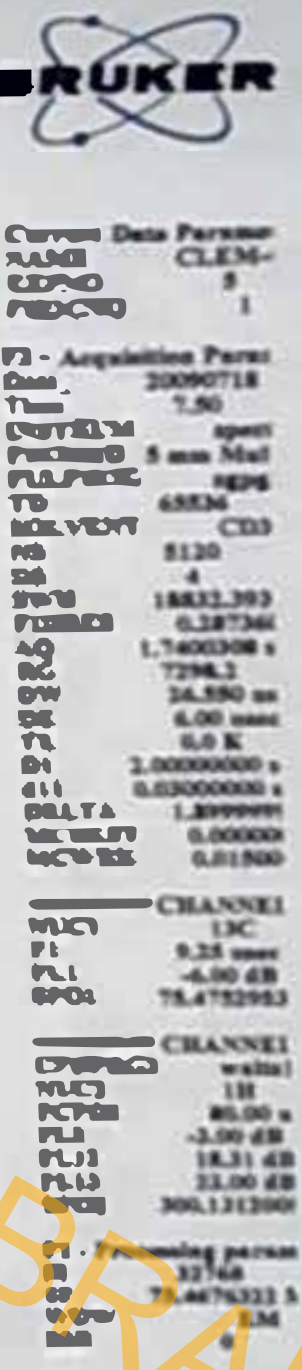

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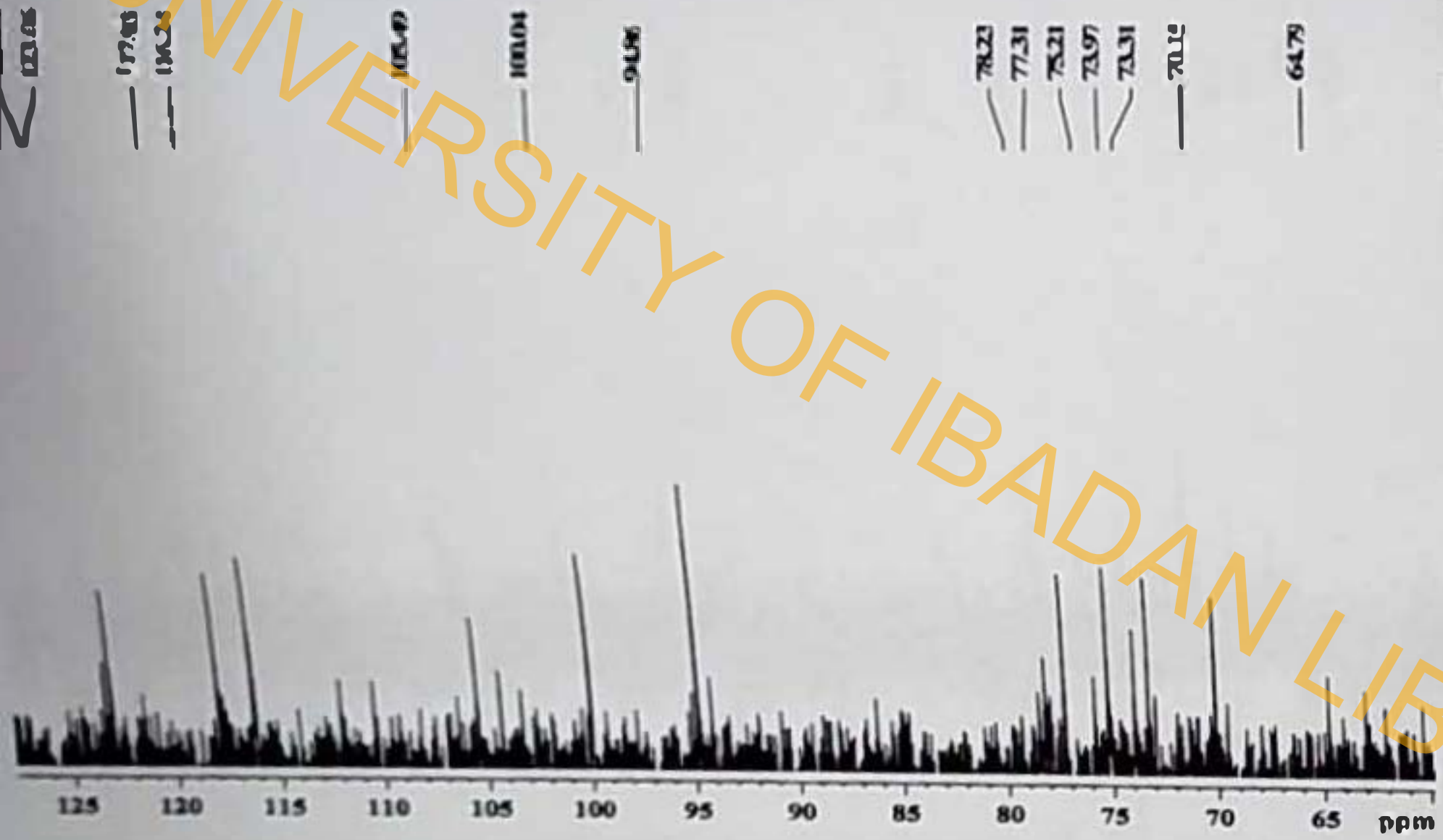

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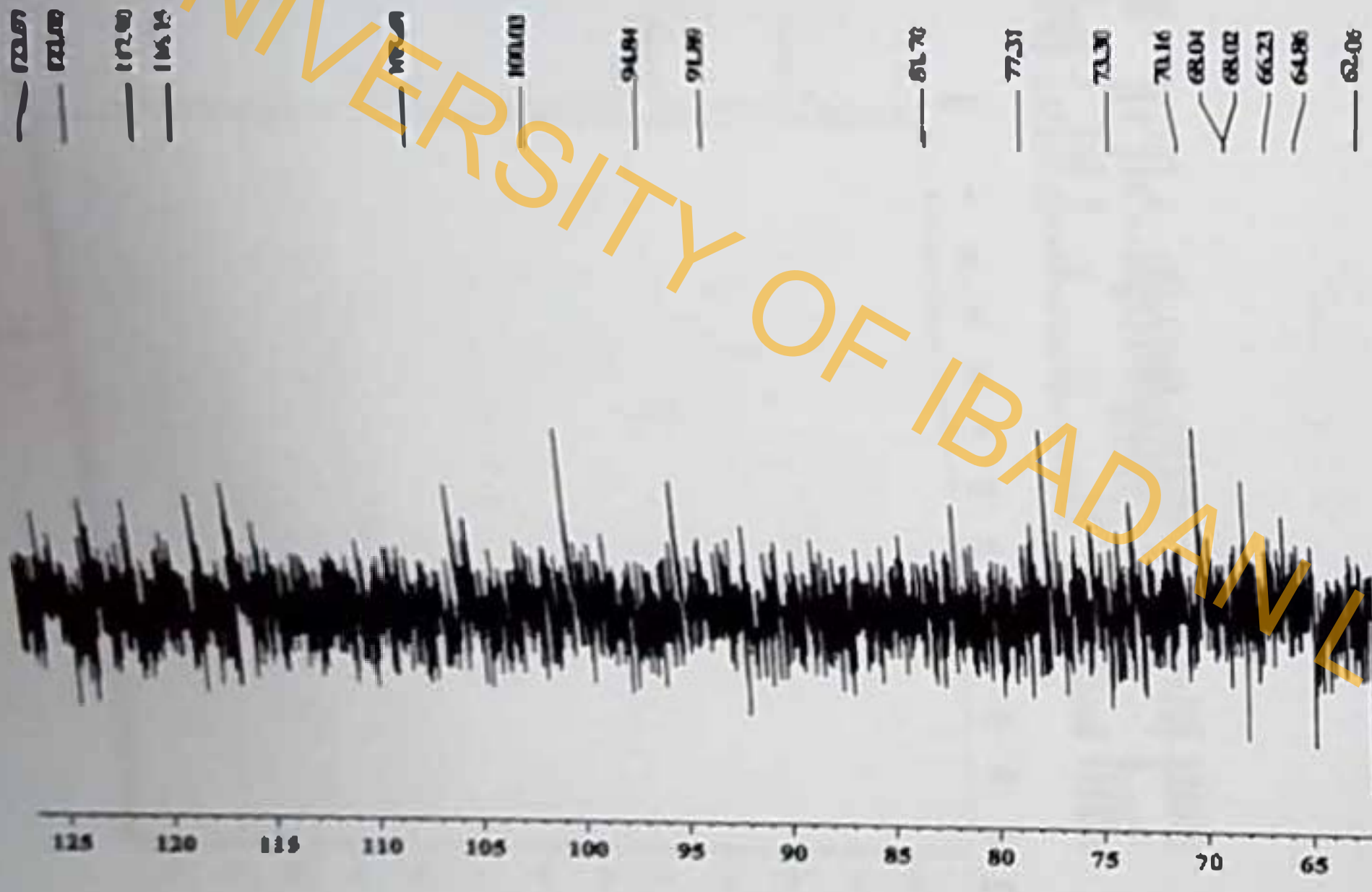

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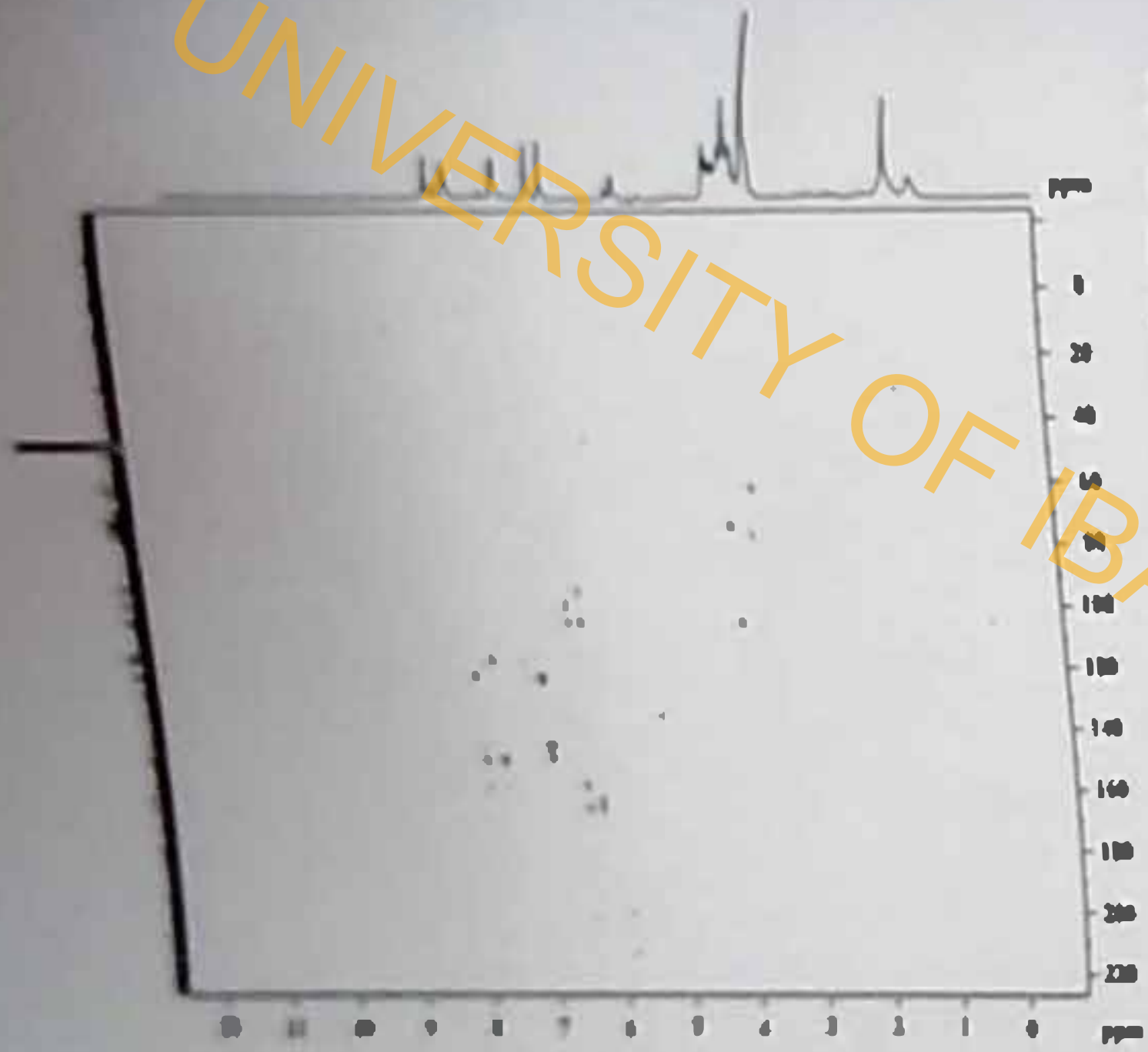

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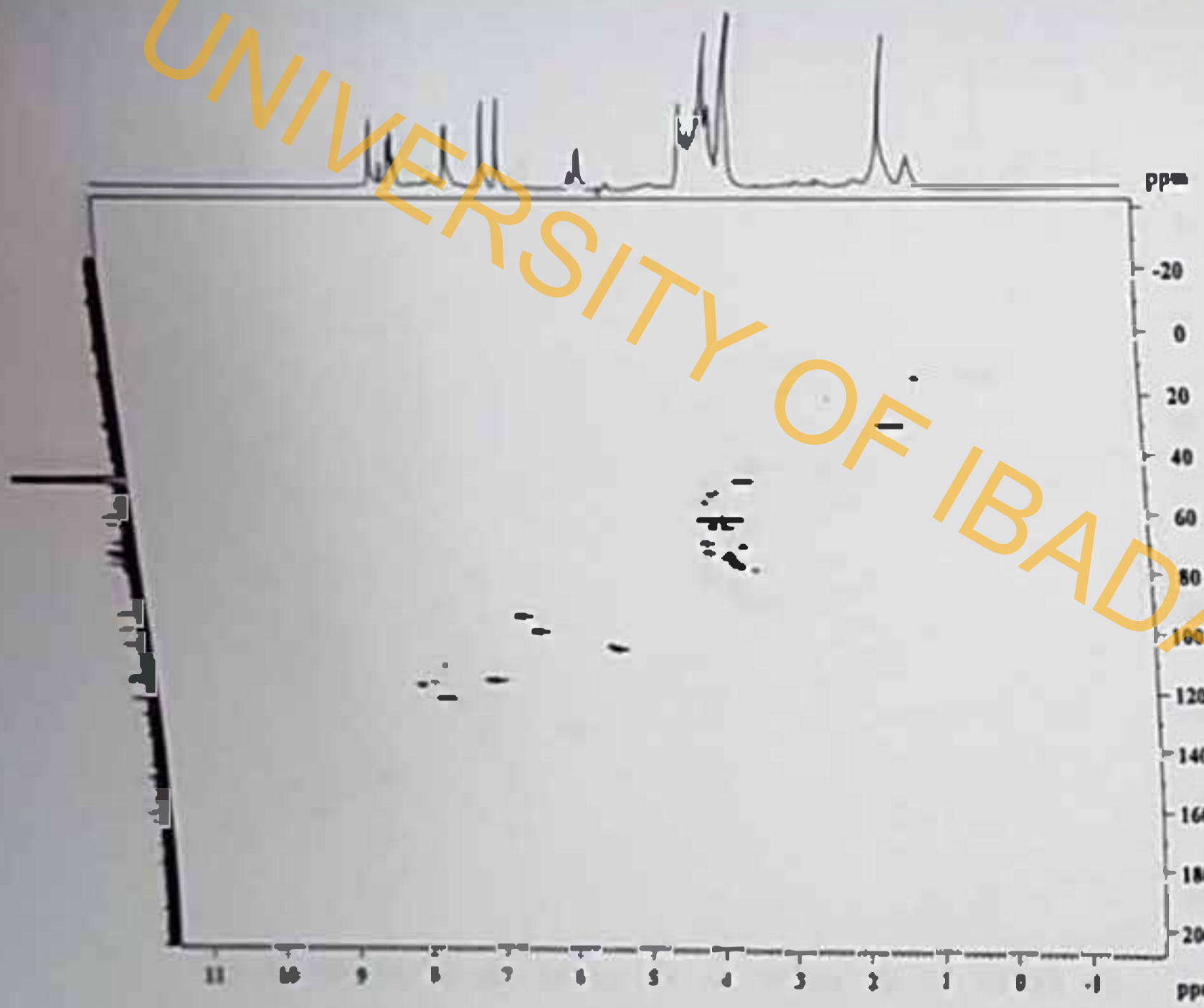

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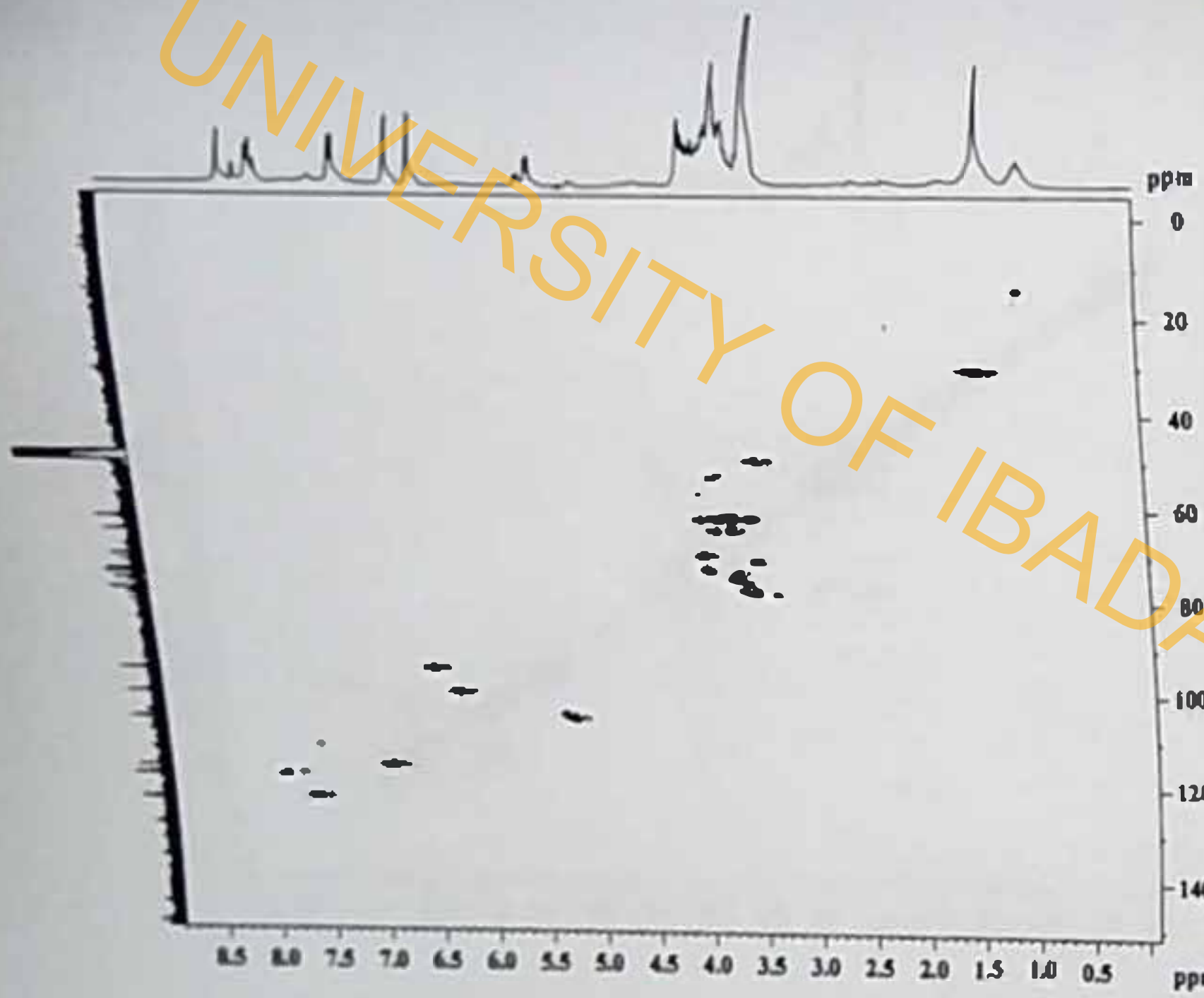

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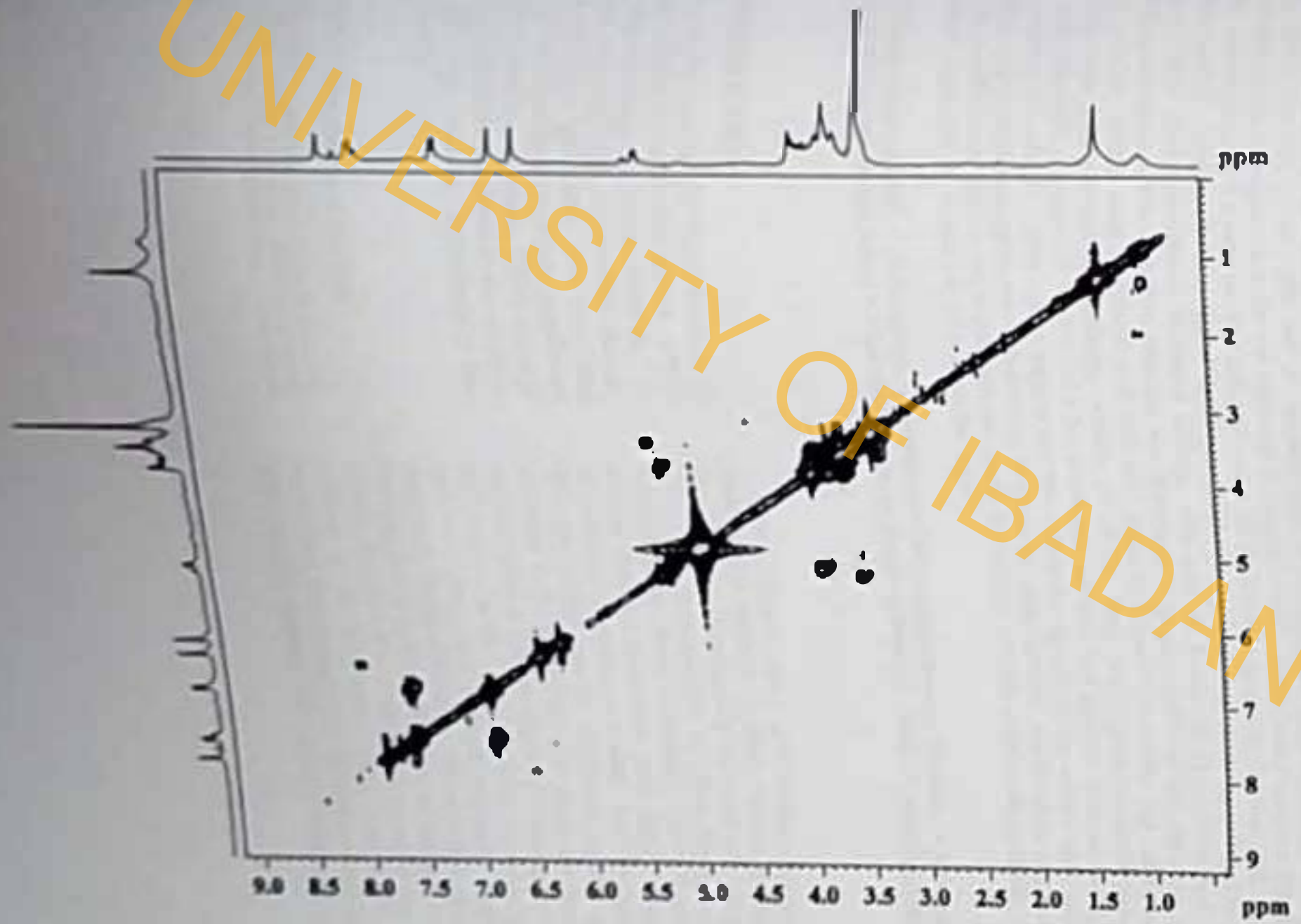

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Evaluation of Antioxidant and Free Radical Scavenging Capacities of Some Nigerian Indigenous Medicinal Plants

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ABSTRACT Methanolic extracts of 10 selected Nigerian medicinal plants—*Psidium guajava*, *Alstonia boonei*, *Cassia [illegible]*, *Newbouldia laevis*, *Spondias mombin*, *Globimetula cupulatum*, *Chromolaena odorata*, *Solanum [illegible]*, *Gongronema latifolium*, *Ocimum gratissimum*, and *Morinda lucida*—widely used in ethnomedicine, were assessed for phytochemical constituents and free radical scavenging activities using seven different antioxidant assay methods. Phytochemical screening tests for steroids, terpenoids, and cardiac glycosides, alkaloids, saponins, tannins, and flavonoids were carried out on extracts. *P. guajava* contained the highest amount of total phenolics (380.08 ± 4.40 mg/L gallic acid equivalents), and the amounts of total flavonoids were found in the leaf extracts of *C. alata* (275.16 ± 1.82 μg/mL quercetin equivalents), *C. odorata* (272.12 ± 2.32 μg/mL QE), and *P. guajava* (269.77 ± 2.78 μg/mL QE). Percentage 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was highest in *S. mombin* (88.58 ± 1.04%) and *P. guajava* (82.79 ± 1.34%) compared with values obtained for ascorbic acid and gallic acid. All the extracts, generally, had low nitric oxide radical scavenging activities, and *G. cupulatum* had the highest hydroxyl radical scavenging activity (63.84 ± 0.07%). The extracts demonstrated high lipid peroxidation inhibitory activity, with only *M. lucida* (38.74 ± 1.09%) and *A. boonei* (0.46 ± 0.59%) being exceptions. The reductive potential was highest in *P. guajava* (0.79 ± 0.04) and least in *S. mombin* (0.26 ± 0.00). DPPH assay correlated well with total phenolic contents ($r^2 = 0.761$) and reductive potentials ($r^2 = 0.81$) and fairly with lipid peroxidation inhibitory activity ($r^2 = 0.511$. There was a good correlation between total phenolic contents and lipid peroxidation inhibitory activity ($r^2 = 0.55$). These results suggest that the methanolic extracts of the studied plant parts possess antioxidant and radical scavenging activities that may be due to the phytochemical content of the plants and as such are potential candidates as natural chemoprophylactic agents. In addition, multiple assay methods should be used to determine antioxidant capacities of samples to have a reliable result.

KEY WORDS: • *antioxidant capacity* • *antioxidants* • *chemoprevention* • *complementary medicine* • *flavonoids* • *free radical scavenging activity* • *medicinal plants* • *Nigerian indigenous plants* • *phenolics* • *reactive oxygen species*

INTRODUCTION

[illegible] of free radicals in the body beyond its [illegible] capacity leads to oxidative stress, which [illegible] in the etiology of many diseases.[1] In [illegible] reactive oxygen species, e.g., superoxide radical, [illegible] and hydrogen peroxide, are important [illegible] in the etiology of several pathological conditions [illegible] protein oxidation, DNA damage [illegible] lipid peroxidation related to cardiovascular disease, [illegible] many diseases, cancer, Alzheimer's disease, and [illegible].[2]

Plants contain many antioxidant compounds that act as their major defense against radical-mediated toxicity by preventing or attenuating the damages caused by free radicals.[3] The consumption of plant foods such as fruits and vegetables has been associated with a lower risk of degenerative diseases that come with aging such as cancer, cardiovascular disease, cataracts, and immune dysfunction.[4] This protection can be explained by the capacity of multiple antioxidants in the plant foods[5] to scavenge free radicals, which are responsible for the oxidative damage of lipids, proteins, and nucleic acids.[6] Studies have reported the carcinogenic and toxic properties of some synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and propylated hydroxyquinone at higher levels.[7] Therefore, current research has unified efforts to the potential application of natural antioxidants from herbs and spices in the fortification of foods against oxidation as well as for therapeutic intervention against free radical-mediated diseases.[8]

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Phytochemicals, especially phenolics in fruits and vegetables, are suggested to be the major bioactive compounds responsible for their health benefits. Most of these beneficial effects are due to the antioxidant and metal chelating abilities of phenolic compounds. Phenolics have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and other free radicals produced by lipid peroxidation.[7,8]

A plethora of methods have come into common use for screening antioxidant activity of various classes of compounds. This is due to the search for novel natural antioxidants in medicinal plants and vegetables that may be relevant in pathologies involving reactive oxygen species, as well as preservation of food substances against oxidation in food industries. These include 2,2-diphenyl-1-picrylhydrazyl (DPPH) reactivity, total phenolic analysis, Trolox equivalent antioxidant capacity, ferric reducing antioxidant power, and oxygen radical absorbance capacity.[9]

In Nigeria numerous food plants are used as herbs and health foods and for therapeutic purposes. This is the first study of its kind to report the antioxidant effectiveness of some Nigerian indigenous plants (*Psidium guajava*, *Alstonia boonei*, *Cassia alata*, *Newbouldia laevis*, *Spondias mombin*, *Globimenda cupulatum*, *Chromolaena odorata*, *Senecio longepedunculata*, *Ocimum gratissimum*, and *Morinda lucida*) (Table 1). Therefore, the antioxidant and free radical scavenging capacities of selected plants from Nigeria, having medicinal properties, have been evaluated by using seven methods. The level of correlation among the methods was also examined.

MATERIALS AND METHODS

Sample preparation and extraction

Plant materials were obtained from farmlands in Akure, Southwestern Nigeria, in the latter part of 2005. They were dried under active ventilation at room temperature, packed in paper bags, and stored. The plant materials were later pulverized with a Retsch Muhle (Haan, Germany) blending machine. The powdered samples (200 g) were extracted by maceration in 500 mL of a solution of methanol and water (4:1 vol/vol) for 72 hours. The mixtures were filtered, first with a mesh and then with Whatman (Maidstone, UK) No. 1 filter paper. The filtrate was concentrated using a rotary evaporator (Resona, Gossau, Switzerland) and then lyophilized with a Modulyo (Edwards, Crawley, UK) SB4 freeze-dryer. The lyophilysates were preserved in dessicators at −4°C.

Phytochemical screening

Extracts were screened for the presence of specific phytochemicals like alkaloids, tannins, cardiac glycosides, terpenoids, flavonoids, and steroids as previously described.[10]

Test for alkaloids

Plant extract (0.5 g) was added to 5 mL of aqueous HCl (1%) on a steam bath. The solution was filtered, and the filtrate was treated with a few drops of Dragendorff's reagent. Turbidity or precipitate indicated the presence of alkaloids.

Test for saponins

Plant extract was shaken with 5 mL of water in a test tube and warmed. Frothing indicated the presence of saponins.

Test for tannins

About 0.5 g of extract was stirred with 10 mL of distilled water. The mixture was filtered, and the filtrate was treated with ferric chloride. A blue-green-black-green precipitate indicated the presence of tannins.

Table 1. Medicinal Plants Used in This Study

Plant species	Common name	Part used	Traditional use
P. guajava	Guava	Leaves	Used for treating fevers and diarrhea and as a tonic in psychiatry
C. alata	Asunwon	Leaves	Laxative, remedy for parasitic skin diseases, ulcers, asthma, and bronchitis
N. laevis	Akoko	Stem bark	Febrifuge, used for the treatment of epilepsy, convulsion, rheumatism, and arthritis
A. boonei	Ahun	Stem bark	For treating malaria, painful micturation, and rheumatic conditions, antivenom, and antihypertensive
G. cupulatum	Aforno	Leaves	Antihypertensive, for treating epilepsy, internal hemorrhages, arthritis, rheumatism, chilblains, leg ulcers, and varicose veins
C. odorata	Akintola	Leaves	For wound dressing, to treat skin infection and stop bleeding
S. longepedunculata	Ipeta	Root	For erectile dysfunction, coughs, colds, fever, backache, toothache, sleeping sickness, and venereal disease
S. mombin	Iyeye	Leaves	Diuretic, emetic, febrifuge, and abortifacient; also used for diarrhea, dysentery, hemorrhoids, and gonorrhea
O. gratissimum	Efinrin	Leaves	Used for the treatment of rheumatism, paralysis, epilepsy, high fever, diarrhea, and mental illness; as an emetic and for hemorrhoids, stomach problems, and eye/throat inflammation
M. lucida	Oruwo	Leaves	Used for malaria, typhoid fever, and jaundice and for treating wound infections, abscesses, and chancre

Test for phlobotannins

The extract was boiled with 1% aqueous HCl. A red precipitate showed the presence of phlobotannins.

Test for anthraquinones

About 0.5 g of extract was shaken with 10 mL of benzene and filtered. Five milliliters of 10% ammonia solution was added to the filtrate. The mixture was shaken. The presence of pink, red, or violet color in the ammoniacal lower phase indicated the presence of free anthraquinones.

Test for steroids

Acetic acid (2 mL) was added to 0.5 g of extract. Two milliliters of H_2SO_4 was then added. A violet to blue-green color showed the presence of steroids.

Test for terpenoids

The extract was mixed with 2 mL of chloroform. Three milliliters of concentrated H_2SO_4 was then carefully added to form a thin layer. A reddish brown coloration at the interface indicated a positive result for terpenoids.

Test for flavonoids

Dilute ammonia solution was added to the extract followed by addition of concentrated H_2SO_4. A yellow coloration that disappeared on standing indicated the presence of flavonoids.

Assay for total phenolic content (TPC)

The TPC of the extracts was assessed as described by McDonald *et al.*[11] Serial dilutions of 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, and 250 mg/L were prepared from a standard gallic acid (Sigma Chemical Co., St Louis, MO, USA) solution. Gallic acid solution (0.1 mL) or extract solution (0.1 mL, 20 mg/mL) was added to 0.2 mL of Folin-Ciocalteu reagent (Sigma) and diluted 10-fold, and 2 mL of distilled water was added. After a few minutes, 1 mL of 15% Na_2CO_3 was thoroughly mixed with the solution. The solutions were then incubated at 40°C for 30 minutes, after which absorbance was read at 760 nm using a Jenway (Jenway, Staffordshire, UK) UV-Vis spectrophotometer. The total content of phenolic compounds in plant metabolic sources was expressed in gallic acid equivalents (GAE) (in mg/L).

Assay for total flavonoid content (TFC)

The TFC of extracts was estimated using the aluminium chloride colorimetric method of Chang *et al.*[12] with a slight modification. Each plant extract (0.5 mL, 1 mg/mL) in ethanol was separately mixed with 0.1 mL of 10% $AlCl_3 \cdot 6H_2O$, 0.1 mL of NaOH, and 2.8 mL of distilled water. The absorbance of the reaction mixture was measured at 415 nm after 30 minutes. TFC was expressed as quercetin equivalents (QE) (in mg/mL).

Evaluation of DPPH radical scavenging activity

The DPPH radical scavenging activity of the extract was determined according to the method of Menson *et al.*[13] DPPH methanol solution (1 mL, 3 mM) was added to 1 mL of 300 µg/mL methanolic solution of extract and allowed to react at room temperature. The absorbance was read after 30 minutes and converted into percentage antioxidant activity.

Evaluation of nitric oxide (NO) radical scavenging activity

NO scavenging activity was determined spectrophotometrically as previously described.[14] In brief, the reaction mixture (3 mL) containing sodium nitroprusside (10 mM) in phosphate-buffered saline and the extract (1 mg/ml) was incubated at 25°C for 150 minutes. Then 0.5 mL of the reaction mixture was removed, and 0.5 mL of Griess reagent was added. The absorbance of the chromophore formed was evaluated at 546 nm. The results were expressed in percentage radical scavenging activity.

Deoxyribose (DOR) assay

Hydroxyl radical scavenging activity was evaluated according to the protocol described by Neergheen *et al.*[15] The method is based on studying the competition between DOR and the extracts for hydroxyl radical generated by the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. The reacting mixture contained, in a final volume of 1 mL, 200 µL of KH_2PO_4-KOH, 200 µL of 15 mM DOR, 200 µL of 500 µM $FeCl_3$, 100 µL of 1 mM EDTA, sample (100 µL, 1.5 mg/mL), H_2O_2 (100 µL, 10 mM), and 100 µL of 1 mM ascorbic acid. The reaction mixture was incubated at 37°C for 1 hour, after which 1 mL of 1% (wt/vol) thiobarbituric acid (TBA) was added to the mixture followed by addition of 1 mL of 2.8% (wt/vol) trichloroacetic acid. The solution was heated in a water bath at 80°C for 20 minutes to develop a pink color characteristic of malondialdehyde-(TBA)$_2$ adduct. This compound was then extracted into 2 mL of butan-1-ol. The absorbance measured at 532 nm was converted into percentage inhibition of DOR degradation.

Evaluation of lipid peroxidation inhibitory activity (LPIA)

A modified TBA-reactive absorbance assay was used to measure the lipid peroxide formed using egg yolk homogenate as lipid-rich medium.[16] Egg homogenate (0.5 mL, 10% [vol/vol]) was added to 0.1 mL of extract (1 mg/mL), and the volume was made up to 1 mL with distilled water. Then 0.05 mL of 0.07 mM $FeSO_4$ was added, and the mixture was incubated for 30 minutes. Thereafter 1.5 mL of acetic acid (pH 3.5, 20%) was added, followed by addition of 1.5 mL of 0.8% (wt/vol) TBA in sodium dodecyl sulfate (1.1%). The resulting mixture was vortex-mixed and heated at 95°C for 60 minutes. After cooling, 5 mL of butan-1-ol was added, and the mixture was centrifuged at 2,300 g for 10 minutes. The absorbance of the organic upper layer was measured at 532 nm and converted to percentage inhibition of lipid peroxidation.

TABLE 2. PHYTOCHEMICAL PROFILE OF EXTRACTS OF STUDY PLANTS

Plant	Phytochemical									
	Alkaloids	Saponins	Tannins	PN	Anth	Steroids	Terpenoids	Flavonoids	CG1	CG2
P. guajava	−	+	+	−	+	+	+	+	+	+
C. alata	+	−	+	+	+	+	+	−	+	+
M. lucida	+	−	+	−	−	+	+	+	+	+
A. boonei	+	+	+	−	−	+	+	+	+	+
G. cupidata	−	+	+	+	+	+	+	+	+	+
C. odorata	+	−	+	−	−	+	+	+	+	+
L. longipedunculata	+	+	−	−	−	+	+	−	+	+
S. mombin	−	+	+	+	+	+	+	+	+	+
O. gratissimum	+	−	+	+	−	+	+	+	+	−
M. lucida	−	+	+	+	−	+	+	+	+	+

PN, phlobatannins; Anth, anthraquinones; CG1, cardiac glycoside with unsaturated ring; CG2, cardiac glycoside with deoxy sugar. (−) = absent; (+) = present.

Evaluation of reductive potential (RP)

The method of Oyaizu[17] was used. Extract (1 mL, 150 μg/mL) was mixed with 2.5 mL each of phosphate buffer and potassium ferricyanide. The mixture was incubated at 50°C for 20 minutes. Trichloroacetic acid (2.5 mL, 10% (wt/vol)) was then added, and the mixture centrifuged at 1,000 g for 10 minutes. Thereafter, 2.5 mL of the upper layer of the solution was mixed with 2.5 mL of distilled water and 0.5 mL of 1% (wt/vol) $FeCl_3$. The absorbance was read at 700 nm. Higher absorbance values indicate higher RP of the extracts.

Statistical analysis

All statistical analyses were performed using the GraphPad InStat version 3 software (GraphPad InStat Software Inc., San Diego, CA, USA). Results are expressed as mean ± SEM values ($n = 3$). One-way analysis of variance was used for data analysis. Significant differences between groups were detected in the analysis of variance using Dunn's multiple-range test at $P < .05$. Statistical differences between mean values of individual tests were detected using independent-sample Student's t test.

RESULTS

Phenolics are one of the largest and the most widely studied groups of phytochemicals. They are widely reported to possess remarkable antioxidant and medicinal properties. Flavonoids account for approximately two-thirds of the phenolics in our diet[18] and are the major focus of researches investigating phenolics. Most of the antioxidant and medicinal properties credited to phenolics have been attributed to the flavonoids. TPC measures the total amount of phenolics, which include flavonoids. TFC is a specific test to quantify the amount of flavonoids. The TPC and TFC of extracts are gross indices of promising medicinal and nutritional benefits. DPPH and hydroxyl radical scavenging activities are different radical scavenging assays. The DPPH radical scavenging assay evaluates the ability of the extracts to quench the DPPH radical, whereas the hydroxyl radical scavenging assay evaluates the ability of extracts to inhibit DOR degradation by the hydroxyl radicals generated via Fenton's reaction.

Phytochemical screening gave positive results for steroids, terpenoids, and cardiac glycosides in all extracts. Alkaloids, tannins, and flavonoids were also detected in many of the extracts (Table 2). The results show that the studied plants

TABLE 3. PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITIES OF EXTRACTS OF STUDY PLANTS

Plant	TPC (mg/L GAE)	TFC (μg/mL QE)	DPPH (% scavenging activity)	NO (% scavenging activity)	DOR (% inhibition of degradation)	LPIA (%)	RP
P. guajava	380.08 ± 4.40^{a}	269.[illegible] ± 2.[illegible]^{a}	82.79 ± 2.84^{ab}	21.68 ± 1.51^{a}	50.60 ± 0.77^{ab}	70.82 ± 0.90^{a}	0.79 ± 0.04^{a}
C. alata	232.68 ± 2.54^{b}	275.16 ± 1.62^{a}	58.80 ± 2.02^{b}	35.01 ± 1.91^{ac}	50.52 ± 0.77^{ab}	61.15 ± 0.13^{b}	0.34 ± 0.01^{b}
M. lucida	139.17 ± 3.49^{c}	76.[illegible] ± 0.93^{b}	24.86 ± 0.85^{c}	26.57 ± 0.27^{acd}	41.09 ± 0.62^{acd}	51.91 ± 0.48^{cd}	0.27 ± 0.00^{b}
A. boonei	83.65 ± 1.49^{d}	44.43 ± 2.02^{d}	41.58 ± 1.43^{d}	44.88 ± 0.55^{bc}	53.84 ± 0.82^{ab}	47.16 ± 0.59^{cd}	0.32 ± 0.01^{b}
G. cupidata	306.20 ± 4.99^{e}	103.99 ± 1.32^{e}	77.79 ± 2.67^{a}	38.69 ± 1.01^{bcd}	63.84 ± 0.97^{b}	69.97 ± 0.68^{a}	0.52 ± 0.01^{c}
C. odorata	213.35 ± 8.43^{b}	27[illegible] ± 2.32^{a}	34.62 ± 1.19^{e}	28.37 ± 1.07^{acd}	56.53 ± 0.86^{ab}	62.60 ± 0.25^{bc}	0.33 ± 0.02^{b}
L. longipedunculata	55.72 ± 2.43^{f}	20.65 ± 2.16^{f}	25.66 ± 0.88^{cd}	43.90 ± 0.04^{bcd}	20.37 ± 0.31^{cd}	53.69 ± 2.21^{bc}	0.26 ± 0.00^{b}
S. mombin	328.56 ± 11.37^{e}	227.96 ± 3.46^{b}	88.58 ± 3.04^{a}	42.95 ± 0.85^{bcd}	55.81 ± 0.85^{ab}	54.03 ± 1.30^{bc}	0.62 ± 0.02^{d}
O. gratissimum	227.35 ± 2.57^{b}	228.84 ± 1.43^{b}	77.81 ± 2.67^{a}	30.57 ± 1.61^{acde}	27.50 ± 0.42^{cd}	59.12 ± 2.23^{bc}	0.55 ± 0.01^{cd}
M. lucida	113.90 ± 2.5[illegible]^{g}	196.00 ± 3.11^{g}	21.37 ± 0.73^{e}	31.33 ± 0.41^{acde}	7.76 ± 0.12^{e}	38.74 ± 1.99^{d}	0.27 ± 0.00^{b}

Data are mean ± SEM values ($n = 3$).
Data with the same superscript letters in a column are not significantly different ($P > .05$).

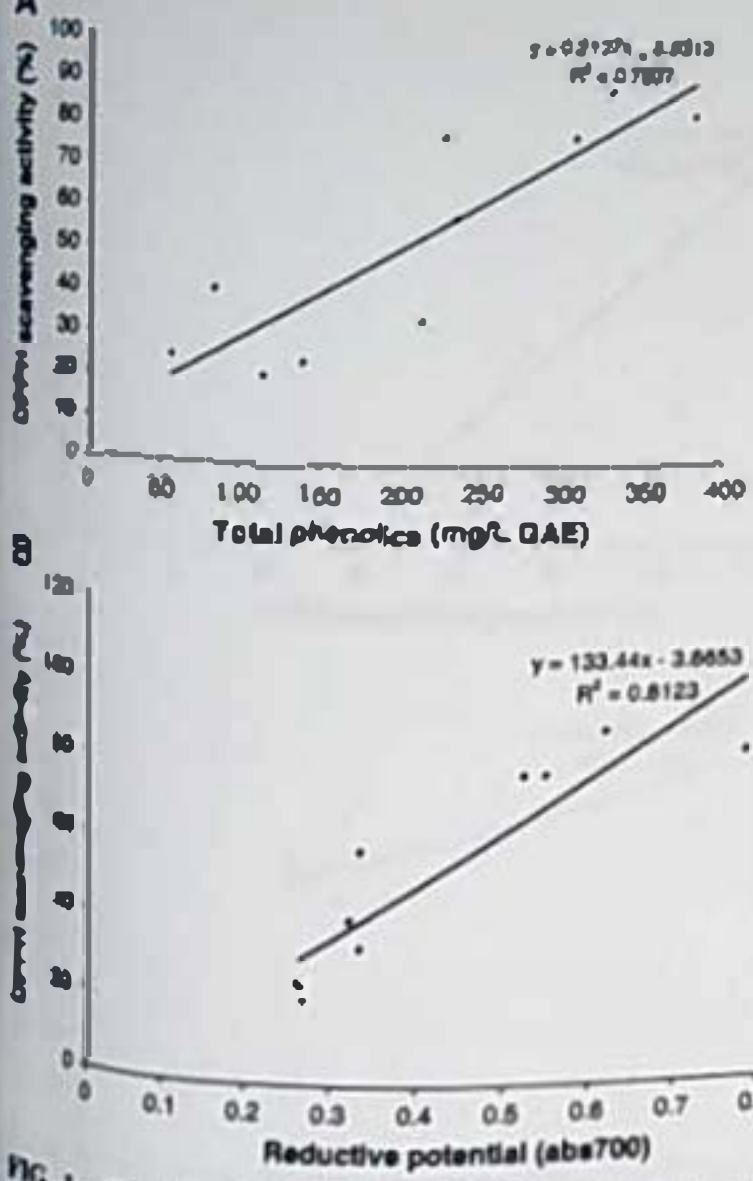

FIG. 1. Relationship between (A) DPPH scavenging activity (%) versus total phenolics (in mg/L GAE) and (B) DPPH scavenging activity (%) versus RP (absorbance at 700 nm [abs700]).

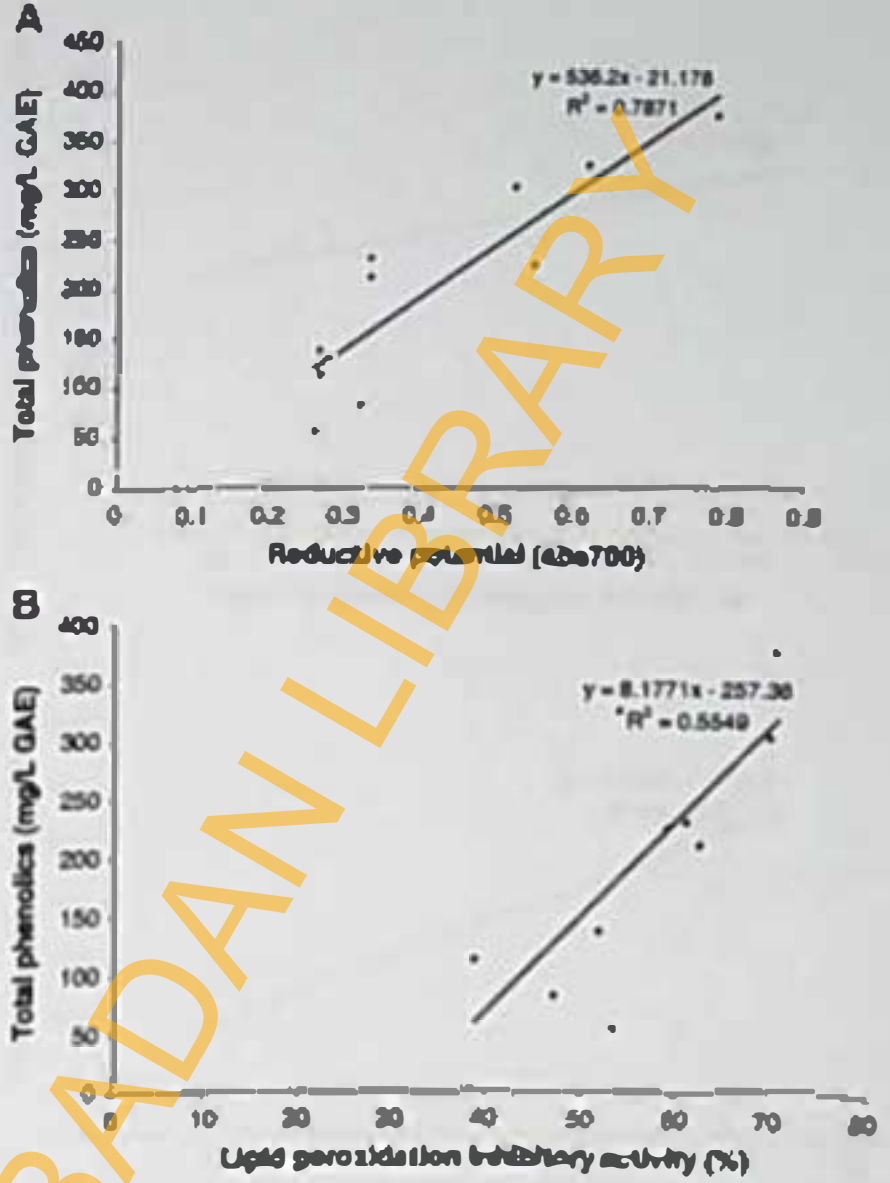

FIG. 2. Relationship between (A) total phenolics (mg/L GAE) versus RP (absorbance at 700 nm [abs700]) and (B) total phenolics (in mg/L GAE) versus LPIA (%).

are rich in diverse phytochemicals, which are probably responsible for their medicinal properties (Table 1).

P. guajava extract showed consistently high values in all assays except NO (21.68 ± 1.51%), where it had the least value among all the studied plants. It had the highest values for TPC (380.08 ± 4.40 mg/L GAE), LPIA (70.82 ± 0.90%), and RP (0.79 ± 0.04). In the DPPH and TFC assays, its values were not significantly different from those of the extracts of *S. mombin* (88.58 ± 3.04%) and *C. alata* (275.16 ± 1.62 μg/mL QE), which recorded the highest values, respectively (Table 3).

S. mombin and *G. cupulata* were second and third, respectively, behind *P. guajava* in order of ranking.

C. alata and *O. gratissimum* also have high values of antioxidant indices in many of the assays. The NO (44.88 ± 0.55%) value for *A. boonei* is remarkably higher than the values for the other plant extract. *C. odorata* showed a remarkably high value for TFC (272.1 ± 2.32 μg/mL QE) and hydroxyl radical scavenging activity (56.53 ± 0.86%). The NO value for *S. longepedunculata* was high (41.90 ± 0.04%). Only *M. lucida* appears to have consistently low values in the assays.

The correlation coefficients confirm that there is a high level of agreement between pairs of some of the assays (Figs. 1–4). DPPH assay had an extremely significant correlation with total phenolic content ($r^2 = 0.76$, $P = .001$) and RP ($r^2 = 0.81$, $P < .05$) (Fig. 1) and a significant correlation with LPIA ($r^2 = 0.41$, $P < .05$). There was also an excellent significant correlation between TPC and RP ($r^2 = 0.79$, $P = .0006$) and a significant correlation between TPC and LPIA ($r^2 = 0.55$, $P = .01$) (Fig. 2). A significant correlation was also observed between TPC and TFC content ($r^2 = 0.43$, $P < .05$) (data not shown). A fair correlation was observed between LPIA and DPPH ($r^2 = 0.50$, $P < .05$) and LPIA and RP ($r^2 = 0.40$, $P < .05$) (Fig. 3), whereas a low correlation was observed between hydroxyl radical scavenging activity and LPIA ($r^2 = 0.31$) and hydroxyl radical scavenging activity and TPC ($r^2 = 0.33$; $P > .05$) (Fig. 4). The values of *P. guajava*, *S. mombin*, *G. cupulata*, *C. alata*, and *O. gratissimum* for DPPH free radical scavenging capacity, TPC, LPIA, and RP reflect this observation. The trend of the results in the four assays for the five plants is apparently the same. However, the levels of agreement between some other pairs of assay methods are insignificant (Tables 3 and 4).

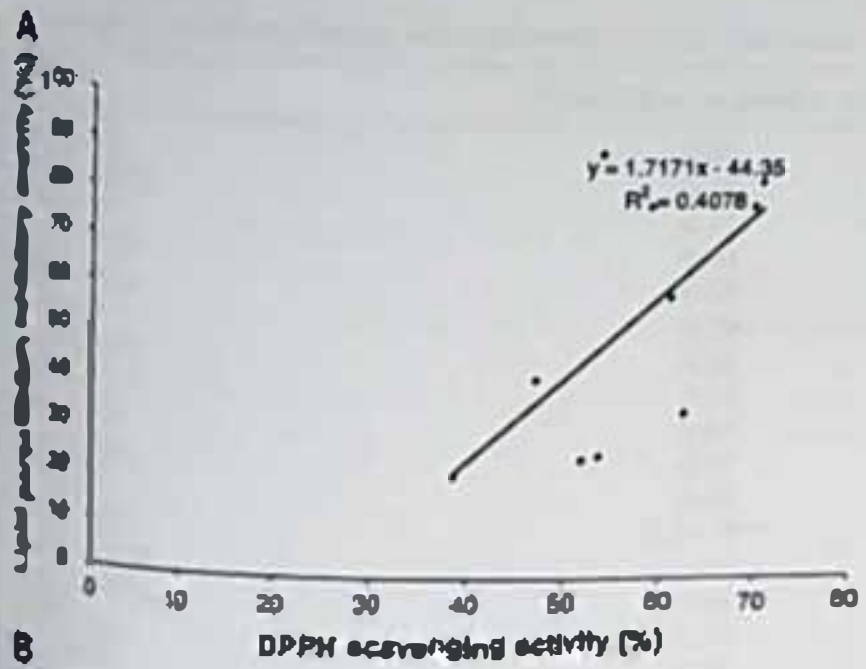

FIG. 3. Relationship between (A) LPIA (%) versus DPPH scavenging activity (%) and (B) LPIA (%) versus RP (absorbance at 700 nm (ab700)).

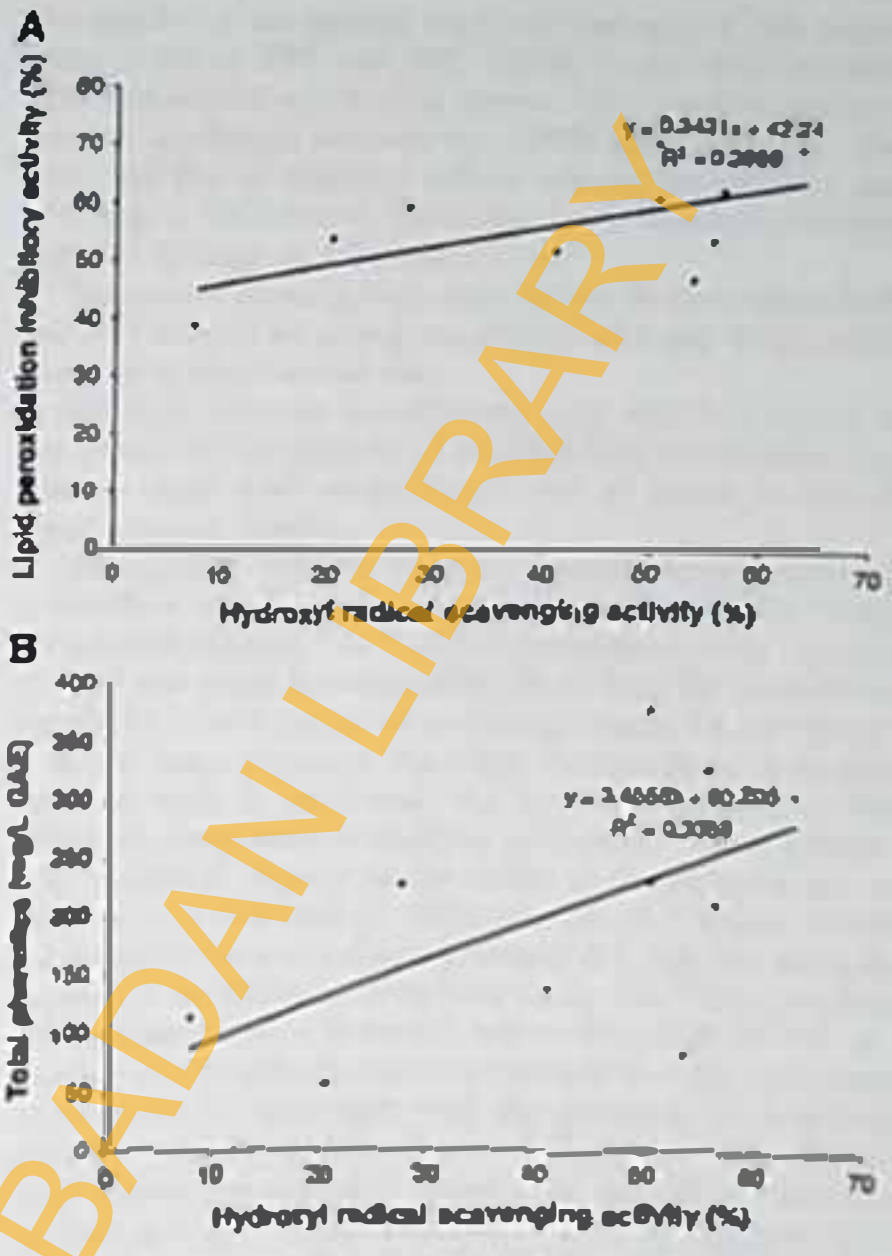

FIG. 4. Relationship between (A) hydroxyl radical scavenging activity (%) versus LPIA (%) and (B) hydroxyl radical scavenging activity (%) versus total phenolics (in mg/L GAE).

DISCUSSION

It is well recognized in plant chemistry that the mode of preparation and administration of herbal remedies are often crucial variables in determining efficacy for pharmacological evaluations.[13] In the traditional use of these plants, decoctions or infusions of the relevant parts are usually made with either water or alcohol as the solvent. The nature of solvent may influence the medicinal or other effects exhibited by plants because solvents extract antioxidant components to different degrees.

Antioxidant activity in higher plants has often been associated with phenolic compounds.[19] In addition to their roles in plants, phenolic compounds in our diet may provide health benefits associated with reduced risk of chronic diseases.[18] Flavonoids are the largest group of phenolics. They have been identified in fruits, vegetables, and other plant parts and linked to reducing the risk of major degenerative diseases. More than 4,000 distinct flavonoids have been identified.[18] The antioxidant activity of plant extracts has been reported to correlate with their phenolic content.[20,21]

Data from the present work indicate that this correlation is dependent on the nature of the antioxidant assay used. The results of this work clearly illustrate that different methodologies can provide completely different responses with respect to the antioxidant capacity of a pure compound or a mixture of compounds.

Significant correlations were observed between some of the assay methods. DPPH free radical scavenging activity had an excellent correlation with TPC and RP (Fig. 1). These three methods have a similar underlying mechanism of reaction. The DPPH assay evaluates antioxidant activity by testing the ability of compounds to act as free radical scavengers or hydrogen donors.[22] The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They also have a metal chelating potential.[23] The RP assay also has to do with the redox properties of substances being investigated. Each assay or group of assays with a similar underlying mechanism may be specific for a particular group of antioxidant substances, and where this group occurs in a substantial amount, such

Table 4. Level of Correlation Between Assay Methods

Assays	Correlation coefficient (r^2)
TPC/TFC	0.43
DPPH/TPC	0.76
TPC/NO	0.12
TPC/DOR	0.34
TPC/LPIA	0.55
TPC/RP	0.79
DPPH/TFC	0.21
TFC/NO	0.29
TFC/DOR	0.03
TFC/LPIA	0.13
TFC/RP	0.22
DPPH/NO	3.0×10^{-5}
DPPH/DOR	0.27
LPIA/DPPH	0.41
DPPH/RP	0.81
NO/DOR	0.01
LPIA/NO	0.10
LPIA/RP	0.40
LPIA/DOR	0.40
RP/NO	0.07
RP/DOR	0.15

tests will yield high values. Exceptions can occur where these groups are bound or masked, leading to their non-detection by the specified assay(s). There are different types of antioxidants in plants. There are metals like selenium, vitamins such as ascorbic acid, and phytochemicals such as carotenoids, phenolics, organosulfur compounds, and nitrogen-containing compounds. The nature and position of functional groups in some antioxidant compounds, *e.g.*, the hydroxyl groups of flavonoids, influence their reactivity and consequently their activity.

Odabasoglu *et al.*[24] reported that there was no correlation between antioxidant activity and TPC of extracts of some lichen species, a contradiction to previous reports.[20,21] The present investigation also clearly contradicts this submission of Odabasoglu *et al.*[24] There were strong correlations between TPC on the one hand and DPPH, RP, and LPIA assays on the other ($r^2 = 0.76$, 0.81, and 0.55, respectively). Odabasoglu *et al.*,[24] however, reported a strong correlation between reducing power and total antioxidant activity. The present investigation also revealed significant correlations between reducing power on one hand and DPPH and TPC on the other. The authors noted that individual phenolics may have distinct antioxidant activities, and there may be antagonistic or synergistic interactions between phenolics and other compounds like carbohydrates and proteins.

Miliauskas *et al.*[23] reported a good correlation between antiradical activity (DPPH) and TPC. Our finding in the present work is in harmony with theirs. The results of the present work also confirm their findings that there was low correlation between TFC and DPPH assay and between TPC and TFC. The values for the correlation coefficients between TPC and DPPH assay and between TPC and TFC in our own study ($r^2 = 0.21$ and 0.43, respectively) were similar to those obtained by Miliauskas *et al.*[23] (0.32 and 0.43, respectively).

The results of the present work showed only a low correlation between TFC and TPC (Table 4) and also between TFC and radical scavenging assays. For example, the correlation coefficient between the DPPH assay and TFC was 0.21, and that of hydroxyl radical scavenging capacity and TFC was 0.03 (Table 4). These results are also in agreement with the findings of Miliauskas *et al.*[23]

The present investigation goes further to show that DOR and NO showed no strong correlation with any of the other assays that were carried out.

Although previous investigators used very few assays or few plants for the purpose of investigating correlations, the present study used seven assays and 10 plants to ensure more accurate results.

Investigators need to be more specific when reporting antioxidant activities of phytochemicals. Terms like "total antioxidant capacity" or "total antioxidant activity" are too general and could be misleading. Tests used for assessment should be clearly indicated to leave no room for ambiguity.

It has been observed that only flavonoids of a certain structure and, in particular, the hydroxyl position in the molecule determine antioxidant properties. These properties, in general, depend on the ability to donate hydrogen or electron to a free radical. Miliauskas *et al.*[23] found, in the same study, some correlation between TPC and flavonoids. In support of the above observations, Choi *et al.*[26] reported that the interaction of a potential antioxidant with DPPH depends on its structural conformation and that this structural requirement is correlated with the presence of hydroxyl groups on the flavonoids. Cos *et al.*[27] reported that allopurinol showed remarkable activity in inhibiting xanthine oxidase and scavenging superoxide radical, whereas tartrifoline showed relatively weak activity. The difference in activities was attributed to variation in the location of the hydroxyl groups and double bonds.

Choi *et al.*[26] found that the scavenging activity of flavonoids on peroxynitrite was governed by the position of the hydroxyl group. *o*-Hydroxyl structures increased the scavenging activity on peroxynitrite. Structural comparison of the flavonols in their study and their scavenging activities clearly shows that the C.3 hydroxyl group plays a pivotal role in the observed scavenging activity. These authors inferred that the higher scavenging potency of galangin compared with galangin 3-*O*-methyl ether may suggest that C.3 methoxylation reduced the scavenging effect of flavonols.

Phytochemicals are complex in nature. Therefore, the antioxidant activities of plant extracts cannot be evaluated by only a single method. The antioxidant defense system of the body is composed of different antioxidant components. The antioxidant capacities of these antioxidant components depend upon which free radicals or oxidants are present in the body.[28] The various methods used in evaluating the antioxidant activity of samples can give varying results depending on the specificity of the free radical being used as a reactant.[28] The results of the present work highlight the diversity and complexity of phytochemicals present in plant extracts and the specificity of different assay protocols for

specific antioxidant species based on the mechanism of reaction.

The DPPH assay appears to be a reliable method of measuring total antioxidant capacity of substances or systems. Its values correlated well with about three other methods used in this study (Fig. 1). As noted by Prakash,[22] it is rapid, simple, and inexpensive, and its value applies to the overall antioxidant capacity of the sample and is not specific to any particular antioxidant component.

Using the frequency of high antioxidant capacity values as the basis, results obtained in this work reveal that *P. guajava*, *S. mombin*, and *G. cupulata* are the plants that demonstrated consistent high activities in the various assays, followed by *C. alata* and *O. gratissimum*. It must be noted that some of the remaining plants have higher activities than the ones in the list above in some of the assays. For example, the NO radical scavenging activity of *A. booneí* (44.88 ± 0.55%) was higher than that of *P. guajava* (21.68 ± 1.51%) and *O. gratissimum* (30.576 ± 1.61%) ($P < .05$).

Overall, the results obtained in this study indicate that Nigerian indigenous medicinal plants could be a source of natural chemoprophylactic antioxidants against reactive oxygen species and as such could be relevant in the treatment of cardiovascular disease, cancer, arthritis, and other pathologies in which free radical mechanisms have been implicated. In view of the potential beneficial properties of the studied plants, our results warrant further investigations on the identification of novel chemoprophylactic compounds in these plants.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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Ramipril-Like Activity of *Spondias Mombin* Linn Against No-Flow Ischemia and Isoproterenol-Induced Cardiotoxicity in Rat Heart

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Abstract The cardioprotective property of *Spondias mombin* (SM) was investigated and compared with that of the ACE inhibitor, ramipril. Alterations to markers of myocardial injury and indices of antioxidant capacity by isoproterenol (ISP) intoxication were significantly corrected in groups treated with SM. The inflammatory index was increased by 24% in ISP-intoxicated group compared with control ($P < 0.001$) but reduced in the groups administered ISP and treated with 100 or 250 mg/kg SM by 17% ($P < 0.001$) and 11% ($P < 0.05$) respectively. Serum lactate dehydrogenase activity and cholesterol level which were significantly increased in ISP-intoxicated group compared with control were reduced in groups administered ISP and treated with SM. Serum phosphate levels in groups administered ISP and treated with SM were significantly lower than values obtained for the ISP-intoxicated group ($P < 0.001$). Tissue catalase and superoxide dismutase activities as well as glutathione level were significantly increased in groups administered ISP and treated with SM compared to ISP-intoxicated group while MDA and nitrite levels were decreased. Disruption in the structure of cardiac myofibrils by ISP intoxication was reduced by treatment with SM. Comparable results were obtained for ramipril. These results are indicative of the potent cardioprotective property of SM.

Keywords *Spondias mombin* · Cardiotoxicity · Ischemia · Isoproterenol · Ramipril · Antioxidant

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Introduction

Cardiovascular diseases (CVD) include coronary heart disease, cerebrovascular disease, hypertension, peripheral artery disease, rheumatic heart disease, and congenital heart disease. Globally, cardiovascular diseases are the number one cause of death, and the situation is not expected to change in the foreseeable future. An estimated 17.5 million people died from cardiovascular diseases in 2005, representing 30% of all global deaths [1]. Acute myocardial ischemia (ischemic heart disease) accounts for the highest percentage of morbidity and mortality in the Western world [2]. Myocardial ischemia results in ATP depletion and accumulation of toxic metabolites, whereas reperfusion leads to the production of reactive oxygen intermediates and calcium overload. The alterations in cellular metabolism and generation of toxic molecules contribute to myocardial ischemia/reperfusion injury [3]. Myocardial ischemia when severe and sustained for prolonged periods results in myocardial infarction [4].

Plants contain many bioactive compounds which counteract free radical mediated toxicity. Inhibition of free

radical generation is usually employed as a facile system to screen for chemotherapeutic agents. A systematic search for useful bioactivities from medicinal plants is considered to be a rational approach in nutraceutical and drug research. Bioprospecting for new plant-derived drugs has been on the increase in recent times because these drugs have fewer side effects than the synthetic ones [5] and many important leads are being discovered [6].

Spondias mombin (SM) is a tree that is native to Africa [7] but also found in other continents of the world. In parts of South western Nigeria, it is used in traditional medicine for the management of diabetes mellitus, the treatment of psychiatric disorders and to gain and retain good memory [8–10]. In other parts of the country, it is used as an aphrodisiac and to treat gonorrhea, fibroid, fever, and other ailments [11]. *Spondias mombin* is also widely used for the management of various diseases in other parts of the world [12–16]. Various pharmacological properties of the plant have been described. These include antioxidant, antimicrobial, antiprotozoal, spasmolytic, abortifacient, antidiabetic, sedative, anticonvulsant, and antipsychotic properties [17–21]. However, there is paucity of reports on its cardioprotective property. In the present study, the cardioprotective property of the plant has been investigated using the ex vivo non-recirculating Langendorff technique and the in vivo isoproterenol-induced myocardial infarction model and compared with that of the ACE inhibitor ramipril.

Materials and Methods

Chemicals and Reagents

Isoproterenol (ISP), ramipril, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide (NADH), sulphosalicylic acid (SSA), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), trichloro acetic acid (TCA), and sulphanilamide were obtained from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals and reagents used were of analytical grade.

Experimental Animals

Adult male Sprague–Dawley (SD) rats weighing 150–200 g were procured from National Animal Laboratory Centre (NALC) of Central Drug Research Institute (CDRI), Lucknow. Animal experiments were conducted after obtaining approval and in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC). Rats were housed in an air-conditioned room and kept in standard laboratory conditions under a 12-h light–dark cycle.

Preparation of Extract

Leaves of *Spondias mombin* (SM) were obtained from farmlands in Akure, South-Western Nigeria and authenticated in the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure. They were dried under active ventilation at room temperature, packed in paper bags and stored. The plant material was later pulverized with a Retsch Muhle blending machine. The powdered sample was extracted by maceration in a methanol aqueous mix (4:1). The methanol filtrate was evaporated in vacuo to give the crude methanolic extract which was used for the assessment of cardioprotective properties.

Anti-Ischemic Studies Using the Langendorff Technique

Extract of SM was investigated for per se and anti-ischemic effects on isolated hearts of male SD rats using the Langendorff non-recirculating technique. Ramipril (10 μM) and nifedipine (1 μM) were used as control standard drugs.

Preparation of Normal HEPES Tyrode (NHT) Buffer

The composition of the physiological salt solution NHT buffer in mM was as follows: NaCl 137, KCl 5.4, HEPES (N-[2-hydroxyethyl] piperazine-N′-2-ethanesulphonic acid) buffer 3.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0, and glucose 11.1. For the preparation of 1 l of NHT buffer, $CaCl_2$ and $MgCl_2$ were dissolved separately in triple distilled water (TDW). The remaining reagents were dissolved separately also in TDW. The two solutions were mixed, and the volume was made up to 1 l. The pH was adjusted to 7.4 using 1 M NaOH. Fresh buffer was prepared on each day of the experiment. Both buffer and solution of extract in buffer were filtered through a 0.22-μM Millipore filter before use.

Experimental Procedure

The animals were anesthetized with chloral hydrate and exsanguinated. Hearts were rapidly excised and rinsed in ice cold perfusion buffer. They were perfused retrogradely through an aortic cannula in the Langendorff mode in a non-recirculating manner at a constant pressure of 80–90 mmHg and a constant temperature of 37°C with continuously oxygenated NHT buffer. The perfusing solutions were led through glass coils enclosed within glass jackets through which warm water was pumped by a recirculating pump (Digitherm circulating pump, Hungary). Spontaneously beating hearts were given a resting tension of 2 g, and contractions were recorded through a force displacement transducer (FT 03, GRASS Instruments Company) on a GRASS polygraph. For the evaluation of ICE as effect,

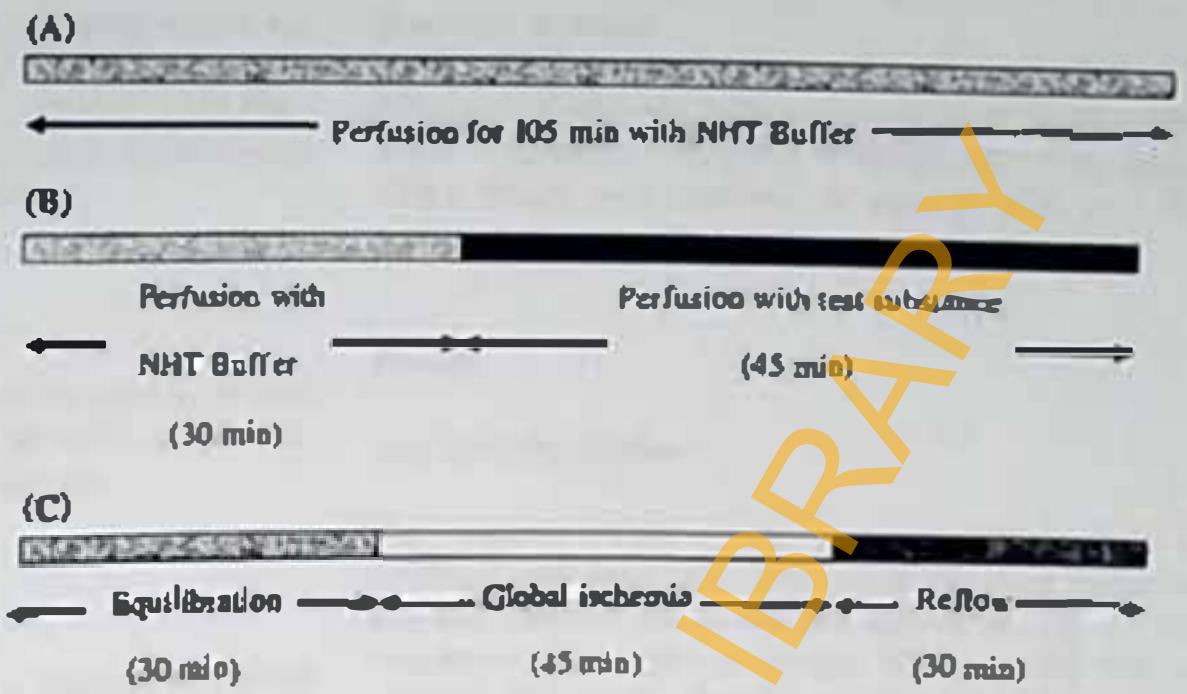

Fig. 1 Experimental protocols for ex vivo cardioprotective studies using the Langendorff technique

after 30 min of equilibration in which perfusion was done with the NHT buffer, the perfusion medium was switched to the solutions of extract or standards (Fig. 1b). Values of cardiac tension (amplitude) and heart rate (HR) for test substances were measured and expressed as percentages of the values for the NHT buffer. For the anti-ischemic study, 30 min of equilibration was followed by 45 min of global (no-flow) ischemia. Reperfusion was carried out for 30 min with NHT buffer or solutions of test substances (Fig. 1c).

Protective Effect Against Isoproterenol-Induced Myocardial Infarction

SM was investigated for in vivo cardioprotective property using the model of isoproterenol-induced myocardial infarction. Ramipril (1.25 mg/kg) was used as the control standard drug.

Experimental Procedure

Male SD rats weighing 150–200 g were randomly allocated to five main groups with $n \geq 9$ animals in each group.

Group 1 (control): Rats received equal volumes of vehicle and had free access to standard pellet diet and water for a month.

Group 2 (ISP): The animals were treated as in Group 1 for a month and in addition received isoproterenol (ISP) on day 29 and 30 at an interval of 24 h.

Group 3 (SM): This was subdivided into two groups

Group 3a (SM 100 mg): animals were administered 100 mg/kg SM

Group 3b (SM 250 mg): animals were administered SM 250 mg/kg

The respective dose of *Spondias mombin* were suspended in 0.2% carboxy methyl cellulose (CMC) and orally fed to the animals once daily.

Group 4 (ISP-challenged, SM-treated group): This group also has two subgroups

Group 4a: (SM 100 mg + ISP)
Group 4b: (SM 250 mg + ISP)

In addition to receiving the treatment given to animals in Group 3, animals in this group received ISP (85 mg/kg) on days 29 and 30.

Group 5 (Ramipril + ISP): Animals in this group were administered ramipril (1.25 mg/kg) and also ISP on days 29 and 30.

SM and ramipril were administered orally while the administration of ISP was done subcutaneously.

Twenty-four hours after the second dose of ISP, animals were anesthetized. Blood was withdrawn by retroorbital vein puncture and used for the estimation of glucose, plasma level of glutathione (GSH), serum lactate dehydrogenase (LDH) activity, and serum cholesterol, phospholipid and malondialdehyde (MDA) concentrations. Rats were sacrificed, hearts excised, freeze in liquid nitrogen and stored at −85°C until used for biochemical analyses.

Hearts stored in liquid nitrogen were weighed. A 10% homogenate was prepared in 50 mM phosphate buffer (pH 7.4). An aliquot was used for the assay of GSH and MDA. The homogenate was centrifuged at 15,000 × *g* at 4°C for 15 min, and the supernatant was used for the estimation of glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and protein.

Myocardial tissue was perfused with saline followed by 10% formaldehyde and then fixed in 4% paraformaldehyde for 24 h. The tissues were routinely processed and embedded

in paraffin. Serial sections were cut, and each section was stained with hematoxylin and eosin. The stained sections were examined under a microscope (Leica DFC 320 Fluorescent microscope, type DM 5000B. Leica Microsystems Ltd) and photomicrographs were taken.

Anti-Inflammatory Potential

The heart weight/body weight ratio of the animals in each group was determined and used as an index of the anti-inflammatory potential of SM and Ramipril.

Biochemical Estimations

Blood glucose was estimated using a glucometer (Accu-chek® Active) with strips supplied by the manufacturer. Serum cholesterol and phosphate were analyzed using the Beckman Coulter Synchron CX9 Pro clinical system with kits supplied by the manufacturer. GSH was estimated according to the method of Anderson [22]. MDA was estimated according to the method of Colado et al. [23]. Nitrite was estimated using the Griess diazotization reaction [24]. Protein was estimated according to the method of Lowry et al. [25].

Superoxide Dismutase

SOD was estimated using the method described by Kakkar et al. [26].

Catalase

Catalase catalyzes the decomposition of H_2O_2 into water and oxygen. The assay for the enzyme was carried out by continuous spectrophotometric rate determination. Briefly, 2.95 ml of buffered substrate (30% H_2O_2 in 10 ml of 50 mM phosphate buffer) was pipetted into a 3-ml cuvette, and the reaction was started at 37°C by the addition of the homogenate. Ten cycles of 15 s each was run at 240 nm. Catalase activity was calculated by dividing the change in absorbance per min by the mg of protein present in the enzyme source.

Lactate Dehydrogenase (LDH)

Serum LDH was estimated according to the method of Seth et al. [27]. Sodium pyruvate (1.84 ml) was added to 10 µl of the sample, and the mixture was incubated at 37°C for 10 min. Then, 50 µl of NADH was added, and the change in absorbance was followed for 3 min at 340 nm.

Statistical Analyses

All statistical analyses were performed using the GraphPad Prism 4 software (GraphPad Software Inc., San Diego, USA). Results were expressed as mean ± SEM. $P < 0.05$ was considered significant.

Results

Anti-Ischemic Studies

The intrinsic effects (evaluated as cardiac tension or amplitude and heart rate) (Fig. 1a) of the NHT buffer on perfused isolated rat hearts are shown in Fig. 2. The evaluation of the per se effects of the buffer was to ascertain that results obtained on treatment with test substances which were dissolved in the buffer were free from interference by the perfusion medium.

Table 1 shows the inotropic (cardiac tension or amplitude) and the chronotropic (heart rate) effects of the extract and standards on isolated rat hearts. The results obtained for the extract is comparable to that of ramipril while nifedipine significantly decreased the amplitude.

Forty-five minutes of global ischemia followed by reflow resulted in the significant reduction of the amplitude of hearts perfused with the NHT buffer alone (control) at both 15- and 30-min post-ischemia (Table 2). *Spermilus mamilla* was effective in reversing the decline in the cardiac tension produced by global (no-flow) ischemia. There

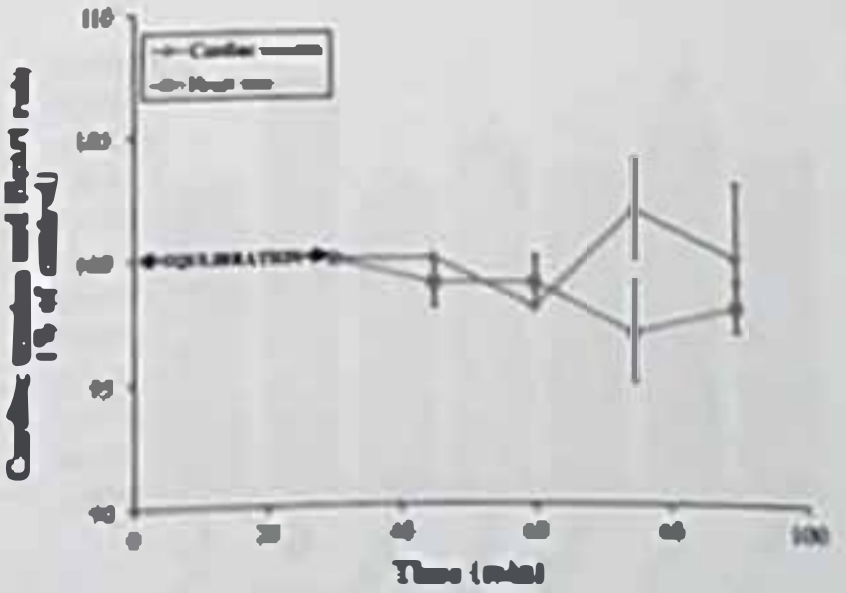

Fig. 2 Cardiac tension and heart rate following perfusion with NHT buffer for 90 min. The cardiac parameters were recorded (n = 6). Values of cardiac tension and heart rate obtained at 30 min were taken as the baseline (as control) and designated 100%. Values obtained at other time intervals were calculated as percentages of the baseline values. There were no significant differences in the values of cardiac tension or heart rate at the different time points compared with their control ($P < 0.05$)

Table 1 Per se effects of extract and standards

Reperfusion medium (concentration)	Control		Test compounds					
	AMP	HR	15′		30′		45′	
			AMP	HR	AMP	HR	AMP	HR
SM (0.001 g/l)	100	100	87 ± 9	92 ± 6	78 ± 9	86 ± 5	84 ± 11	86 ± 1
SM (0.005 g/l)	100	100	110 ± 2	93 ± 4	118 ± 1	88 ± 3*	ND	ND
SM (0.01 g/l)	100	100	119 ± 6	90 ± 5	121 ± 7	83 ± 5	121 ± 7	80 ± 4*
SM (0.02 g/l)	100	100	89 ± 4*	104 ± 3	98 ± 5	95 ± 5	110 ± 4*	100 ± 7
Ramipril (10 μM)	100	100	101 ± 3	92 ± 6	105 ± 5	86 ± 5	107 ± 9	80 ± 3
Nifedipine (1 μM)	100	100	47 ± 14	81 ± 10	57 ± 13*	83 ± 11	54 ± 13	88 ± 19

Results are presented as mean ± SEM ($n = 6$)

AMP Amplitude, *HR* Heart rate, *15′*, *30′*, *45′* 15th, 30th, 45th min of reperfusion, *SM* Spondias mombin, *ND* not determined

* Significantly different from control ($P < 0.05$)

Table 2 Global (no-flow) ischemia followed by reflow with buffer or extract

Compound used for reflow	Pre-ischemic value		Post-ischemic value			
	Amp	Rate	15′		30′	
			Amp	Rate	Amp	Rate
NHT Buffer	100 ± 0	100 ± 0	66 ± 6*	113 ± 10	73 ± 8*	109 ± 3
SM (0.005 g/l)	100 ± 0	100 ± 0	122 ± 3*	110 ± 19	122 ± 7*	104 ± 10
SM (0.01 g/l)	100 ± 0	100 ± 0	87 ± 28	105 ± 4	130 ± 17	104 ± 7
SM (0.02 g/l)	100 ± 0	100 ± 0	84 ± 21	141 ± 7	121 ± 4*	113 ± 9

Results are presented as mean ± SEM ($n = 6$). *15′*, *30′* 15th, 30th min of reperfusion

* Significantly different from pre-ischemic value

Significantly different from NHT Buffer ($P < 0.05$)

were no statistically significant differences in heart rate in the NHT perfused and *Spondias mombin*-treated groups at 0.001 and 0.02 g/l at all the time points. The reduction in heart rate shown in ramipril- and nifedipine-treated groups was not significant ($P > 0.05$), while that shown by SM (0.005 g/l) at the 30th min post-ischemia and SM (0.01 g/l) at the 45th min post-ischemia was significant ($P < 0.05$).

Protective Effect of SM Against Isoproterenol-Induced Cardiotoxicity

Figure 3 shows that ISP administration led to an increase in the heart weight/body weight ratio indicating a possible inflammation of the heart. SM extract and ramipril were able to reduce the increased heart weight/body weight ratio. Administration of the plant extract alone had no adverse effect on the heart weight/body ratio.

Figures 4 and 5 illustrate the disruption caused by ISP administration to the antioxidant defense system of the animals. The endogenous antioxidant GSH was depleted

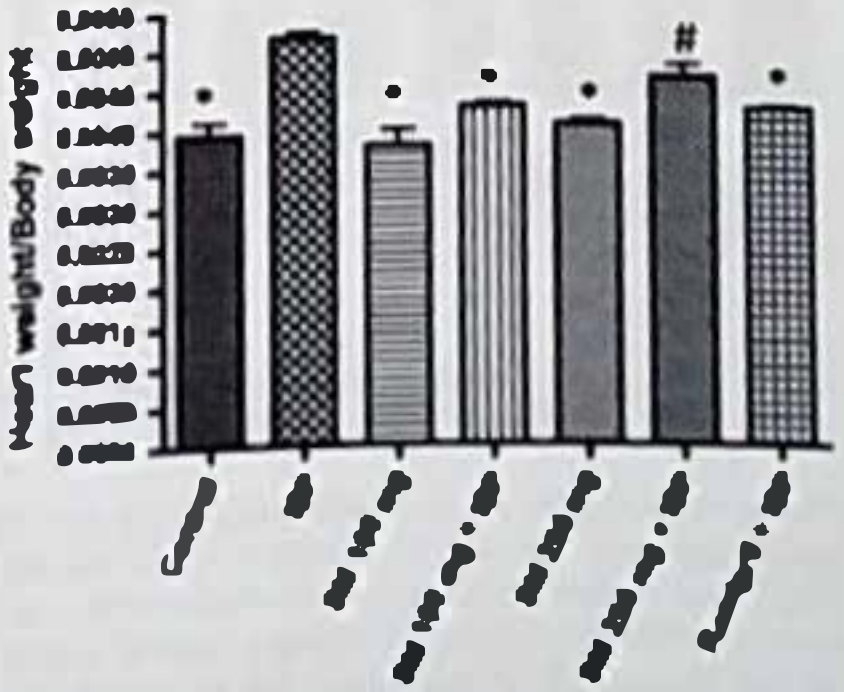

Fig. 3 Heart weight/body weight ratio of experimental animals. Results are presented as mean ± SEM ($n = 6$). * significantly different from ISP ($P < 0.001$). # significantly different from ISP ($P < 0.05$)

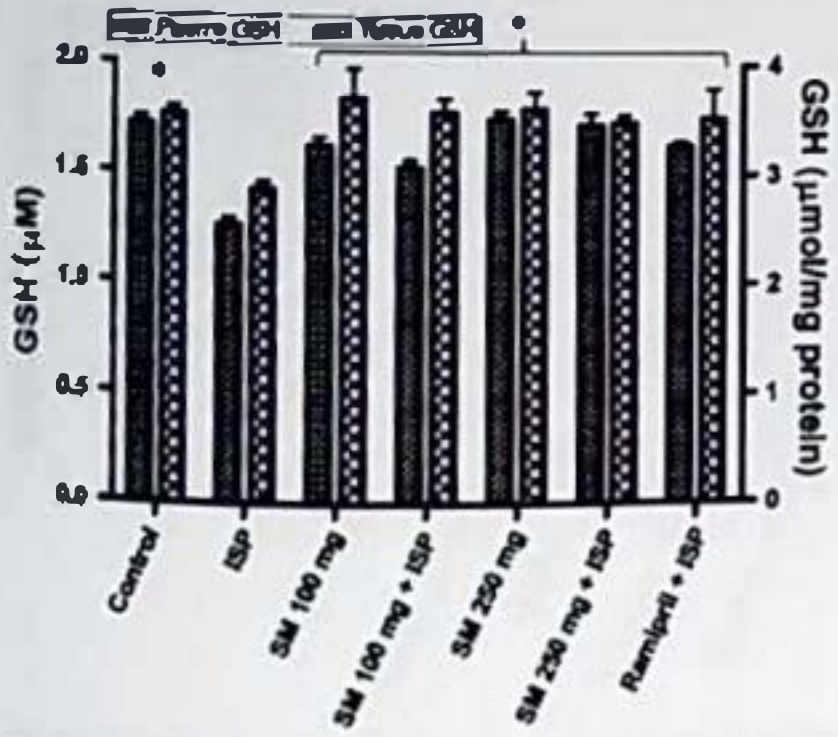

Fig. 4 Plasma GSH (μM) and tissue GSH (μmol/mg protein) levels in control and test groups. Results are presented as mean ± SEM ($n = 5$). *Significantly different from ISP ($P < 0.05$)

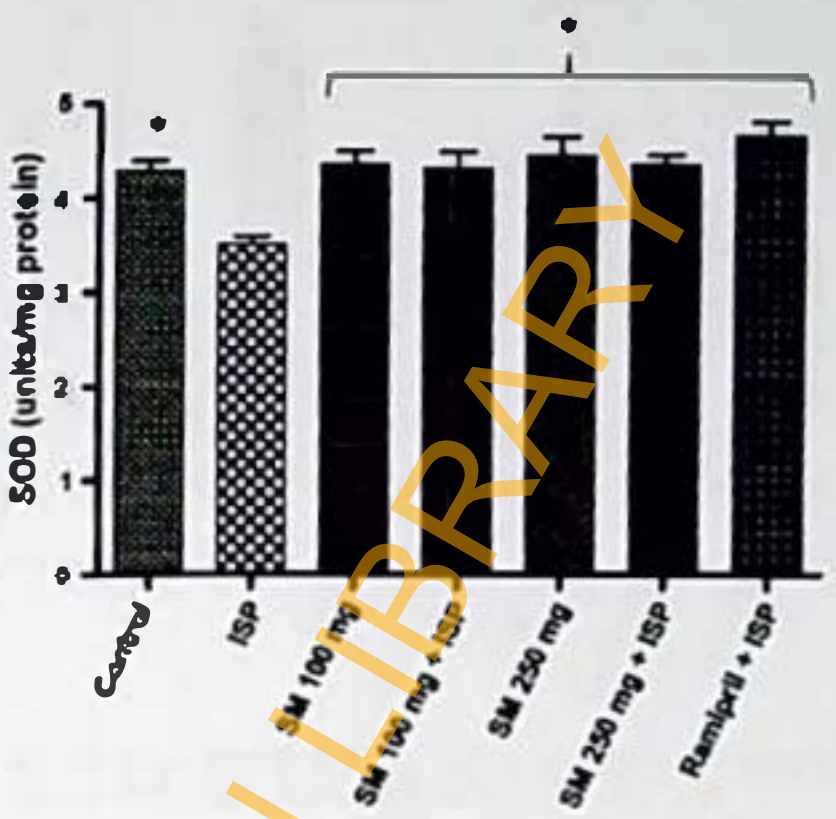

Fig. 6 Tissue SOD activity. Results are presented as mean ± SEM ($n = 5$). *Significantly different from ISP ($P < 0.05$)

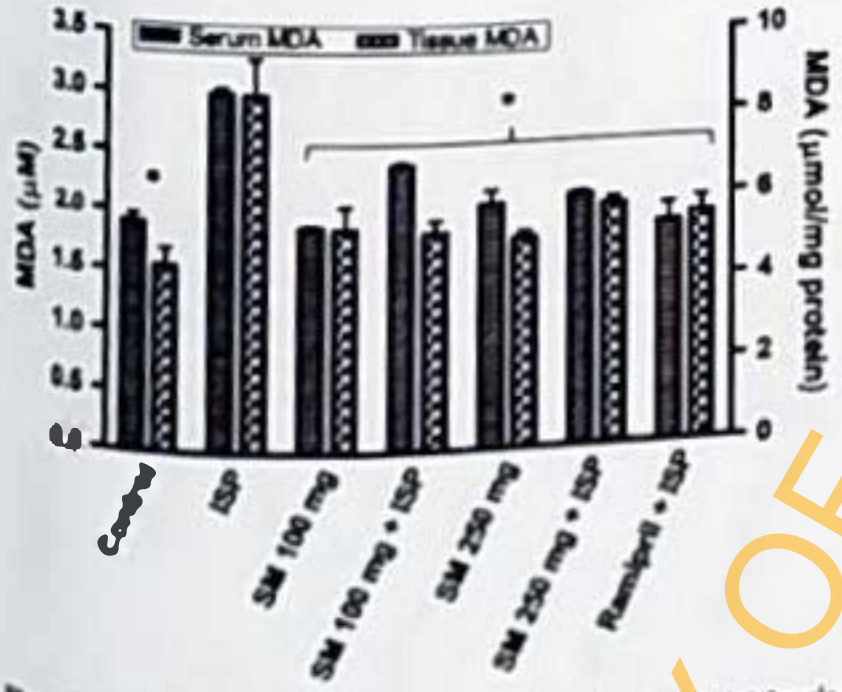

Fig. 5 Serum MDA (μM) and tissue MDA (μmol/mg protein) levels in control and test groups. Results are presented as mean ± SEM ($n = 5$). *Significantly different from ISP ($P < 0.05$)

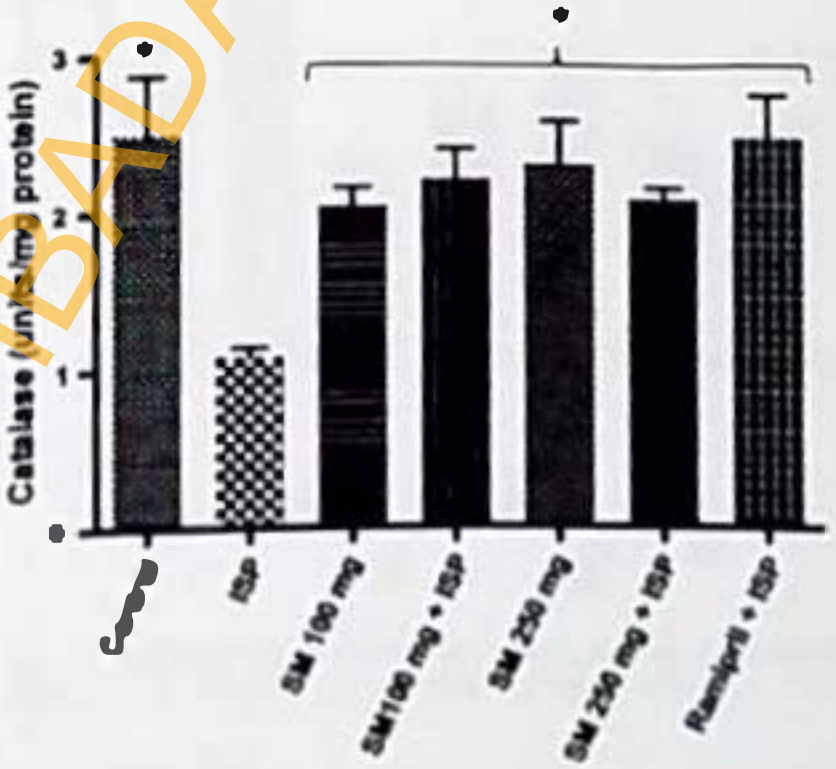

Fig. 7 Tissue catalase activity. Results are presented as mean ± SEM ($n = 5$). *Significantly different from ISP ($P < 0.05$)

while production of MDA was exacerbated. Administration of SM extract and ramipril significantly counteracted these deleterious trends in the serum as well as the heart tissue. Moreover, decreases in tissue SOD and CAT activities occasioned by ISP intoxication were significantly corrected by treatment with extracts and ramipril (Figs. 6 and 7), while the increase in tissue nitrite concentration observed in the ISP-intoxicated group was significantly decreased in the extract and ramipril-treated groups (Fig. 8).

ISP administration probably caused disturbances in glucose metabolism resulting in hyperglycemia (Fig. 9).

Groups administered extract alone did not show reduced glucose levels compared to the ISP-challenged group ($P > 0.05$). However, glucose concentrations were significantly reduced in groups challenged with ISP and treated with 250 mg/kg SM extract or ramipril compared with the ISP-challenged group. Serum cholesterol level was also elevated as a result of ISP administration but decreased in groups challenged with ISP and treated with 250 mg/kg

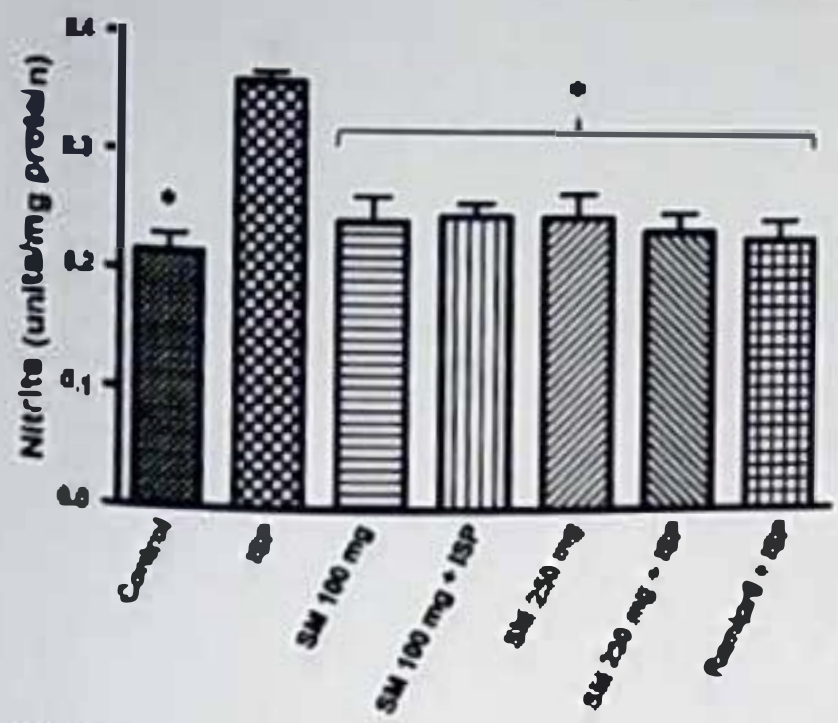

Fig. 8 Tissue nitrite levels. Results are presented as mean ± SEM ($n = 5$). *Significantly different from ISP ($P < 0.05$)

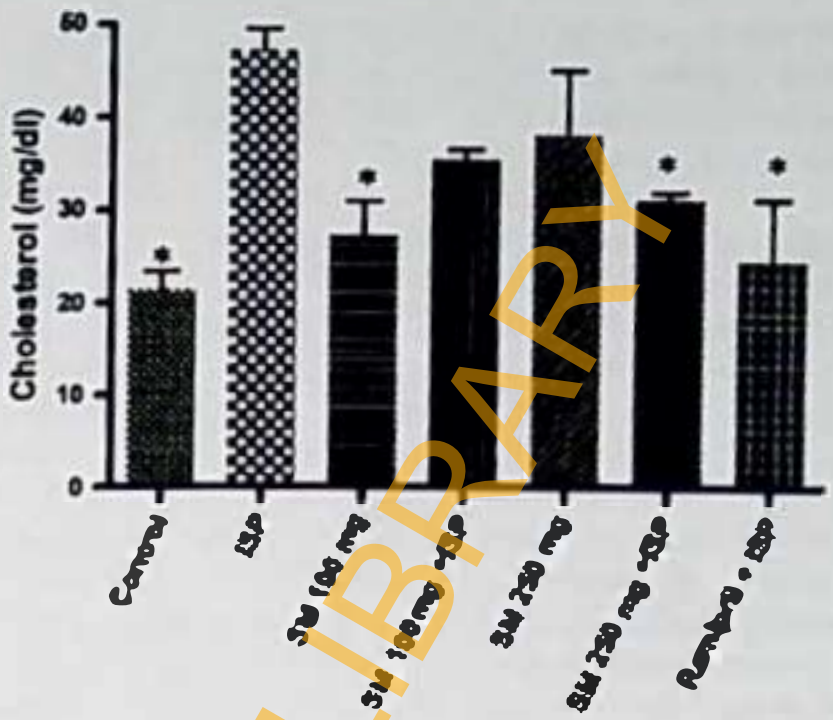

Fig. 10 Serum cholesterol levels. Results are presented as mean ± SEM ($n = 5$). *Significantly different from ISP ($P < 0.05$)

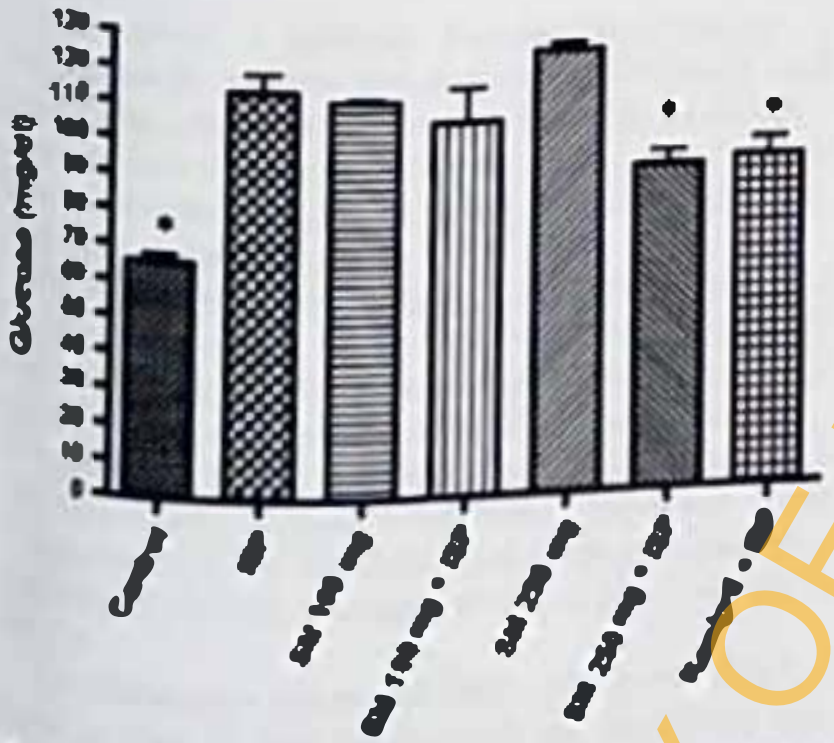

Fig. 9 Blood glucose level in all groups. Results are presented as mean ± SEM ($n = 5$). *Significantly different from ISP ($P < 0.05$)

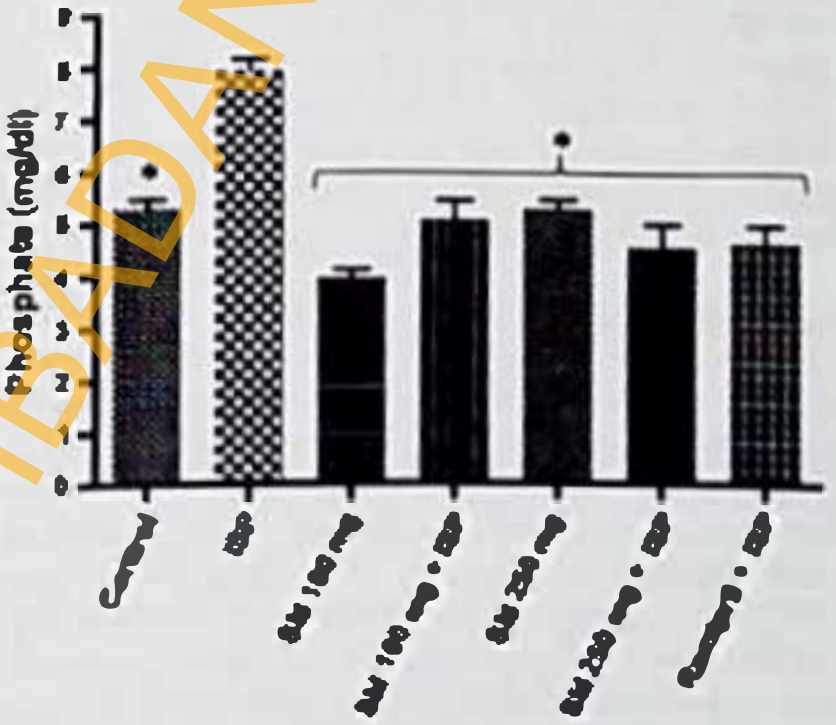

Fig. 11 Serum phosphate levels. Results are presented as mean ± SEM ($n = 5$). *Significantly different from ISP ($P < 0.05$)

SM or ramipril ($P < 0.05$) (Fig. 10). The elevated phosphate level in ISP-intoxicated group was reduced by SM at the two doses employed ($P < 0.05$) (Fig. 11). ISP intoxication also led to massive leakage of LDH into the serum (Fig. 12). Both SM extract and ramipril were effective in decreasing the elevated LDH levels ($P < 0.001$).

There was extensive disruption and fragmentation of heart myofibrils in ISP administered animals (Fig. 13a), but administration of SM (250 mg/kg) remarkably mitigated the ISP-induced damage (Fig. 13b). The myofibrillar structure of the heart in the group treated with 250 mg/kg extract alone seemed to be enhanced (Fig. 13c).

Discussion

Figure 2 indicates that excised hearts retrogradely perfused in the Langendorff mode with NHT buffer can maintain the mean cardiac contractility function for more than 90 min. Table 1 together with Fig. 3 shows that *Spondias mombin* had no significant deterioration or toxic effect on the isolated rat hearts, and Table 2 shows that *Spondias mombin* leaf extract improved the contractility of the heart which has been severely injured by ischemia. The Langendorff studies therefore demonstrated that *Spondias mombin* leaf extract possessed significant positive inotropic and antiarrhythmic properties

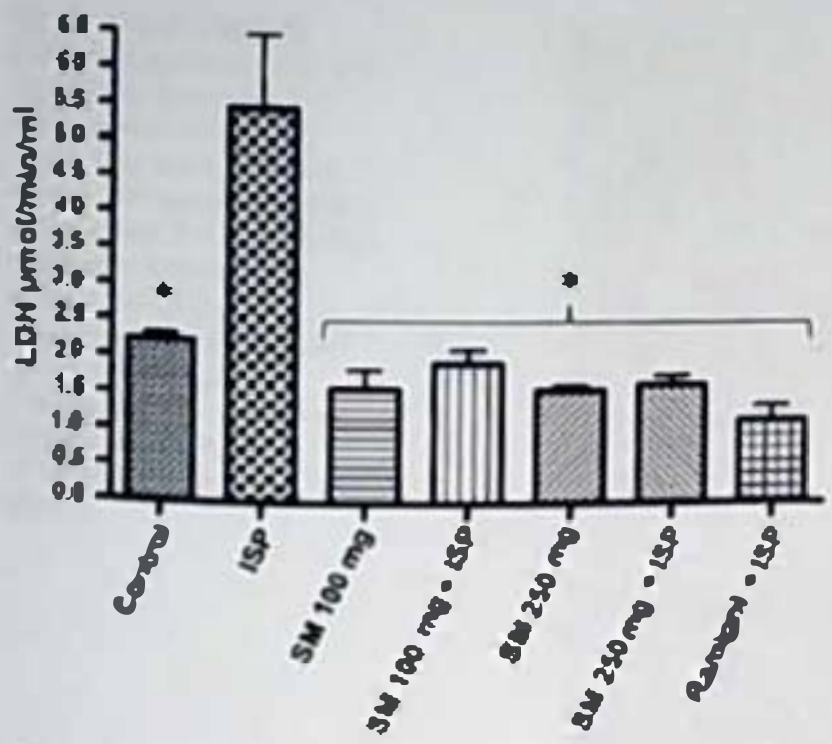

Fig. 12 Serum LDH levels. Results are presented as mean ± SEM (n = 6). *Significantly different from ISP ($P < 0.001$)

Isoproterenol, a synthetic β-adrenoceptor agonist, is well known to generate free radicals and stimulate lipid peroxidation, which is a causative factor for irreversible damage to the myocardium. It also increases the levels of serum and myocardial lipids, which in turn leads to coronary heart disease [28, 29].

Figure 3 reveals that administration of SM leaf extract had no adverse effect on the heart weight/body ratio and suggests that it may possess anti-inflammatory potential since it was able to significantly reduce (by 17%, $P < 0.001$ and 11%, $P < 0.05$ for 100 and 250 mg/kg of SM respectively) the heart weight/body weight ratio which was elevated by ISP intoxication. A weak anti-inflammatory activity has been reported for extract from the bark of SM [30].

The endogenous antioxidant GSH and MDA which is a major product of lipid peroxidation are established biomarkers of oxidative stress. Changes in the serum or tissue level of GSH are a reflection of changes in the activities of the enzymes associated with its turnover. Figures 4, 5, and 8 show that SM significantly protected against ISP-induced oxidative stress, in both serum and heart tissue, to a level comparable with the effect of ramipril. The significant increases in the activities of SOD and CAT in the SM and ramipril-treated groups compared with the ISP-intoxicated group ($P < 0.05$) further confirm the in vivo antioxidative property of SM (Figs. 6 and 7). Also, the reduction in the leakage of LDH into the serum in the SM and ramipril-treated groups compared with the ISP-intoxicated group (Fig. 12) demonstrates that SM protected against the destruction of cardiomyocytes.

Elevated serum phosphate concentration has been linked with cardiovascular and renal diseases, specifically with calcification of the coronary arteries and aorta as well as cardiovascular and all-cause mortality in the setting of end-stage renal disease (ESRD) [31–33]. ISP intoxication led to a significant increase in the phosphate level which was decreased by treatment with SM (Fig. 11).

The result of the evaluation of blood glucose concentration is hard to explain. The antidiabetic activity of SM has been reported [34]. However, in previous studies, the period of exposure to SM was ≤24 h in animals with induced diabetes. In the present investigation, SM was administered to normoglycemic animals for 30 days. It may be that SM produces differential effects on glucose metabolism depending on the duration of treatment and the health status of the animals. The reduction in glucose concentration in animals challenged with ISP and treated with SM compared with the elevation of glucose concentration in animals treated with SM alone may suggest a complex interplay of various factors in glucose metabolism. Cholesterol concentration was reduced in all groups compared to the ISP-challenged group, albeit not significantly in the group administered only 250 mg/kg SM and the one challenged with ISP and treated with 100 mg/kg SM. Thus, SM could be said to be effective in lowering elevated serum cholesterol level (Fig. 10). The histological analyses show convincingly that SM was able to prevent the disarray of cardiac myofibrils caused by ISP in rats (Figs. 13).

In the present study, SM showed similar effects to that of ramipril, an angiotensin-converting enzyme (ACE) inhibitor. ACE inhibitors have been shown to prevent ischemic events related to ischemia/reperfusion injury and atherosclerosis [35–37]. SM may also possess ACE inhibitory activity. Phenolics and flavonoids in foods and extracts from plants have been reported to possess anti-ACE activity [38], and phenolics are present in SM [30]. Oxygen-free radical scavenging properties of ACE inhibitors have been postulated to contribute significantly to the reduction of myocardial infarction [39]. The excellent antioxidative activity shown by SM in this study and its anti-ischemic activity may be pointers to a possible ACE inhibitory activity.

Taken alone, individual antioxidants studied in clinical trials do not appear to have consistent preventive effects as the isolated compound may not behave the same way as the compound in extracts or fractions [40]. Bioactivity may be as a result of synergistic interactions among diverse phytochemicals. For these reasons, results of bioactivity studies on isolated compounds should be juxtaposed with those of extracts or fractions to ascertain which pharmacological formulations would be more beneficial.

To the best of our knowledge, this is the first major study exploring the cardioprotective property of SM. The mechanism of SM cardioprotection seem to involve

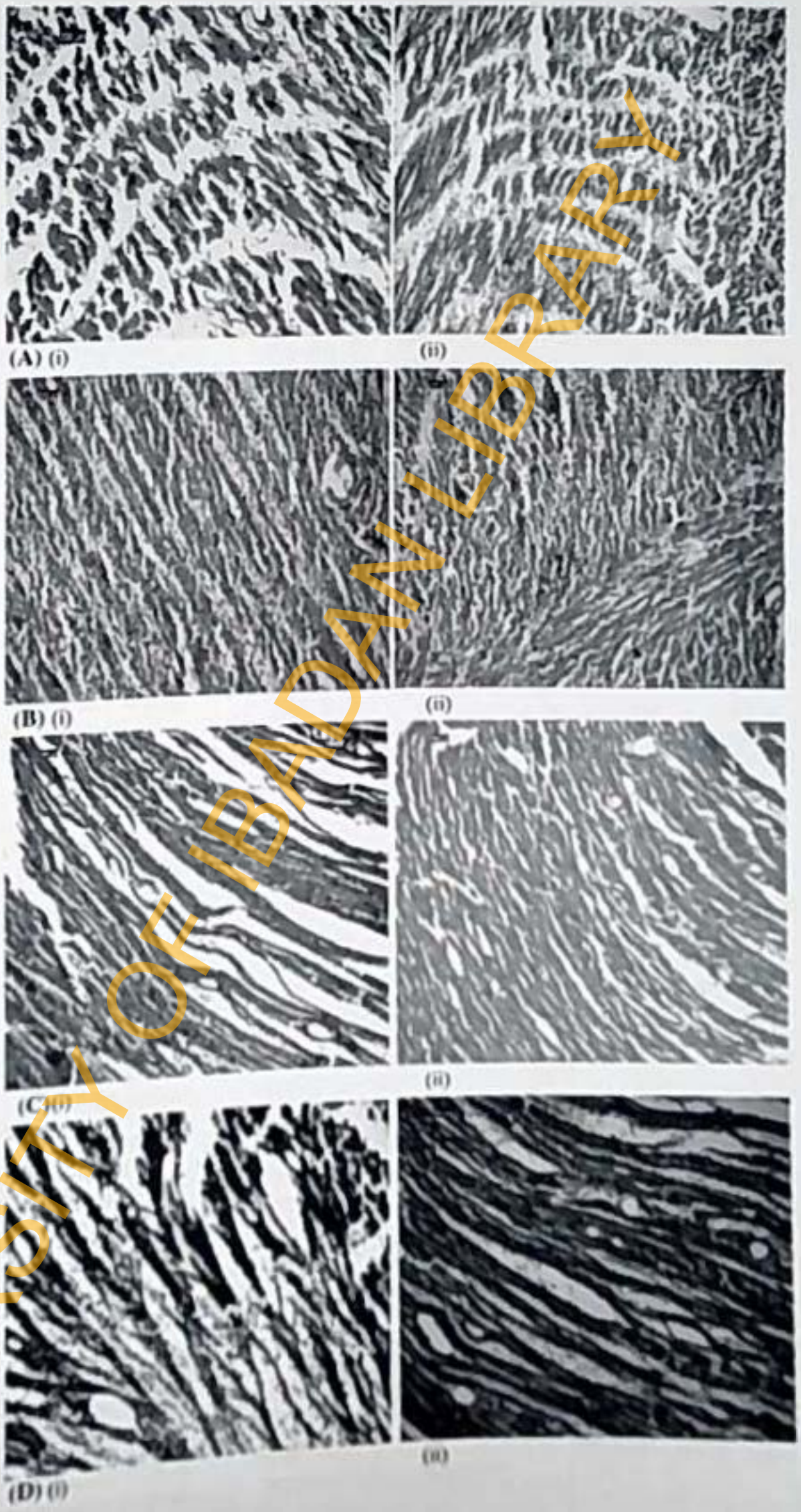

Fig. 13 H and E stained microscopy sections. **a** *(i) and (ii)* Massive disruption and fragmentation of the myofibrillar structure of the heart in ISP-intoxicated group; **b** *(i) and (ii)*: SM (250 mg/kg) remarkably mitigated the damage caused by ISP administration; **c** *(i) and (ii)*: Group treated with SM (250 mg/kg) only did not show negative morphological alterations; **d** *(i) and (ii)* Control group

improvement of cardiac contractile function, prevention of the disruption of cardiac myofibrils, preservation of the integrity of cardiomyocyte membrane, and reduction of oxidative stress. Prevention of atherosclerotic plaque formation may also be a contributory mechanism. Further research is needed to unravel the precise mechanisms and bioactive principles responsible for the cardioprotective property of SM and to ascertain whether cardioprotection by SM is attributable to an individual compound or a group of phytochemicals. Investigations along these lines are ongoing in our laboratories.

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