Modulation of Gentamicin- and Cisplatin- induced Nephrotoxicity and Acute Renal Failure by Methanolic Extracts of Soybean (*Glycine max (L.) Merr.*) in Rats

BY

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CERTIFICATION

I certify that this work was carried out by EKOR Martins under my supervision in the Drug Metabolism & Toxicology unit of the Department of Biochemistry, University of Ibadan, Nigeria.

20-8-2009

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DEDICATION

To my father, Mr. Robert O. Ekor, of blessed memory, who provided the template and did not deny me the love of a father;

To my mother, Mrs Esther N. Ekor, who joyfully traversed the lonely valleys of soul poverty and abnegation of all things to provide love and care;

To my darling wife, Oluwayemisi, in whom I have continued to enjoy true companionship and love without diminution when I left my father and mother.

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I am grateful to my supervisor and the Head of Department, Prof. E.O. Farombi, for leading me this far and on whose wings I've been able to attain this height, for "no bird soars too high if he soars on his own wings". I consider myself extremely privileged to have trained under a distinguished academic, an erudite scholar and an outstanding researcher and teacher. Sir, I realised over the years that gold is the only object you appear to value and you treat your students like one. Not many distant observers realise this or have sufficient thoughtfulness to appreciate the pleasant contradiction of purifying gold by taking it through fire. But in this I have seen a perfect balance of love and discipline. Thank you for the painstaking supervision, the corrections, your insistence on thoroughness and high premium on diligence. I thank you sir for letting me into the challenges and tasks that have helped in developing my academic muscles. You became a source of inspiration and a model even at such a time when mediocrity is inadvertently celebrated and excellence viewed with illusion. Words would fail me to qualify you with precision, but I'm proud to find a mentor in you.

It is with utmost sense of gratitude and profound appreciation that I acknowledge Prof. G.O. Emerole, who admitted me into the Drug Metabolism & Toxicology unit, and started the supervision of this work and continued to follow up even after retiring from active service. Sir, you introduced me into the world of research and true academics. Your zero tolerance to laziness and uncompromising attitude to discipline, decency, moral rectitude and fidelity will remain my guiding principles. You laid the foundation of an enviable legacy in the unit. You never meddled with mediocrity neither did you tolerate complacency. You showed us how to reach the top through diligence, dedication and honesty. In spite of environmental limitations and challenges, you distinguished yourself as an academic. Only time and eternity would tell the impact and influence you have made. Thank you for giving yourself to us. God bless you Sir.

I also wish to acknowledge with deep sense of appreciation the immense contribution of Prof. O.O. Olorunsogo. Thank you, sir for treating me like a son and for giving me access to your laboratory. Your commitment to academics, your students and to work generally remains an invaluable source of inspiration and encouragement to me. Thank you for your interest in academic matters of the department and your wonderful contributions at seminars. I'm also grateful to other professors and senior academic members of staff of the department, Prof. A.O. Uwaifo, Prof. M.A. Fafunso, Prof. E.N. Maduagwu, Dr. (Mrs) A.O. Odetola and Dr. (Mrs) O.A. Odunola for their contributions.

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My special thanks to my head of Department, Prof. (Mrs) A.F. Aderounmu of the Department of Pharmacology, Olabisi Onabanjo University, who has been more of a mother than a boss. Thank you for all your support and encouragement over the years. Dr. P.I. Aziba of the Department of Pharmacology of the Olabisi Onabanjo University remains an invaluable source of inspiration. I really cannot quantify the support and encouragement which always inspire hope and strength to keep forging ahead. I will continue to remain grateful to him. The family of Dr. and Mrs O.A. Osonuga has particularly been pretty wonderful beyond description. A model and an ideal family everyone would aspire to experience. Thank you for all the support, the love and hospitality at all times, the prayers and above all for treating me like a member of the family. The good Lord shall continue to reward the entire family for all her labour of love.

I sincerely appreciate the kind support and encouragement I enjoyed from my friends and colleagues, all of whom I cannot itemize for the purpose of brevity. It is with profound sense of gratitude that I appreciate the kind gestures and support of Mrs. O.A. Adesanoye. Your thoughtfulness and unreserved magnanimity beat my imagination. Thank you for joyfully sharing the comfort of your office with me. Special thanks to Dr. (Mrs) T.O. Bamidele who has been a very wonderful sister and colleague. I appreciate your support, prayers and the fellowship of love. I just must not fail to acknowledge the invaluable contributions of Mr. T.O. Idowu and Dr. M. Obuotor of the Faculty of Pharmacy, and Department of Biochemistry respectively, of the Obafemi Awolowo University, Ile-Ife. My profound gratitude to Mr A.O. Odewabi of the Department of Chemical Pathology of the Olabisi Onabanjo University for his invaluable contribution and support and also to Dr. O. Adaramoye for his concern and encouragement. It is with due sense of humility that I

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It is with joy and profound sense of gratitude that I appreciate my sweet mother. Thank you for your many prayers, patience, understanding and support. Also to my elder sister, Mrs. Felicia Onyebolise and immediate younger brother, Mr. Maxwell Ekor, both of whom have been very wonderful in every way. To my darling wife, Oluwayemisi, you have been simply wonderful. Thank you for your priceless support, prayers, encouragement and above all, your love.

Finally, I recall to mind the words of the great sage, William Shakespeare, who thoughtfully acknowledged that "our wills and fates do so contrary run that our devices still are overthrown. Our thoughts are ours, their ends none of our own". It is on this backdrop that I acknowledge with deep reverence and adoration The One who only can "build" and without whom "they labour in vain that build it", The Almighty, The God of grace and mercy. Without any iota of doubt, The Lord has been "merciful and gracious, slow to anger and plenteous in mercy". To a sensible man, there is no such thing as chance; for nothing can exist without a cause. Truly, "fate holds the strings, and men like children move, but as they're led; success is from above.

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ABSTRACT

Cisplatin and gentamicin are potent chemotherapeutic agents whose clinical efficacies have been demonstrated in many human malignancies and life-threatening bacterial infections respectively. Nephrotoxicity, however, limits the full clinical utility of these drugs. This study investigated the protective effect of methanolic extract of soybean (MESB) against this adverse effect and the mechanisms for protection.

Nephrotoxicity was induced by the administration of gentamicin (80 mg/kg/day subcutaneously for 12 days) and cisplatin (2 mg/kg/day intraperitoneally for 5 days) one-hour after oral administration of 250-1000 mg/kg/day MESB in rats. *In vitro*, the antioxidant activity of MESB was investigated in the trolox equivalent antioxidant capacity (TEAC) [using 2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonate] (ABTS)], ferric reducing antioxidant power (FRAP) and reducing power (RP) assays, as well as in Fe^{3+}/ascorbate- and 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH)-induced lipid peroxidation (LPO) models. The radical scavenging activity (RSA) of MESB on hydroxyl ($OH^{\cdot}$), superoxide (O^{-}_2), nitric oxide ($NO^{\cdot}$) and 1,1-dipheny-2-picrylhydrazyl (DPPH$^{\cdot}$) radicals was also evaluated. MESB was fractionated by column chromatography over silica gel60 and sephadex LH-20. Separated fractions were subjected to thin-layer chromatography. Data were analyzed by ANOVA.

Cisplatin-associated increase in Blood Urea Nitrogen (BUN) was significantly ($p<0.05$) decreased by 49.8%, 59.0%, 21.1% and creatinine by 34.7%, 62.1%, 24.6% following 250, 500 and 1000 mg/kg MESB administration respectively. Elevation in BUN caused by gentamicin was significantly ($p<0.05$) decreased by 76.5%, 76.6% and creatinine by 78.6%, 77.7% following treatment with 500 and 1000 mg/kg MESB respectively. Doses of MESB at 250, 500 and 1000 mg/kg increased significantly ($p<0.05$) cisplatin-associated decreases in the activities of superoxide dismutase (SOD) by 103.9%, 219.5%, 28.6%, catalase (CAT) by 11.6%, 27.8%, 8.4% and glutathione-s-transferase (GST) by 103.0%, 110.4%, 53.7% respectively. Decrease in glutathione (GSH) level induced by cisplatin also increased significantly ($p<0.05$) by 145.6%, 250.7% and 34.0% respectively at these doses. Similarly, gentamicin-associated reduction in the activities of SOD increased by 11.2% and 33.2%, CAT by 11.7% and 95.0%, GST by 44.7% and 89.4% while GSH level increased significantly ($p<0.05$) by 184.6% and 161.5% at 500 and 1000 mg/kg doses respectively. Also, MESB prevented gentamicin-associated decreases in renal activities of γ-glutamyltransferase by 33.8% and 84.7% and aspartate aminotransferase by 11.9% and 13.5%. Furthermore, MESB prevented cisplatin-associated increases in urinary N-acetyl-β-

D-glucosaminidase excretion by 37.7%, 49.2%, 14.6%, serum NO production by 35.5%, 36.4%, 33.8%, LPO by 46.4%, 54.6%, 41.6% and activities of mycloperoxidase and xanthine oxidase by 26.8%, 40.6%, 7.4% and 0%, 2.2%, 17.9% respectively in the rats. *In vitro*, the antioxidant activity of MESB ranged from 24.8±5.6 to 228.0±13.6µmol trolox equivalent in the TEAC assay at 125-500µg and also demonstrated marked RP. Fe^{3+}/ascorbate- and AAPH-induced LPO were significantly ($p<0.001$) inhibited by 46.3% and 40.0% at 800µg respectively. MESB exhibited maximum RSA of 24.1% (50µg), 68.2% (50µg), 55.6% (1mg), and 11.6% (50µg) on $NO^{\cdot}$, $O^{\cdot}_2$, $OH^{\cdot}$ and DPPH$^{\cdot}$ radicals respectively ($p<0.05$). Phytochemical analysis revealed the presence of antioxidant phenolics with dihydroxy and trihydroxy groups.

Methanolic extract of soybean protected against cisplatin- and gentamicin-associated renal dysfunction and injury probably by antioxidant, free radical scavenging and anti-inflammatory activities attributable to the presence of phenolic compounds.

Key words: Nephrotoxicity, Cisplatin, Gentamicin, Antioxidants, Soybean extract.

Word count: 500

ABBREVIATIONS

AAPH	2,2,-azobis(2-amidinopropane) hydrochloride
ABTS	2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
ADR	Adrenaline
AKI	Acute kidney injury
AKIN	Acute kidney injury network
AMP	Adenosine monophosphate
ARF	Acute renal failure
AST	Aspartate aminotransferase
ATN	Acute tubular necrosis
BBI	Bowman-Birk inhibitor
BUN	Blood Urea Nitrogen
CAT	Catalase
CC	Column chromatography
CDDP	*cis*-diammine-dichloroplatinum (II)
CDNB	1-Chloro-2,4-dinitrobenzene
DMTU	Dimethylthiourea
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl radical
DTNB	5',5'- dithiobis-2-nitrobenzoic acid
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
ET-1	Endothelin-1
Fe-NTA	Ferric nitrilotriacetate
FOX	Ferrous oxidation-xylenol orange
FRAP	Ferric reducing antioxidant power
G6Pase	Glucose -6-phosphatase
GFR	Glomerular filtration rate
GM	Gentamicin
GSH	Reduced glutathione
GST	Glutathione-S-transferase

STS	Sodium thiosulfate
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
TKI	Tyrosine kinase inhibitor
TLC	Thin-layer chromatography
TMP	Tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
TPTZ	2,4,6-Tri(2-pyridyl)-S-triazine
TXA_2	Thromboxane A_2
UPR	Unfolded protein response
WHO	World Health Organization
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
γ-GT	γ-glutamyltransferase

CHAPTER ONE

1.1 INTRODUCTION

Nephrotoxicity can be defined as renal injury or dysfunction that arises as a direct or indirect result of exposure to drugs and industrial or environmental chemicals (WHO, 1991). Over the last 30 years, it has become increasingly obvious that the kidney is adversely affected by many chemicals. The level of exposure varies from minute quantities to very high doses. Exposure may be chronic or limited to a single event, and it may be due to a single substance or to multiple chemicals. The circumstances of exposure may be inadvertent, accidental, or intentional overdose or of therapeutic necessity. Some chemicals cause an acute injury and others produce chronic renal changes that may lead to end-stage renal failure and renal malignancies. The extent and cost of clinically relevant nephrotoxicity became apparent during the last two decades. However, the full extent of the economic impact of chemically induced or associated nephropathy is difficult to define because the diagnosis of early injury and the documentation of the cascade of secondary degenerative changes have not been adequately identified. Instead many chemically associated renal diseases are only identified as acute renal failure or as chronic renal failure at a very late stage when therapeutic intervention is impossible. More importantly at this stage, the etiology may be obscured by lack of reliable information on the likely causative agents, the levels and duration of exposure, and other possible contributing and exacerbating factors.

Epidemiological evidence indicates that nephrotoxicity leading to acute and/or chronic renal failure represents a substantial financial burden to society (Nuyts *et al.*, 1989, WHO, 1991). Indeed, there is some indication that chemical exposure could play a much greater role in the very high incidence of end-stage renal disease encountered in nephrology and dialysis clinics than is currently considered to be the case.

Owing to its diverse functions and small mass in relation to the resting cardiac output that it handles, the kidney is a target both for chemicals that are pharmacologically active and for toxic metabolites. The nephron and its related cells perform a diversity of physiological functions. It is the major organ of excretion and homeostasis for water-soluble molecules. Because it is a metabolically active organ, it can concentrate certain substances actively. In addition, its cells have the potential to inactivate chemicals and metabolically activate a variety of compounds. There are a

number of other processes that establish the potential for cellular injury. Specific physiological characteristics are localized to specific cell types. This makes them susceptible to, and the target for, chemicals. The effect of any chemical on a cell may be pharmacological and toxicological, in which case the effect is dose related and occurs only as long as the concentration of the effector is high enough to be active. The cell responds to injury by repair and the kidney responds to cellular lesion by renal and extrarenal adaptation to compensate for loss of that cell function. Although there is a substantial capacity within the kidney for repair, there are also several circumstances where damage may be irreversible. In general, the proximal and distal tubules and urothelia can be repaired, but the glomeruli and medulla may have a significantly lower repair facility. It is, therefore, possible to initiate a series of degenerative changes as a result of interfering with one or more of the normal physiological processes.

The Environmental Health Criteria monographs normally focus on industrial chemicals, but at present most of the experimental and human information on nephrotoxicity is based on therapeutic substances (WHO, 1991). These data are most useful because there are animal and human comparisons for specific chemicals where the levels of exposure and the nephrotoxicological consequences are well documented. From these data it has been possible to glean some understanding of mechanisms of primary injury and the long-term consequences and health significance. Thus, these compounds are generally well studied, and the more rational understanding of the mechanism of their nephrotoxicity in animals and man provides the basis for validating extrapolation between species and making rational risk assessment.

Clinically, a vast number of nephrotoxicants can produce a variety of clinical syndromes- acute renal failure, chronic renal failure, nephrotic syndrome, hypertension and renal tubular defects. The evolving understanding of the pathophysiology of toxicant-mediated renal injury has implications for potential therapies and preventive measures.

Acute renal failure (ARF) may occur in 2% to 5% of hospitalized patients and is frequent in intensive care units, affecting up to 30% of patients (Brivet *et al.*, 1996; de Mendonca *et al.*, 2000; Liano *et al.*, 1998). The mortality of acute renal failure is approximately 50% (Joseph and Nally Jr., 2002). From large but separate databases of US hospitalizations in the past 10 to 15 years, there is evidence for a marked increase in the incidence (Waikar *et al.*, 2006; Xue *et al.*, 2006).

Nephrotoxins (in form of therapeutic drugs, industrial or environmental chemicals) may account for approximately 50% of all cases of acute and chronic renal failure. Aminoglycosides (e.g. gentamicin) and platinum coordination complexes (e.g. cisplatin) are among the most common drugs associated with nephrotoxic injury in hospitalized patients. About 30% of patients treated with gentamicin for more than 7 days show some signs of nephrotoxicity (Atcssahin *et al.*, 2003) and this side effect and other complications that arise from the use of this drug limit its clinical usefulness. Similarly, despite the use of optimal methods for administering cisplatin, such as the use of active hydration (Cvitkovic *et al.*, 1977) or sodium chloride as the vehicle (Ozols *et al.*, 1984), approximately 30% of patients still manifest nephrotoxicity. Renal injury arising from these and other therapeutic agents is widely recognized to lead to high morbidity, increases the length of hospital stay, increases costs, and high rates of mortality in hospitalized patients (Brivet *et al.*, 1996; de Mendonca *et al.*, 2000; Liano *et al.*, 1998; Mcinitz *et al.*, 2002). Interestingly, the mortality rate has not decreased or changed significantly over the last 40 years. Management of ARF in critically ill patients remains primarily supportive until renal function recovers. Thus, any therapeutic strategy which prevents or reduces either the incidence or morbidity of ARF will be of benefit. Therefore, the need for urgent and effective methods of reducing the predisposition to, or mitigating renal injury arising from the use of therapeutic agents becomes imperative. Except for a few isolated studies, the vast majority of animal and clinical studies have yet to demonstrate conclusively the benefit of pharmacologic treatment of acute kidney injury.

Therapy which embraces the consumption of plant products rich in flavonoids have been documented to provide benefits without the adverse effects of pharmacological agents used in clinical practice. Polyphenolic compounds are widely distributed in edible plants (Sergedienc *et al.*, 1999) and have been suggested to protect against a variety of diseases (Johnson *et al.*, 2005). Recent investigations suggest that polyphenolic components of higher plants may act as antioxidants or via other mechanisms contributing to disease processes (Wang *et al.*, 2000). Recent findings have also demonstrated that polyphenols cross intestinal barriers and are sufficiently absorbed to have the potential to exert biological effects (Williamson & Monach, 2005). In the light of these, the possible therapeutic potential of soybean (rich in polyphenolic compounds or isoflavones) was evaluated in experimental models of

nephrotoxicity and acule renal failure induced by gentamicin and cisplatin in rats in this study.

CHAPTER TWO

LITERATURE REVIEW

2.1 KIDNEY STRUCTURE AND FUNCTION

2.1.1 THE NEPHRON

The kidney is divided into three main regions, cortex (outer), medulla (inner), and pelvis (Figure 1). Within the cortex arise the renal corpuscles, defined as superficial, midcortical or juxtamedullary depending on the anatomical location of the renal corpuscle in the cortex. The nephron is the functional unit of the kidney and consists of a continuous tube of highly specialized heterogeneous cells, which show sub-specialization along the length of nephrons and between them. There are marked structural and functional differences between the nephrons arising in the cortex and those arising in the juxtamedullary regions. The total number of nephrons varies between different species and within any one species as a function of age. The macroscopic differentiation of the kidney into distinct zones arises not only from the regional vascularity but also from the way different functional parts of the nephron are arranged within the kidney. A more detailed account of the ultrastructure of the morphologically definable regions of the nephron and their functional inter-relationship has been provided by Moffat (1981, 1982), Bohman (1980), and Maunsbach *et al.* (1980). The nephron nomenclature has been standardized by the Renal Commission of the International Union of Physical Sciences (Kriz and Bankir, 1988).

2.1.1.1 Cellular heterogeneity and cell-cell interaction

There are well over 20 morphologically different cell types (based on light microscopy alone) in the kidney, and when histochemical and immunohistochemical methods are applied to renal tissue sections the diversity of cell types is even more apparent. The spectrum of biochemical (and structural and functional) characteristics in these cells demonstrates the very marked heterogeneity that is the hallmark of the kidney. It is well established that the expression of many of these biochemical characteristics is an integral of the functions of that particular region of the kidney, and there is the potential to change the expression of these characteristics in terms of the demands on the kidney. These include water and electrolytes, dietary factors, and chemicals with pharmacological and toxic effects, or may be as a result of chemical and

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other types of injury. More importantly, the characteristics of a cell may make it either resistant or sensitive to the target selective toxicity of a chemical.

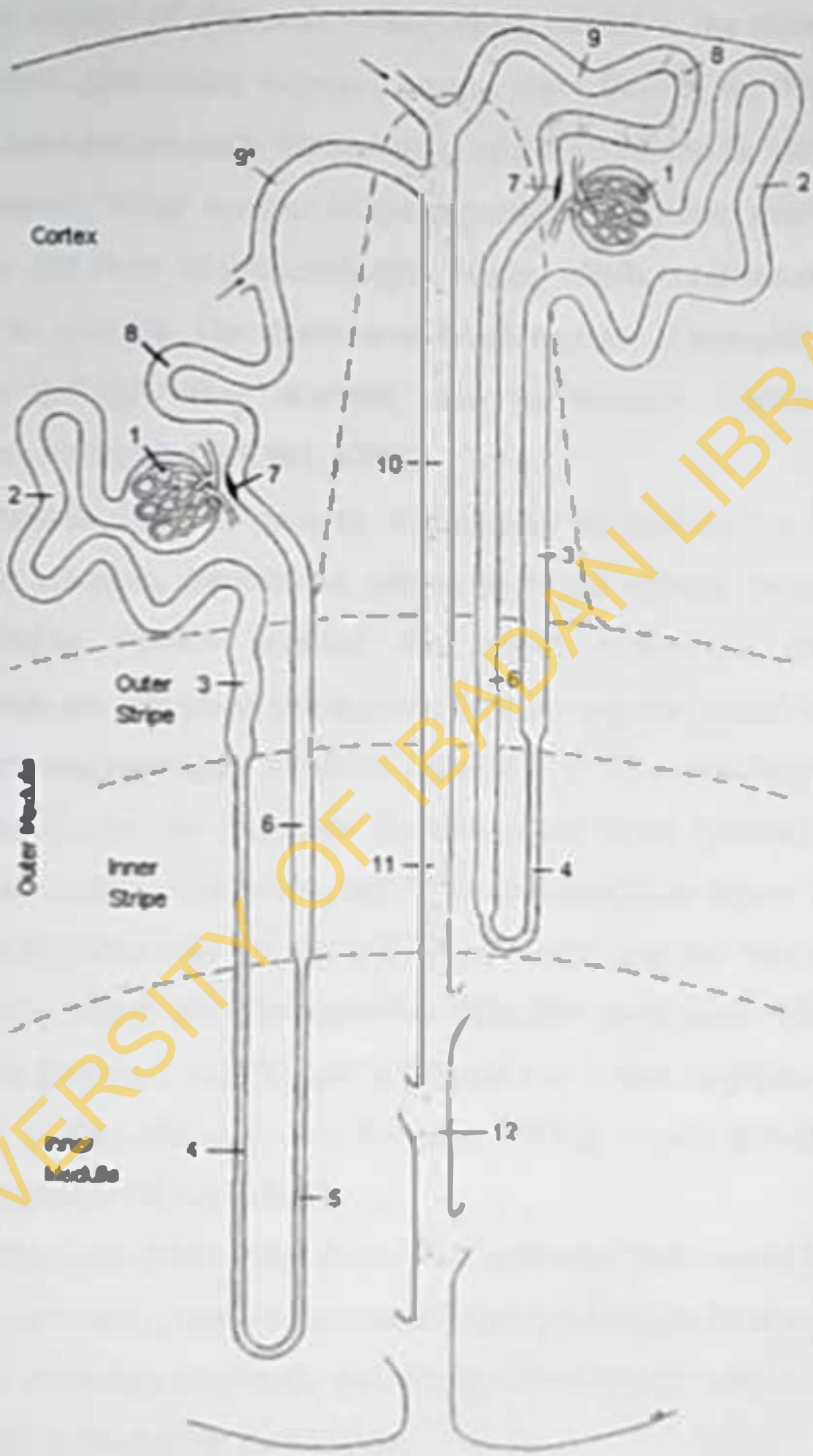


Figure 1: Scheme of nephron. This scheme depicts a short-looped and a long-looped nephron together with the collecting system. Within the cortex a medullary ray is delineated by a dashed line. 1 = renal corpuscle including Bowman's capsule and the glomerulus (glomerular tuft), 2 = proximal convoluted tubule, 3 = proximal straight tubule, 4 = descending thin limb, 5 = ascending thin limb, 6 = distal straight tubule (thick ascending limb), 7 = macula densa located within the final portion of the thick ascending limb, 8 = distal convoluted tubule, 9 = connecting tubule of the juxtamedullary nephron that forms an arcade, 10 = cortical collecting duct, 11 = outer medullary collecting duct, 12 = inner medullary collecting duct. From: Kriz & Bankir (1988).

2.1.1.2 The glomerulus

The glomerulus forms the initial part of the nephron and functions as a relatively poorly selective macro-molecular exclusion filter to the hydrostatic pressure of the blood. The number of glomeruli is, in general, related to the mass of the species, and the size of each glomerulus depends, among other factors, on the environmental water balance. Three anatomically distinct types of glomeruli can be identified: those in the superficial cortex, which are part of the superficial nephrons; those arising in the midcortical area; and those of juxtamedullary origin, which continue as nephrons that loop down into the medulla. The structure of the glomerulus is complex (Figure 2) and has only been defined using scanning and transmission electron microscopy (Maunsbach *et al.*, 1980; Moffat 1981, 1982).

The glomerular "tuft" is made up of a number of capillary branches that arise from the afferent arteriole, anastomose, and drain to the efferent arteriole. There are also communicating vessels between the branch capillaries. The fenestrated endothelium cannot prevent plasma molecules from leaving the lumen, but a negatively charged cell coat imparts some selective permeability. The capillaries are in direct contact with the glomerular basement membrane (or basal lamina), which, when viewed under the electron microscope, can be divided into three layers: the lamina rara interna on the endothelial side; the central lamina densa; and the lamina rara externa, which is in direct contact with the epithelial cells (the podocytes). The basal lamina contains collagen (mostly Type IV) and sialic acid and is rich in glycosaminoglycans, mainly heparan sulfate (Kanwar and Farquhar, 1979), which provides a strongly anionic macromolecular filtration barrier.

The capillary tuft (ensheathed in its basal lamina) is surrounded by a number of podocytes, each of which gives rise to several primary processes (trabeculae). These in turn give rise to secondary processes, and, finally, to numerous tertiary foot processes that are embedded in the lamina rara externa.

The foot processes of one podocyte interdigitate with those of an adjacent epithelial cell for adjacent trabeculae. The surfaces of the podocytes are covered by a strongly anionic cell coat that extends to the spaces between the foot processes. It is through these spaces that the glomerular filtrate reaches the lumen of Bowman's space. Thus, the podocyte provides a structural support for the basal lamina and may also serve to provide additional anionic forces for the process of biological ultrafiltration. It

has been suggested that podocytes may have phagocytic properties and undergo contraction (Moffat, 1981).

The axial regions of each glomerulus contain mesangial cells. These cells undergo contraction and may thus control glomerular blood flow via biogenic amine or hormonal control. Of equal importance is the observation that these cells take up large molecules (such as colloids, immune complexes, and protein aggregates), which may eventually be disposed of via the renal lymphatic system.

The driving force for filtration is provided by the glomerular capillary hydrostatic pressure (which is controlled mainly by the vascular tone of the afferent and efferent arterioles), minus both the plasma osmotic pressure and the hydrostatic pressure in the Bowman's space. The resulting "effective filtration pressure" across the basal lamina is about 1.2-2.0 kPa (10-15 mmHg). Selective filtration is achieved primarily on the basis of size restriction by the basement membrane, which impedes the passage of macromolecules with an effective radius greater than 1.8 nm and completely prevents the filtration of macromolecules with an effective radius greater than 4.5 nm. In addition, the presence of fixed negative charges on the endothelial, epithelial, and basement membranes hinders the filtration of anionic macromolecules while facilitating the passage of cationic macromolecules. The selectivity of filtration is, in part, a consequence of the anionic nature of the basement membrane, which blocks or slows the passage of negatively charged or neutral macromolecules and leaves those carrying a cationic charge and small molecules (irrespective of charge) to pass unimpeded.

2.1.1.3 The proximal tubule

The proximal tubule is found only in the cortex or subcortical zones of the kidney. Anatomically each proximal tubule can be divided into the convoluted portion (pars convoluta) and the shorter straight descending portion (the pars recta), which then continues to become the descending limb of the loop of Henle. It may be sub-divided, by a number of morphological and functional features, into three segments, S_1, S_2, and S_3.

The proximal tubule plays a decisive role in maintaining homeostasis. This is achieved when sodium and chloride ions flux from the tubule lumen to the peritubular capillaries under the control of a number of processes such as nonspecific electrophysiological gradients and selective active transport mechanisms. Water follows the ions by osmotic effects. In addition, hydrostatic pressure, attributable to the

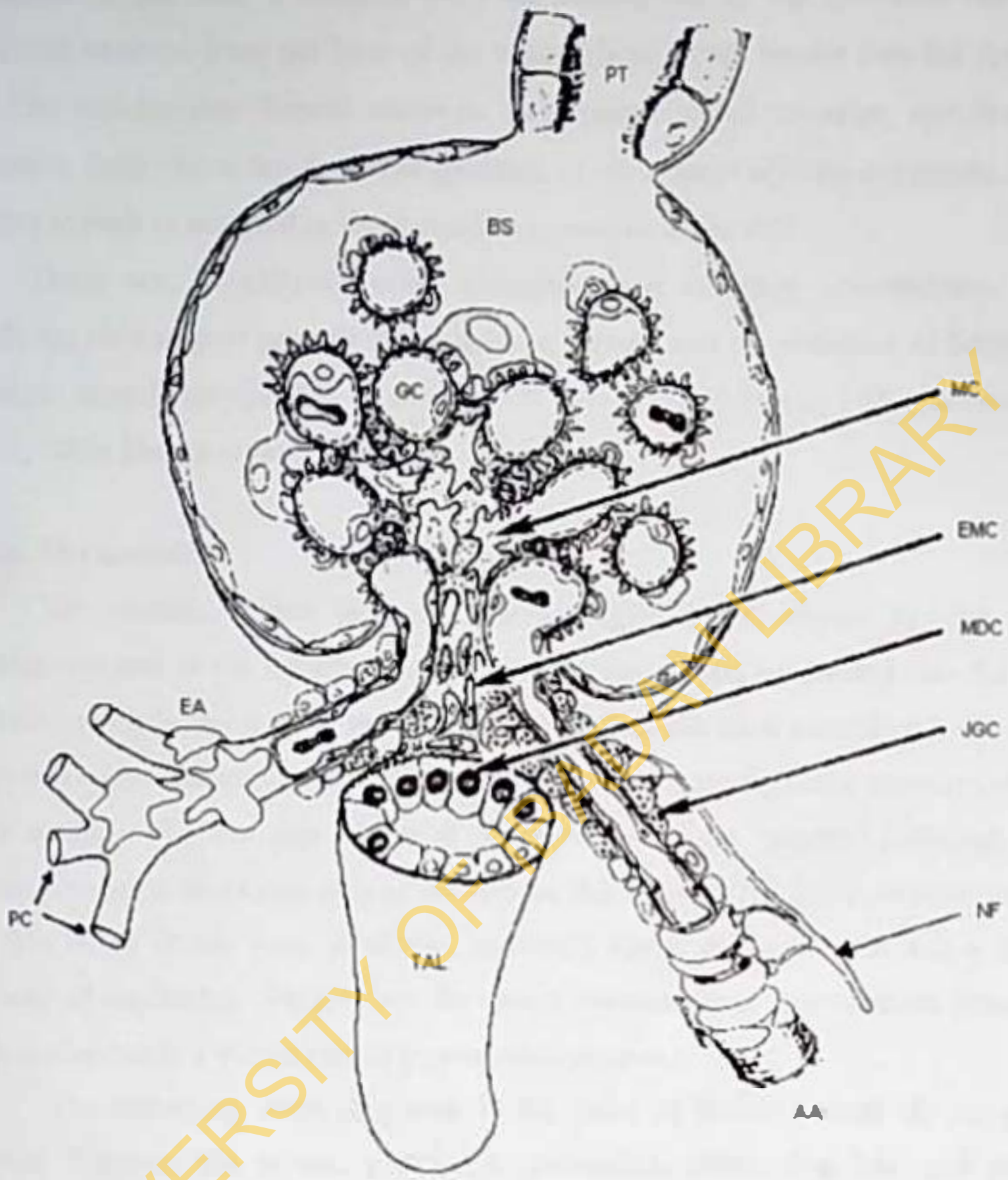


Figure 2: Glomerulus and juxtaglomerular complex, consisting of afferent arteriole (AA) with the granular cells (JGC) of the juxtaglomerular apparatus, the exuraglomerular mesangial cells (EMC), the macula densa (MDC) segment of the ascending loop of Henle, and the efferent arteriole (EA). Also shown are the proximal tubule (PT), Bowman's space (BS), glomerular capillaries (GC), peritubular capillaries (PC), mesangial cells (MC), and nerve fibres (NF). From: Schrier & Gottschalk (1987).

presence of both proteins and glycosamino-glycans (Wolgast *et al.*, 1973), contributes to water movement from the epithelial cell to the interstitium and thence, by an osmotic gradient, into the capillaries (Valtin, 1973). The flux of ions within the proximal tubule, including the absorption and secretion of HCO_3^- and H^+ and the "lumen trapping" of ammonium ions, controls renal acid-base regulation (Valtin, 1973).

Those proteins that have passed from Bowman's capsule (a significant amount of albumin in the case of normal rats) are reabsorbed in the proximal tubule by pinocytotic removal from the base of the microvillous brush border into the epithelial cells. The vesicles thus formed combine, form protein-filled vacuoles, and fuse with lysosomes, from which the digestion products of the protein diffuse, eventually, to the capillary system or are used in the metabolic processes of the cell.

There are, in addition, other absorptive and secretory mechanisms. These include the co-transport process that reabsorbs glucose and the secretion of both acidic and basic organic compounds (Valtin, 1973; Orloff and Berliner, 1973; Brenner and Rector, 1986; Berndt, 1989).

2.1.1.4 The medulla

The medulla differs from the cortex (Figure 1 and Figure 3) both at the macroscopic and at the microscopic levels. This region can be divided into the outer medulla (which is made up of the thin descending and the thick ascending limbs of the loops of Henle, collecting ducts, the vasa recta, and a dense capillary network) and the inner medulla, the free part of which is referred to as the "papilla" (although some researchers apply that name only to the apex of this region). The inner medulla contains the thin limbs of the loops of Henle, collecting ducts, the vasa recta, and a diffuse network of capillaries. Packed into the spaces between these structures are interstitial cells embedded in a matrix rich in glycosaminoglycans.

The collecting ducts terminate as the ducts of Bellini around the tip of the papilla. Whereas the mouse, gerbil, rat, guinea-pig, rabbit, dog, cat, and primate kidneys have only a single papilla, the pig and man have multi-papillate kidneys. There are between 9 and 20 papillae in each human kidney (Burry *et al*., 1977), of which there are two anatomically distinguishable types. The conical non-refluxing papillae, where the surface orifices of the ducts of Bellini are slit-like, close when there is an increase in the "back-pressure" of urine from the bladder and so prevent intrarenal reflux when reflux occurs from the bladder. These papillae occur predominantly in the mid zone. The refluxing papillae occur predominantly in the polar regions, and, as they have flattened tips, the collecting duct orifices are wide and prone to retrograde flow of urine into the tubules during vesico-ureteric reflux (Ransley and Risdon, 1979). The microscopic and ultrastructural features of the medulla have been described by several researchers (Moffat, 1979, 1981, 1982; Bohman, 1980; Maunsbach *et al*., 1980).

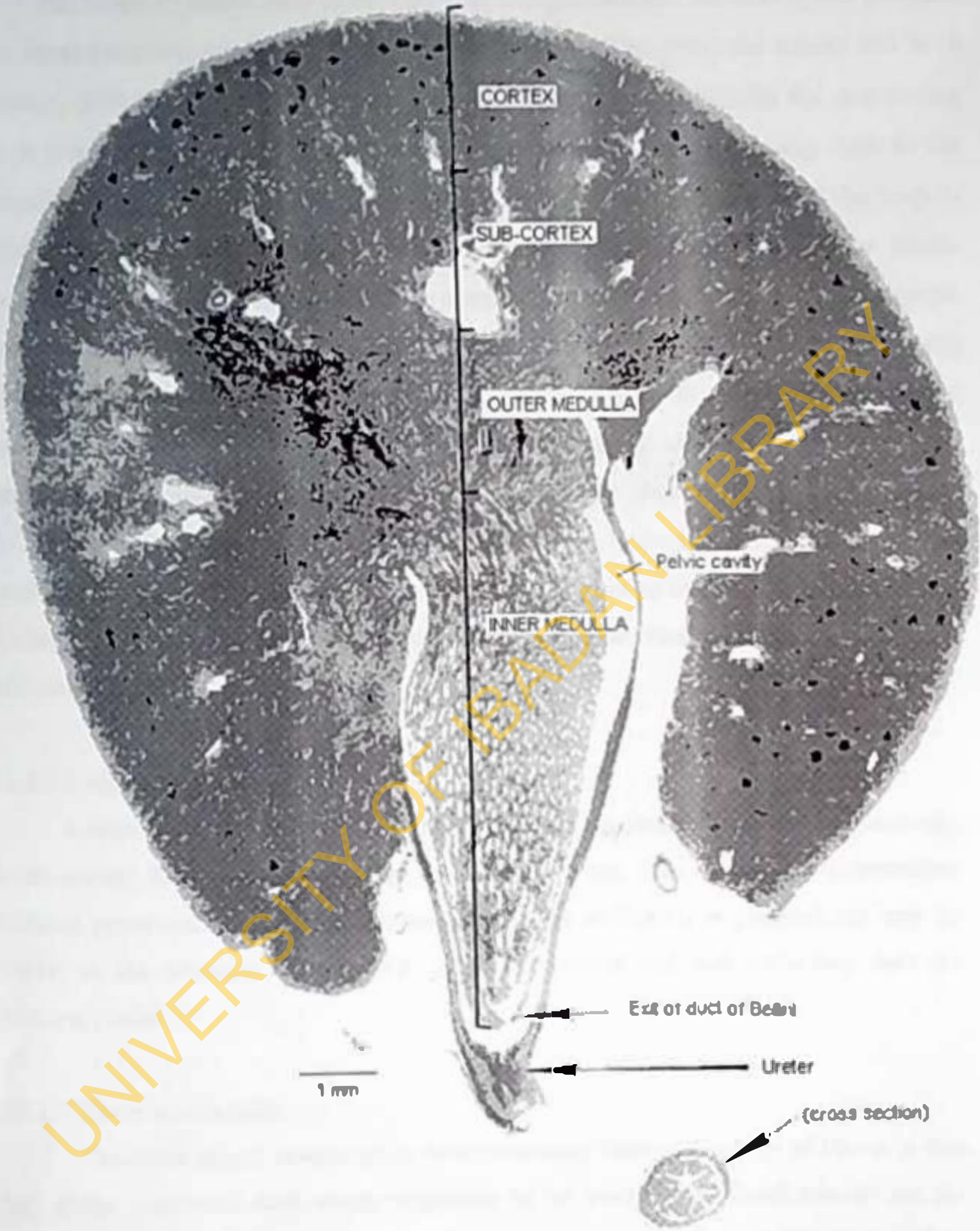


Figure 3. Coronal section through a normal rat kidney. Toluidine Blue; bar line = 1mm). From: Bach & Bridges (1985a)

2.1.1.5 The loops of Henle

The loops of Henle may be divided into two populations on anatomical grounds. Short loops penetrate no further than the outer medulla. The proximal tubule and thick ascending limb are closely associated in the cortex, but in the medulla the descending limb is intimately related to the ascending vasa recta, and the ascending limb to the collecting duct. The association of the ascending and descending limbs of the loop of Henle with the vascular system or with the collecting ducts provides a multi-dimensional network in which solutes or water may undergo countercurrent exchange. These exchanges may either provide a shunt that excludes selected solutes (and water) from the inner medulla or, alternatively, solutes (e.g., sodium chloride and urea) may be trapped in this zone. This exclusion of water and trapping of sodium chloride, urea, and osmolytes helps maintain the osmotic gradient along the inner medulla. In long loops (the length is proportional to the renal concentrating potential), the loop of Henle penetrates the inner medulla. Only about a third of the ascending and descending limbs of long loops lie together; in the other instances the ascending limbs are nearer to collecting ducts than to descending limbs.

2.1.1.6 Collecting ducts

Collecting ducts consists of three identifiable segments, which lie, respectively, in the cortex, the outer medulla, and the inner medulla. These segments demonstrate different permeabilities to water and osmolytes. The difference in permeability may be related to the presence of two cell types, the intercalated and collecting duct (or principal) cells.

2.1.1.7 The distal tubule

The distal tubule connects the thick ascending limb of the loop of Henle to that part of the collecting duct which originates in the cortex. The distal tubules are involved in both ion and water reabsorption, but play a much less significant role than the proximal tubules. The underlying mechanisms responsible for reabsorption appear, in essence, to be similar to those already outlined. The major differences include a stronger Na^+ gradient against which to "pump", the ability to reabsorb sodium without reabsorbing water, the controlling effects of anti-diuretic hormone (ADH) and aldosterone (among other mediators), and the very limited (or lack of) protein reabsorption. The secretion of potassium ions appears to be under the control of an

active transport mechanism, the regulating factors of which are many and complex (Valtin, 1973; Orloff and Berliner, 1973; Brenner and Rector. 1986).

2.1.1.8 The countercurrent multiplier system and urine concentration

Less than 1% of the glomerular filtrate leaves the kidney as urine (unless there is a state of diuresis), the remainder having been reabsorbed. The process of urine concentration is complex and depends (at least in part) on the countercurrent multiplier system, which establishes a steep osmotic gradient along the inner medulla. The high osmolality is a consequence of the differential permeability of the limbs of the loops of Henle and the collecting ducts to water and ions. The thick ascending limb is thought to have an active mechanism which transports chloride and sodium out of the lumen and into the interstitium, but the limb remains impermeable to water. As a consequence the osmolality decreases in this part of the tubule (the diluting segment). The descending limb, on the other hand, is freely permeable to water, but probably not to sodium ions. The high ion concentration in the interstitium would draw water out of the descending limb, increasing the osmolality towards the turn of the U-loop. This is augmented by urea and other osmolytes that leave the collecting ducts and enter the ascending limb via the interstitium, thus being recirculated to the medulla.

The collecting ducts regulate the final urine concentration by controlling the amount of water that is reabsorbed. The passage of water out of the ducts is thought to be mediated largely by cyclic adenosine-monophosphate (cAMP), the synthesis of which is stimulated by ADH, which increases the permeability of the luminal cell membrane to water. Osmotic effects draw the water out of the cell (through the basement membrane) into the hyperosmotic interstitium. In the absence of ADH the collecting duct is thought to be impermeable and relatively little water is reabsorbed from it. The interstitial osmotic gradient is assumed to be maintained by the effective removal of water via the ascending vasa recta, which have both a greater radius than the descending vasa recta and are about twice as numerous. The countercurrent exchange associated with the loops of Henle arising from cortical nephrons offers an important "barrier" zone, which is thought to facilitate solute trapping in and solvent exclusion from the inner medulla, and thus helps to maintain the hyperosmolality in this "compartment".

There are a number of other factors that control, alter, or contribute to urine concentration. Medullary blood flow is complex, as are the factors controlling it.

Increased blood flow rates will decrease the efficiency of countercurrent exchange in the outer medulla, as a consequence of which the high osmotic gradient in the inner medullary compartment will be "washed out", and urine will not be concentrated. Diuresis is associated with increased blood flow rates (Earley and Friedler, 1964, 1965; Chuang *et al.*, 1978).

A unique feature of the vasa recta is their permeability to macromolecules, a consequence of which is that the medulla contains a large pool of albumin. The factors controlling the rapid turnover of this milieu are poorly understood. It is generally assumed that (together with the glycosaminoglycans) these proteins provide an interstitial osmotic pressure that facilitates water reabsorption (Brenner and Rector, 1986).

2.1.1.9 The interstitinl cells

Interstitial cells occur in most organs. Three types of interstitial cells have been described in the medulla of the rat kidney (Bohman, 1980). Type I cells are the most abundant and represent the typical renal medullary cells. Type 2 medullary interstitial cells are generally round and lack lipid droplets, while Type 3 cells correspond to the pericytes. Types 2 and 3 are sparsely distributed and are often overlooked between the tubules, ducts, and blood vessels. In the inner medulla, however, Type I cells are numerous and especially prominent because they are set in a dense matrix of glycosaminoglycans (previously referred to as mucopolysaccharides or acidic mucopolysaccharides).

The medullary interstitial cells have been described by Moffat (1979, 1981, 1982), Bohman (1980), and Maunsbach *et al.* (1980). The number of cells and the amount of matrix substance occupies 10-20% of the tissue volume in the outer medulla, and 40% near the apex of the inner medulla (Bohman, 1980). The cells, which are arranged in a regular pattern perpendicular to the tubules and vessels, are irregular in shape and have many long slender processes. These come into close contact with adjacent interstitial cells, capillaries, and the limbs of the loop of Henle, but there is no such relationship with the collecting ducts.

One of the most characteristic features associated with the Type I cells is the presence of lipid inclusion droplets, which occupy at least 2-4% of the total cell volume. The lipid content is largely triglycerides, with variable amounts of cholesterol esters and phospholipids. A number of conditions have been described where there are

marked changes in the size and number of lipid droplets. The pathophysiological significance of these changes is difficult to interpret because of varied experimental approaches, species variation, and contradictory reports (Bohman, 1980).

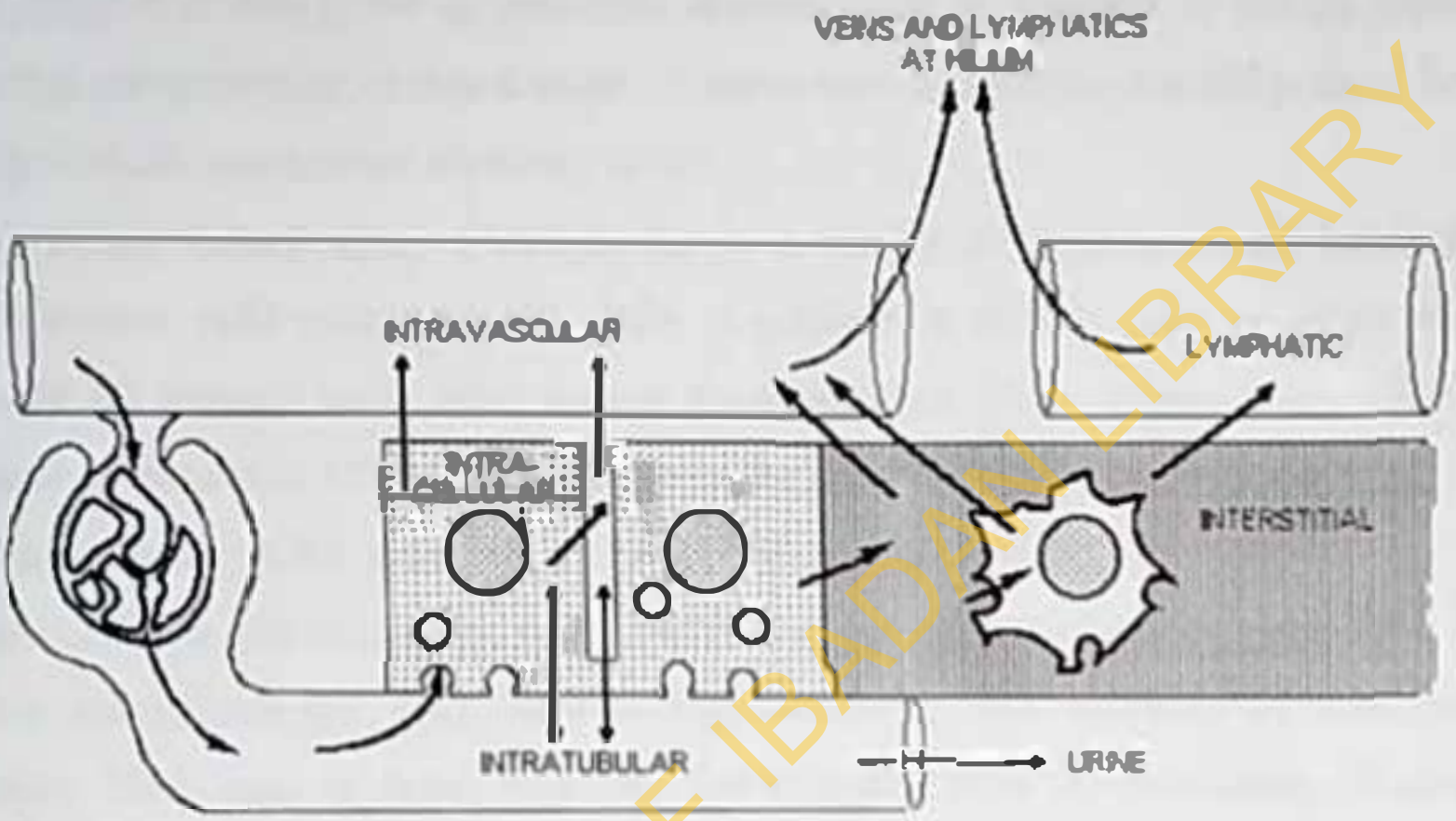


Figure 4: Schematic representation of the different cellular and extracellular components in the kidney. From: Moffat (1982).

According to the Acute Kidney Injury Network (AKIN) group proposed consensus definition, Acute kidney injury (AKI), inclusive of clinical terms such as acute renal failure (ARF), acute tubular necrosis (ATN), and delayed graft function, is being defined as functional or structural abnormalities or markers of kidney damage, including abnormalities in blood, urine, or tissue tests or imaging studies present for <3 months (AKIN Amsterdam Meeting, 2006).

Acute kidney injury (AKI) is due to a variety of conditions and has serious consequences. AKI occurs in 5 to 10% of patients in tertiary care hospitals and in virtually all patients early after kidney transplantation (Star, 1998). From large but separate databases of US hospitalizations in the past 10 to 15 yr, there is evidence for a marked increase in the incidence of AKI (Waikar *et al.*, 2006; Xue *et al.*, 2006). This, in part, reflects the increasing comorbidity and age of patients who have AKI. It is widely recognized that AKI leads to high morbidity and mortality in hospitalized patients. There may be hope, however, that mortality rates are decreasing (Waikar *et al.*, 2006; Xue *et al.*, 2006); nevertheless, mortality rates remain unacceptably high, and there is an urgent need for effective therapy (Star, 1998). Except for a few isolated studies, the vast majority of animal and clinical studies have yet to demonstrate conclusively the benefit of pharmacologic treatment of AKI.

2.2.1 Pathogenesis of AKI

The pathogenesis of AKI is complex. Ischemia and nephrotoxins are major factors that precipitate injury, and although the initiating events may be dissimilar, subsequent injury responses likely involve similar pathways. As an example, AKI that is associated with ischemia is due to a reduction of renal blood flow below the limits of blood flow autoregulation. A variety of molecular responses that are "maladaptive" and stereotypical then occur. These responses lead to endothelial and epithelial cell injury after the onset of reperfusion (Sutton *et al.*, 2002). Pathogenic factors such as vasoconstriction, leukostasis, vascular congestion, apoptosis, and abnormalities in immune modulators and growth factors have formed the basis of rational therapeutic interventions. However, many of these targeted therapies have failed, are inconclusive, or have yet to be performed (Conger, 1995; Ronco and Bellomo, 2003). Given the complexity of the pathogenesis of AKI, it may be naive to expect that one therapeutic

intervention would have success unless that intervention focuses on prevention of AKI and targets a specific initiating cause. There are encouraging results from therapeutic interventions in the prevention of contrast nephropathy: Saline (Solomon *et al.*, 1994), sodium bicarbonate (Merten *et al.*, 2004), low and isosmolar contrast (Rudnick *et al.*, 1995; Aspelin *et al.*, 2003), and theophylline (Ix *et al.*, 2004). Given the multiple overlapping pathways that are involved in AKI, therapies may need to target multiple pathways simultaneously to achieve success (Kelly and Molitoris, 2000).

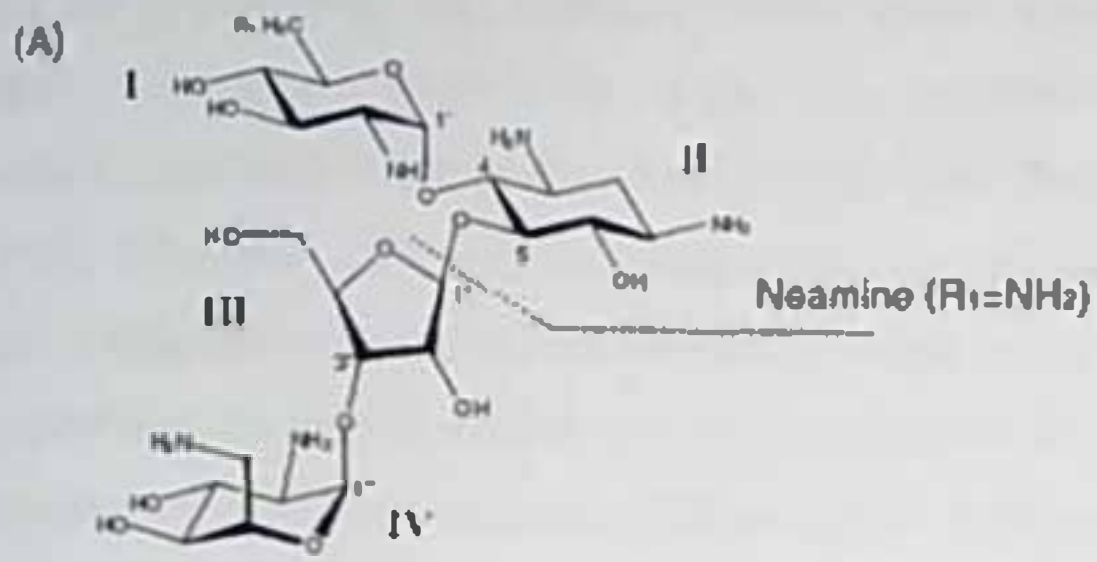
Defining clinically significant AKI is critical. In the past, clinical trials have used widely varying definitions that ranged from a 20 to 30% rise in serum creatinine to the need for dialysis, which has led to reported incidence of AKI of 1 to 25% (Chertow *et al.*, 1998; de Mendonca *et al.*, 2000) and mortality rates that varied from 28 to 90% (Metnitz *et al.*, 2002; Cosentino *et al.*, 1994). The absence of consensus on a definition for AKI led to the Acute Dialysis Quality Initiative (ADQI) and the development of the Acute Kidney Injury Network (AKIN), which represents the efforts of workgroups that seek to develop consensus and evidence-based statements in the field of AKI. ADQI used a set of criteria called the RIFLE (Risk, Injury, Failure, Loss, and End stage) criteria (Bellomo *et al.*, 2004), which were modified recently by AKIN (AKIN Amsterdam Meeting 2006). Validation of the classification and staging system of AKI will be required in future clinical studies and holds promise that this classification scheme can improve the design of trials.

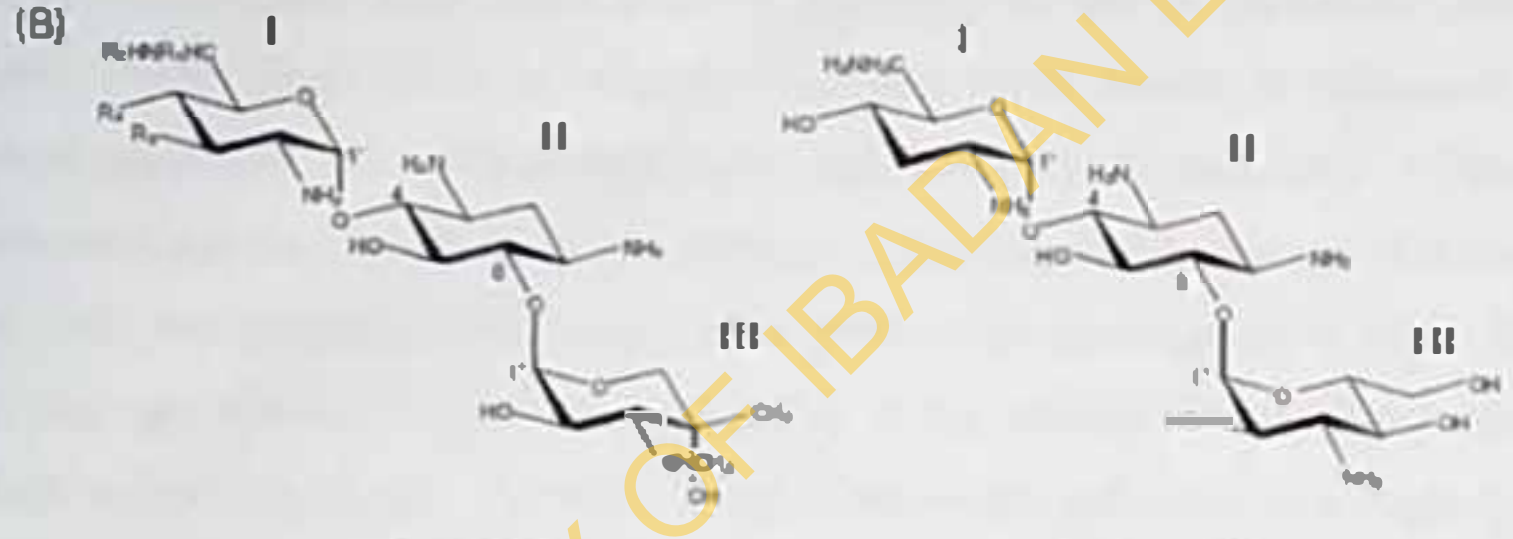
2.3 AMINOGLYCOSIDE ANTIBIOTICS AND NEPHROTOXICITY

Aminoglycosides have long been one of the commonest causes of drug-induced nephrotoxicity (Walker and Duggin, 1988). Although a clear recognition of the patient- and treatment-related risk factors (Moore *et al.*, 1987), combined with the once-a-day schedule and effective monitoring procedures (Prins *et al.*, 1996), have definitely improved the situation over what prevailed in the early 1980s (Smith *et al.*, 1980), we are still short of having brought the safety of aminoglycosides to that of the main other wide-spectrum antibiotics. Research aimed at obtaining intrinsically less toxic compounds has met with only modest success, and few of the other approaches proposed to reduce the toxicities of the available agents have reached practical clinical applications. Yet, because aminoglycosides are very effective antibiotics well suited to the treatment of severe infections (Gilbert, 1995), it seems important to maintain and even develop efforts to improve their therapeutic indices. The chemical structures of some clinically useful aminoglycoside antibiotics are shown in figure 5.

2.3.1 GENERAL FEATURES OF AMINOGLYCOSIDE - INDUCED NEPHROTOXICITY

Nepbrotoxicity induced by aminoglycosides manifests clinically as nonoliguric renal failure. with a slow rise in serum creatinine and a bypoosmolar urinary output developing after several days of treatment. Aminoglycosides are nephrotoxic because a small but sizable proportion of the administered dose (approximately 5%) is retained in the epithelial cells lining the S1 and S2 segments of the proximal tubules (Vandewalle *et al.*, 1981) after glomerular filtration (Fabre *et al.*, 1976). Aminoglycosides accumulated by these cells are mainly localized with endosomal and lysosomal vacuoles (Sandoval *et al.*, 1998; Silverblatt and Kuehn, 1979) but are also localized with the Golgi complex (Sandoval *et al.*, 1998). They elicit an array of morphological and functional alterations of increasing severity. The effects of low dose and high dose have been distinguished since there is probably more than a quantitative difference between the changes seen under these two conditions (Tulkens, 1986).





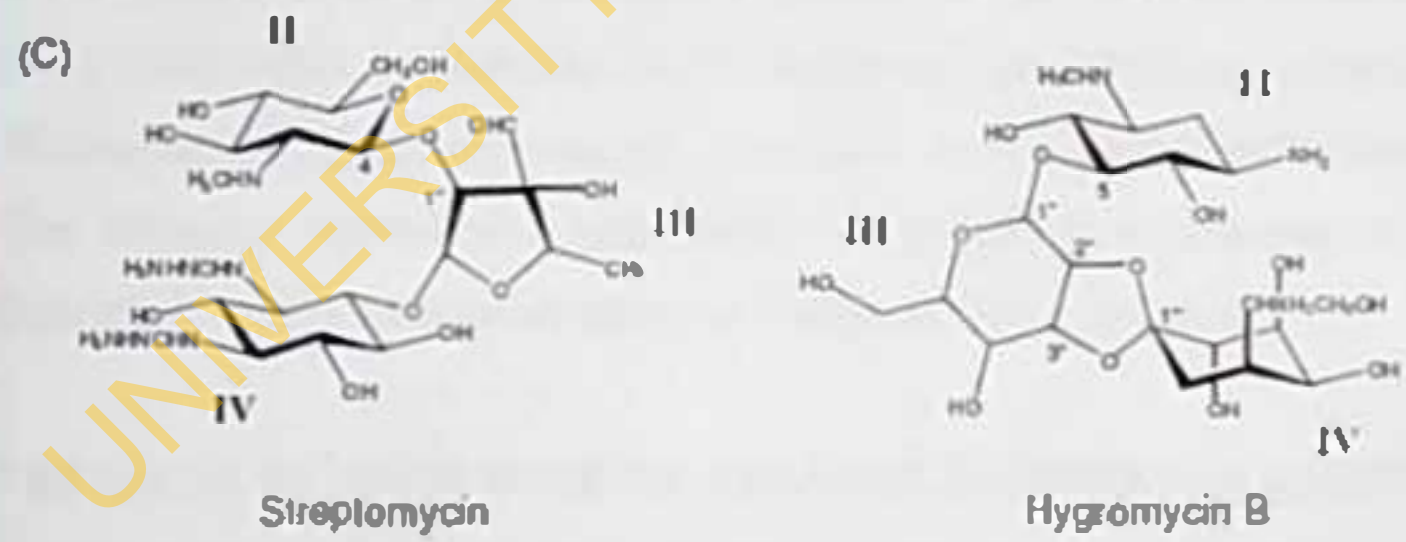


Figure 5. Conformational structures of some aminoglycoside antibiotics. (A) The 4,5-disubstituted class of aminoglycosides, paromomycin and neamine. (B) The 4,6-disubstituted class of aminoglycosides, gentamicin and tobramycin. Gentamicin is a mixture of gentamicin C1a (R1=H, R2=H, R3=H and R4=H), gentamicin C1 (R1=CH3, R2=CH3, R3=H and R4=H) and gentamicin C2 (R1=H, R2= CH3, R3=H and R4=H). Geneticin (R1=CH3, R2=OH, R3=OH and R4=OH) is also categorized into the gentamicin subclass. (C) Structurally dissimilar aminoglycosides, streptomycin and hygromycin B.

2.3.2 EFFECTS OF CLINICAL DOSES OF AMINOGLYCOSIDES IN ANIMALS AND HUMANS

After only a few days of administration of clinical doses to humans or of low multiples of the human therapeutic dose to animals (typically 10 to 20 mg/kg of body weight for a laboratory rat), aminoglycosides induce conspicuous and characteristic changes in lysosomes of proximal tubular cells consistent with the accumulation of polar lipids (myeloid bodies) (Begg and Barclay, 1995; De Broe *et al.*, 1984; Kosek *et al.*, 1974; Watanabe, 1978). Thesechanges are preceded and accompanied by signs of tubular dysfunctions or alterations (release of brush-border and lysosomal enzymes; decreased reabsorption of filtered proteins; wasting of K^+, Mg^{2+}, Ca^{2+}, and glucose; phospholipiduria; and cast excretion (Gilbert, 1995). In humans, the occurrence of these signs may be followed by the development of overt renal failure characterized mainly by a nonoliguric and even often polyuric hypoosmotic fall in creatinine clearance (Gilbert, 1995). Progression to oliguric or anuric renal failure is infrequent, and recovery upon drug discontinuation is most often observed. Occasionally, a Fanconi's syndrome (Casteels-Van Daele *et al.*, 1980) or a Bartter's-like syndrome (Landau and Kher, 1997) has been observed. A correlation between the development of these clinical signs and the severity or rate of progression of the subclinical alterations remains difficult to establish mainly because of large interpatient variations. Consequently, the usefulness of monitoring the subclinical changes to detect individuals at risk has remained questionable. In animals, tubular alterations have clearly been associated with the development of focal necroses and apoptoses in the tubular epithelium, together with an extensive tubular and peritubular cell proliferation (Laurent *et al.*, 1983; Toubeau *et al.*, 1986), without an apparent change in kidney function.

2.3.3 EFFECTS OF HIGH DOSE OF AMINOGLYCOSIDES IN ANIMALS

High doses (40 mg/kg or more for gentamicin) are necessary in animals to rapidly induce extended cortical necrosis and overt renal dysfunction (Kosek *et al.*, 1974; Parker *et al.*, 1982). At this stage, a large number of structural, metabolic, and functional alterations are observed in tubular cells, and several of these alterations have been claimed to be responsible for cell death or dysfunction. Many of the changes observed at the level of the apical membrane (Elliott and Patchin, 1992; Hishida *et al.*, 1994; Hori *et al.*, 1992; Skopicki *et al.*, 1996; Somermeyer *et al.*, 1983) could, however, be merely mediated by a direct effect of the drug on this structure during its

initial stages of uptake in proximal tubular cells. Conversely, other effects, such as inhibition of protein synthesis and modulation of gene expression, mitochondrial alterations, or inhibition of enzymes located on the cytosolic side of the pericellular membrane, must involve uptake and intracellular distribution of the drug to the corresponding targets.

2.3.4 REDUCING OR PROTECTING AGAINST AMINOGLYCOSIDE - INDUCED NEPHROTOXICITY

The goal of reducing or protecting against aminoglycoside nephrotoxicity has attracted much effort and attention over the last decade. These efforts can be subdivided into several types of approaches.

i. Decreasing or preventing aminoglycoside accumulation by kidneys. Decreasing or preventing aminoglycoside accumulation by the kidneys would represent one of the most simple and radical approaches to reduce aminoglycoside nephrotoxicity, since it should lead to success whatever the targets of aminoglycosides are in the kidney. Aminoglycoside accumulation could be reduced either by impairing their uptake or by enhancing their release. Reduction of uptake has been obtained by two strategies. The first one is aimed at complexing the aminoglycosides extracellularly, and the second one is aimed at competing with or decreasing drug binding to the brush-border membrane. Unfortunately, these approaches could not be translated into clinical applications because of a lack of efficacy and/or because of intrinsic toxicity (Cohen *et al.*, 1988; Malis *et al.*, 1984). Yet, the strategy based on competition for binding eventually led to the recognition that aminoglycosides could be their own competitors. Early studies with animals (Reiner *et al.*, 1978) indeed revealed that administration of the daily dose of gentamicin as a single dose (thus creating one high daily peak level) was considerably less toxic than administration of the same daily dose divided into three doses per day or by continuous infusion (Giuliano *et al.*, 1984). An explanation for this unexpected behavior came from the finding that aminoglycoside uptake by kidney tubular cells is saturable (Giuliano *et al.*, 1986), so that much of the drug that passes in the lumen will not be reabsorbed if the drug is too concentrated. Because saturation was shown to occur at a clinically meaningful range of concentrations, this observation triggered a large number of studies comparing the toxicities of various drug administration schedules. Almost at the same time, animal and clinical studies

demonstrated that a high, transient peak level in serum was also a critical determinant of aminoglycoside efficacy (Gilbert, 1995). The once-daily dosing mode of administration of aminoglycosides was therefore clinically tested in the late 1980s in a limited series of clinical trials that were at first cautious (Sturm, 1989; ter Braak *et al.*, 1990; Tulkens *et al.*, 1988), but thereafter, it was tested with almost all indications for aminoglycosides (Gilbert, 1995). The once-daily regimen is now widely accepted (Gilbert, 1997), and it is in the official package insert recommendations for netilmicin and amikacin in several countries in Europe and elsewhere, even though discordant voices are still heard (Bertino and Rotschafer, 1997). Beyond its potential impact on the toxicity and activity of aminoglycosides, the once-daily dosing regimen also offers interesting pharmacoeconomic and practical advantages (Nicolau *et al.*, 1995) and makes drug monitoring easier (Prins *et al.*, 1996). A further development could be the implementation of once-daily administration at a specific hour of the day since there might be important circadian variations in the glomerular filtration rate and, hence, the availability of the aminoglycoside to the kidney (a previous clinical study has indicated that the administration of gentamicin during the midnight to 7 a.m. period was probably more likely to cause toxicity than administration during other periods of the day (Beauchamp *et al.*, 1997; Prins *et al.*, 1997).

ii. Preventing or decreasing the lysosomal phospholipidosis induced by the cell-associated aminoglycosides. A reduction in lysosomal phospholipidosis could be achieved either by use of an aminoglycoside modified to bind less tightly to phospholipids at an acidic pH or by the administration of an agent that would prevent the binding of the antibiotic to phospholipids. Both strategies have been followed.

a. Aminoglycoside modifications. A series of 1-*N*-substituted derivatives of gentamicins and kanamycins were synthesized in the late 1970s to obtain molecules resistant to the bacterial enzymes that inactivate the parent compounds. Retrospectively, it was found that all derivatives in which the N-1 atom has been made nonionizable (i.e., by substitution of the amino function with an acyl side chain such as in amikacin) show reduced levels of binding to acidic phospholipids together with a lesser inhibitory potency toward lysosomal phospholipases (Carlier *et al.*, 1983). Among them, amikacin, isepamicin, and arbekacin have been successfully developed and have been proved to cause less intense renal changes in animals as well as in humans when they are tested under strictly comparable conditions with other currently used

aminoglycosides (Blum, 1995; De Broe *et al.*, 1984; Kondo, 1994; Laurent *et al.*, 1982; Miller *et al.*, 1978). For amikacin and isepamicin, this effect has been ascribed to a lesser degree of interaction of the amino functions of these drugs with the phospho group of negatively charged phospholipid (Carrier *et al.*, 1997; Schanck *et al.*, 1992) and to changes in the orientation of the drug bound to the lipid layer (Tulkens *et al.*, 1990) (these models, however, have been challenged [Carrier *et al.*, 1997]). Efforts have therefore been made accordingly to rationally design new aminoglycosides with similar and, it is hoped, even more favorable properties. This led to the synthesis of derivatives of kanamycin B substituted at position C-6" with halogen atoms, a pseudo-halogen group (azido), or increasingly bulkier alkyl chains via an intermediate N, S, or O atom, yielding the corresponding 6"-amino, -amido, -thioalkyl, or -alkoxy derivatives (Mingeot-Leclercq *et al.*, 1991), as well as to derivatives of netilmicin and kanamycin A substituted at positions N-1 and N-6' with amino acids (Kotretsou *et al.*, 1995), but with only modest success. In a similar context, the disaccharidic aminoglycosides astromicin (fortimicin A) and dactimicin (2-*N*"-formimidoyl-astromicin) have been shown to bind less tightly to phospholipid bilayers and to be weaker inhibitors of lysosomal phospholipases (Lambricht, 1992). Their lesser nephrotoxicities observed in animals could, however, mainly be due to their lower levels of accumulation in the renal cortex (Inouye *et al.*, 1989).

Of greater interest are probably the derivatives of tobramycin, dibekacin, arbekacin, or kanamycin with a fluorine atom at position 5, 3', or 3"' (Kobayashi *et al.*, 1992; Shitara *et al.*, 1992; Takahashi *et al.*, 1993; Tsuchiya *et al.*, 1993; Tsuchiya *et al.*, 1985). These were originally made to confer resistance to aminoglycoside-inactivating enzymes, but for these derivatives chemical and biophysical considerations suggest a reduced level of binding to phospholipids because of a decreased basicity of the vicinal amino group through an inductive effect. These compounds showed increased 50% lethal doses, but experimental data on their binding to phospholipids are not available.

b. Prevention of aminoglycoside binding to phospholipids. Polyaspartic acid has emerged as a very successful protectant against aminoglycoside-induced nephrotoxicity from the screening of various polymers that are likely to impair the binding of aminoglycosides to kidney membrane vesicles (Williams and Hottendorf, 1985). In experimental studies with animals, the coadministration of polyaspartic acid with gentamicin or amikacin was shown to protect against the development of phospholipidosis and phospholipiduria (Kishore *et al.*, 1992), as well against all early

and late signs of aminoglycoside nephrotoxicity (Elliott and Patchin, 1995; Gilbert *et al.*, 1989). Actually, both polyaspartic acid and the aminoglycoside reach the lysosomes by endocytosis (Kállay and Tulkens, 1989) and form ion-pair complexes within these organelles due to the acidic pH prevailing therein. *In vitro* studies demonstrated that polyaspartic acid prevents aminoglycoside binding to negatively charged phospholipids bilayers and thereby makes the drug unable to inhibit the activities of lysosomal phospholipases (Kishore *et al.*, 1990). Further studies showed that polyaspartic acid also protects against gentamicin-induced alterations of phospholipid metabolism in cultured cells (Ramsammy *et al.*, 1990) and of electrophysiological alterations in cultured human proximal tubular cells (Todd and Hottendorf, 1997). Polyaspartic acid also prevents impairment by gentamicin of homotypic fusion of renal cell endosomes (Hammond *et al.*, 1997) and blocks the process of aminoglycoside-induced aggregation of negatively charged liposomes (Van Bambeke *et al.*, 1995), all events which had been directly related to the binding of gentamicin to phospholipids. *In vivo* studies have now defined the limits and the duration of the protection afforded by polyaspartic acid (Swan *et al.*, 1993). Moreover, pharmacokinetic evaluations have shown that polyaspartic acid increases the penetration of gentamicin in the so-called deep peripheral compartment (which most likely represents the intracellular drug-polyaspartic acid complex (Whitfein *et al.*, 1996) and which suggests that the antibiotic is stored in a nontoxic form). A protective effect of polyaspartic acid against ototoxicity has also been demonstrated (Hulka *et al.*, 1993). A movement toward large-scale toxicological studies and clinical applications of polyaspartic acid therefore appears to be warranted but is still hindered by the lack of a clear definition of the precise type of polymer which needs to be used. It must indeed, at the same time, be filtratable through the glomerulus, bind effectively to gentamicin (Kohlhepp *et al.*, 1992), remain sufficiently stable in the kidney to afford significant protection (Kishore *et al.*, 1992), and not causing renal toxicity per se, as was shown for polymers that are too stable (Kishore *et al.*, 1990). Daptomycin (LY 146032), which contains three Asp residues, also colocalizes in the lysosomes of the renal cortex with gentamicin and protects against lysosomal alterations *in vivo* (Thibault *et al.*, 1994). *In vitro*, it increases the negative charge density of membranes, while at the same time affecting the lipid packing (Gumani *et al.*, 1995), two effects which counteract those of gentamicin and facilitate the access of the catalytic site of the phospholipases to their lipidic substrate. Torbafylline (HWA-448), an analog of the vasculoactive agent pentoxifylline, also

protects against gentamicin-induced phospholipidosis, but its mode of action is unknown (Ford *et al.*, 1995).

iii. Other means of protection. Among the other main approaches used so far to reduce or to protect against aminoglycoside nephrotoxicity, the most consistent effects have been observed with the use of antioxidants and especially deferroxamine. On the basis of the finding that gentamicin forms complexes with mitochondrial Fe^{2+} to catalyze the formation of free oxygen radicals, iron chelators were tested and were proven to be effective in the prevention of aminoglycoside-induced ototoxicity (Schacht, 1997; Song *et al.*, 1997). Extension of this finding to nephrotoxicity appears to be possible (Walker and Shah, 1987), but biophysical and biochemical considerations (Priuska and Schacht, 1997) suggest that the protective effect of deferroxamine may be critically dependent on the dosage of gentamicin. Other compounds were also used on account of their antioxidant effects, but the mechanisms have not always been unambiguously established. Means of protection based on a correction of the functional abnormalities or on an increase in cell regeneration capabilities have also been attempted, but no clinical application has so far been made.

2.3.5 GENTAMICIN – INDUCED NEPHROTOXICITY IN HUMANS AND ANIMALS

Gentamicin is an aminoglycoside antibiotic that is still commonly used in the treatment of life-threatening infections. Their broad-spectrum activity against aerobic gram+ve and gram-ve organism, their chemical stability, and their rapid bactericidal action has often made them first-line drugs in a variety of clinical situations (Singenthaler *et al.*, 1986; Appel, 1990). However, high concentrations of these antibiotics are nephrotoxic. In some cases, this side effect is so severe that the use of the drug must be discontinued. It has been estimated that up to 30% of patients treated with aminoglycosides for more than 7 days show some signs of nephrotoxicity (Mathew, 1992). Although newer agents, for example, third-generation cephalosporins and aztreonam, may be as therapeutic and cost-effective as the aminoglycosides without the nephrotoxicity associated with the latter agents, the aminoglycoside remain among the most widely used antimicrobial agents. This may be due to their rapid effectiveness, as well as to the fact that more is known about their pharmacology, toxicity and therapeutic properties than about other newer agents. The

pharmacokinetics, pathology and clinical pattern of gentamicin-induced kidney damage have been studied extensively in both man and animal. However, there is no unanimity in the literature regarding the possible mechanism(s) of action of these, or the factors that can modulate the nephrotoxicity. (Kacew and Bergeron, 1990; Appel, 1990). The value of aminoglycosides, including gentamicin, in clinical practice would be greatly enhanced if some means could be found to protect the kidney from this undesirable side effect. Several factors have been identified to increase the risk of aminoglycoside nephrotoxicity that cannot be modified readily by the clinician (e.g., sex, obesity, preexisting liver or renal disease and underlying diseases) and factors that the clinician may be able to modify (drug dosage/interval/duration, specific aminoglycoside, hypokalaemia, hypomagnesaemia, metabolic acidosis, volume depletion and concurrent medications) (Kaloyanides, 1993).

In the last two decades several review articles have appeared which address various aspects of gentamicin nephrotoxicity in man and animal (Kosek *et al.*, 1974; Luft *et al.*, 1975; Hottendorf and Williams, 1986; Humes, 1988; Bennett, 1989; Kacew and Bergeron, 1990).

2.3.5.1 Gentamicin Pharmacokinetics in Humans

Gentamicin, and other aminoglycosides, are polycations and highly polar. They are not absorbed in the gastrointestinal tract but are rapidly absorbed after intramuscular or subcutaneous injection. They can also be given intravenously or intrathecally. Binding to plasma protein is minimal. Gentamicin crosses the placenta but does not cross blood-brain barrier into the central nervous system or penetrate the eye. Tissue levels are low except in the renal cortex. The plasma half-life is 2-3 hr. There are no known gentamicin metabolites. Elimination is virtually entirely by glomerular filtration in the kidney (Bennett, 1989; Rang and Dale, 1987). Concomitant treatment with the nephrotoxic drug cisplatin reduces gentamicin excretion in rats resulting in increased antibiotic level in plasma and tissues for at least a week (Engineer *et al.*, 1987). It has been suggested that the cytotoxicity of aminoglycosides may be mediated by a toxic metabolite generated by the hepatic cytochrome P-450 drug metabolizing enzymes (Huang and Schacht, 1990; Crann *et al.*, 1992). More recently, however, this could not be confirmed by Sanders *et al.* (1993), who pretreated rats with an inhibitor of drug metabolism prior to administration of gentamicin and found that

this inhibition of drug metabolism does not alter gentomicin nepbrotoxicity. This indicates that the toxicity is due to the drug itself and not a metabolite thereof.

2.3.5.2 Pathology and Pathophysiology of Gentamicin – induced Renal Injury

Gentamicin is incorporated and accumulated in proximal tubule lysosomes, and its accumulation is thought by some workers to explain aminoglycoside nephrotoxicity (Papanikolaou *et al.*, 1992). However, the concentration of the antibiotic in the tubules is not always necessarily correlated with the nephrotoxicity. It has been reported that some agents (e.g., polyaspartic acid and Sairei-to) can protect against gentamicin nephrotoxicity despite the fact that the gentamicin renal concentration is not different or actually 10 times more than in the controls (Bennett *et al.*, 1988b; Ohno *et al.*, 1993). On the other hand reduction in gentamicin accumulation in the kidney is not always indicative of protection. For example, in rats treated concomitantly with gentamicin and 5-pyridoxal phosphate, renal gentamicin level was reduced by about 40% compared to rats treated with gentamicin alone. This, however, was not associated with protection against nephrotoxicity (Kacew, 1989). Phospolipids administration reduced the nephrotoxicity of gentamicin without affecting significantly the gentamicin concentration in the renal cortex (Chan *et al.*, 1991). More recently superoxide dismutase (SOD) and dimethylthiourea (DMTU) were reported to protect against gentamicin nephrotoxicity without affecting the renal cortical gentamicin content (Nakajima *et al.*, 1994). These reports do not totally negate the value of measuring the antibiotic concentration in the kidney for assessing the extent of nephrotoxicity but indicates that the presence of the drug in the kidney per se does not always indicate nephrotoxicity. Accumulation of the drug in specific target organelles in the renal cortex may be the critical step in the nephrotoxicity (Kacew and Bergeron, 1990). Currently, it is generally agreed that gentamicin produces dose-dependent proximal renal tubular necrosis, which can be dissociated from intracellular accumulation (Bennett, 1989).

Gentamicin is considered an "obligatory nephrotoxin" and even small doses have been reported by several authors to produce nephrotoxicity in man and animals. It has been suggested that nephrotoxicity from aminoglycoside may be inevitable to some extent in every patient, and only in its most severe form does the damage to the kidney reach a level allowing clinical diagnosis (Bennett, 1989).

After gentamicin is filtered in the glomerulus, a small proportion of the filtered drug (2-5%) binds to and is actively transported into proximal tubular cells. The drug can be taken up into the cell from both the luminal and basolateral surfaces, although binding to brush border membrane phospholipids with absorptive pinocytosis predominates (Bennett, 1989).

The basolateral uptake is quantitatively less significant than brush border transport. However, the latter is more critical to nephrotoxicity because it allows for drug exposure to critical sites in key organelles such as mitochondria and microsomes (Weinberg *et al.*, 1985). Gentamicin is taken up at the apical pole of the tubule cells binding to anionic sites on the brush border membranes (Nassberger *et al.*, 1990). After the penetration of the cytoplasm by endocytosis, the drug accumulates in the lysosomes.

Using a model of proximal renal tubule epithelial cells derived from transgenic mouse, the specific uptake mechanism of gentamicin in renal cortex has been recently reported (Kaunitz *et al.*, 1993). This uptake mechanism has been shown to be inhibited by L-lysine but not by any other amino acid. It was therefore concluded that gentamicin and L-lysine share a specific uptake mechanism located in the apical membrane of renal proximal tubule cells. These results are at variance from earlier results (Williams *et al.*, 1985), which indicate that L-lysine was ineffective in inhibiting gentamicin binding to rat renal cortical membrane vesicles.

The effect of gentamicin on electrolytes has received relatively little attention (Foster *et al.*, 1992). Briefly, gentamicin produces disturbances in electrolyte homeostasis resulting in hypomagnesamia, hypocalcaemia and hypokalaemia (Ali *et al.*, 1992a; Foster *et al.*, 1992). These electrolyte disturbances are thought to result from a failure of the kidneys to adequately conserve cations due to an adverse renal action of the drug.

The pathophysiology of the impairment of glomerular filtration rate (GFR) in gentamicin-induced nephrotoxicity was studied extensively but its mechanism remains unclear. It has been ascribed to proximal tubular injury, abnormalities or renal circulation or reduction of glomerular capillary ultrafiltration (Nakajima *et al.*, 1994). It was suggested that the abnormalities of the renal circulation rather than tubular damage are paramount in the reduction of GFR that occurs following gentamicin administration. Although it is generally accepted that gentamicin adversely affects the proximal tubule function, there is evidence that the increased urine and electrolyte

excretion associated with acute gentamicin treatment is at least partially n consequence of decreased transport in the thick ascending limb of Henle's loop (Kidwell *et al.*, 1994).

During and after gentamicin-induced injury and regeneration, apoptosis (programmed cell death) could be seen in the cortical distal tubules and parallel increased proliferative activity in the distal convoluted tubules. This was suggested to be a consequence of tubular obstruction during the first days after induction of acute renal failure (Nouwen *et al.*, 1994).

2.3.5.3 Mechanisms of Gentamicin-induced Nephrotoxicity

Several hypotheses have been advanced to elucidate the possible mechanism(s) of gentamicin nephrotoxicity. In view of the evidence supporting these apparently different suggested mechanisms, it is reasonable to conclude that the nephrotoxicity may be produced through more than one mechanism. Some of the suggested mechanisms include:

(1) Hydroxyradicals. Hydroxyradicals are strong mediators of tissue injury. They can react with metal chelators via the Fenton reaction. and they can oxidize a wide variety of organic compounds including polyunsaturated fatty acids (Pryor. 1986; Ross, 1988; Aruoma, 1994a,b), leading to cell membrane injury and protein degeneration.

Walker and Shah (1988) and Shah and Walker (1992) have shown that gentamicin enhances the production of hydrogen peroxide by rat renal cortical mitochondria, and that the hydroxyl scavengers and iron chelators could prevent gentamicin-induced acute renal failure. Thus, they believed that hydroxyl radical plays an important role in gentamicin nephrotoxicity. Other reports also lend support to the contention of the involvement of hydroxyradicals in gentamicin nephrotoxicity. Administration of metallothioncin, which is a hydroxyl radical scavenger, protects against gentamicin nephrotoxicity (Yang *et al.*, 1991). Also, selenium and Vitamin E administration, both of which can scavenge free radicals, were also beneficial in gentamicin nephrotoxicity (Ademuyiwa *et al.*, 1990). Reduced cortical glutathione (GSH) concentration was reported to be decreased by gentamiein treatment (Ali *et al.*, 1992). As GSH is a hydroxyradical scavenger (Ross, 1988), it was concluded that gentamicin nephrotoxicity involves depletion of cortical GSH. Other workers, however, have reported that gentamicin treatment produces nephrotoxicity without affecting

significantly renal GSH concentration (Wu *et al.*, 1990). It should be mentioned that in the latter work gentamicin had reduced GSH from 2.05 ± 0.39 µmol/g to 1.71 ± 0.06 µmol/g. Depletion of GSH concentration failed to affect gentamicin nephrotoxicity (Wu *et al.*, 1990) negating a role for the oxyradical scavenger GSH in gentamicin nephrotoxicity. Other reports have also failed to confirm a causal relationship between GSH depletion and gentamicin nephrotoxicity (Swann and Acosta, 1988). Inhibition of lipid peroxidation failed to protect against gentamicin nephrotoxicity (Ramsammy *et al.*, 1987). However, others have reported that iron (Fe) supplementation to the diet or intramuscular administration can aggravate gentamicin nephrotoxicity (Kays *et al.*, 1992; Ben-Ismail *et al.*, 1994). Fe is known to play a critical role in initiating free radical oxidation process leading to lipid peroxidation (Braughter *et al.*, 1986). Increased Fe intake may be associated with increased kidney Fe concentration (Johnson and Hove, 1986). This could potentially enhance the free radical oxidation process during gentamicin therapy and possibly hinder recovery. Although the Fe-antagonist deferoxamine was found to ameliorate gentamicin nephrotoxicity (Walker and Shah, 1988), this could not be confirmed in a study by Ben-Ismail *et al.*, (1994). Another piece of evidence supporting the idea of the involvement of oxyradicals in gentamicin nephrotoxicity is the report of Ben-Ismail *et al.*, (1994) who found that at a dose of 100 mg/kg ascorbic acid was found to, at least partially, protect against gentamicin nephrotoxicity. At double that dose, however, it exacerbated the nephrotoxicity probably because the vitamin at high doses may act as a pro-oxidant (Ben-Ismail *et al.*, 1994).

Further, previous evidence for the involvement of oxyradicals is the works of Yang *et al.*, (1991), Ueda *et al.*, (1993), Nakajima *et al.*, (1994), Hishida *et al.*, (1994). And Ali and Bashir (1996), which indicated that the free radical scavenger superoxide dismutase (SOD) activity in the kidney is reduced by gentamicin, and that treatment with the enzyme ameliorates the nephrotoxicity.

(2) Increase in the renal cortical phospholipidosis This has been suggested as one mechanism by which gentamicin may exert its nephrotoxicity (Laurent *et al.*, 1982, Ramsammy *et al.*, 1989, Abdel Gayoum *et al.*, 1993). It has been suggested that gentamicin nephrotoxicity is a phospholipidosis due to the inhibition by gentamicin of

enzymes responsible for phospholipid metabolism, for example, phospholipase and sphingomyclinase (Laurent *et al.*, 1982).

As the gentamicin-induced nephrotoxicity is mainly a consequence of damage to cell membranes, and one of the essential constituents of cell membranes is phospholipids, Chan *et al.* (1991) investigated the influence of phospholipids administration on the nephrotoxicity. It was found that, phospholipids treatment, whether started before or concurrently with gentamicin, reduced the induced nephrotoxicity, without affecting gentamicin renal concentration. The protection was ascribed to an enhanced recycling of membrane phospholipids and accelerated regenerative activity of renal tubular cells. It is also possible that phospholipids may have protected phospholipase and sphingomyelinase from inhibition by gentamicin. The results of Chan *et al.* (1991) on phospholipid protection may be of some clinical value as treatment of humans with phospholipids (for other conditions) was without significant ill effect. Moreover, the fact that phospholipids do not reduce the renal accumulation of gentamicin is advantageous as the agents that reduce the renal concentration of gentamicin usually also decrease the antimicrobial action of the drug. Thirdly, co-treatment and pretreatment with phospholipids gave similar results. This has obvious clinical advantage, as it eliminates the need for pretreatment in patients requiring treatment with the antibiotic, especially in life-threatening infections. In rabbits, however, Hanslik *et al.* (1994) found that administration of an antagonist of platelet activity factor (PAF) (BN 52021) does not affect gentamicin nephrotoxicity in rabbits, suggesting that PAF (which is an ubiquitous phospholipid, probably has no role in gentamicin nephrotoxicity. This conclusion is at variance with that of Rodriquez-Barbero *et al.* (1992) who reported that the above antagonist of PAF reduces some of gentamicin nephrotoxic signs in rats, thus suggesting a role for the phospholipid PAF in the toxicity.

(3) Inhibition of Na^+- K^+ - ATPase. In cortical homogenates from rats treated chronically with gentamicin, Na^+-K^+-ATPase activity is decreased (Williams *et al.*, 1984; Cronin and Newman, 1985; Ali *et al.*, 1995a), and this change is considered instrumental in gentamicin nephrotoxicity. The site of action for inhibition of Na^+-K^+-ATPase has, however, not been localized. Although gentamicin *in vivo* inhibits the activity of the enzyme in the proximal convoluted tubule (PCT) and not in the thick ascending limb of Henle (mTAL), work *in vitro* has shown that gentamicin inhibits the

enzyme both in PCT and mTAL cells when cells were permeabilized to allow the drug to enter the cells. It was concluded that gentamicin inhibits Na^+-K^+ - ATPase in renal tubule cells when it has access to the cytoplasm (Fukuda *et al.*, 1991). It was further suggested that the loss of integrity of the Na^+-K^+-ATPase enzyme contributes to gentamicin nephrotoxicity (Fukuda *et al.*, 1991) as this enzyme regulates intracellular electrolyte and cell volume.

(4) Thromboxane A_2 (TXA_2) and prostaglandins (PGE). These have recently been suggested by Papanikolaou *et al.*, (1992) to be involved in the acute renal failure (ARF) induced by gentamicin (200 mg/kg/day for 3 days) in Wistar rats. The gentamicin ARF was associated with increased urinary TXA_2 excretion. Also, the ratios of urinary PGE/TXB_2 and 6- keto-$PGF_{1\alpha}/TXB_2$ excretions significantly decreased following gentamicin administration.

Administration of OKY-046, the TXA_2 synthetase inhibitor protected rats against gentamicin nephrotoxicity. It was thus concluded that gentamicin may induce ARF by increasing TXA_2 synthesis. This is in agreement with previous studies that have indicated that increased renal TXB_2 synthesis occurs during the development of glycepol-induced ARF. In addition to TXB_2, other vasoconstrictor agents and additional mechanisms could be involved in the development of gentamicin ARF. Earlier, Assael *et al.* (1985) have reported that gentamicin significantly increases prostaglandin (PG) E_2 production after 10 days of treatment. Coadministration of gentamicin and aspirin abolishes this effect. It was hypothesized that the increase in PGE_2 causes vasodilatation in the kidney to maintain normal renal blood flow and GFR during the development of gentamicin nephrotoxicity.

(5) Effect on microsomal protein synthesis. The idea that aminoglycoside might damage proximal tubular cells by mechanisms similar to those by which they act as antibiotics was ignored for many years because the concentrations of drug required for such effects was 10-20-fold those achieved in serum (Bennett, 1989). However, Buss and Piatt, (1985) clearly showed kidney specific changes in microsomal protein synthesis with concentrations of drug readily achieved in the renal cortex of rats. After discontinuation of gentamicin, given in long-term low doses, there is a marked renal cell proliferative response (Houghton *et al.*, 1986). Bennett *et al.* (1988a) also provided evidence that

processes similar to those responsible for bacterial killing could be involved in experimental gentamicin nephrotoxicity before overt cellular necrosis.

(6) Lysosomal injury. Due to the accumulation in the proximal tubule lysosomes, impaired function of these organelles may be an important mechanism leading to proximal tubular activity (Kaloyanides and Pastoriza-Munoz, 1980; Powell and Reindenberg, 1983).

It has been reported that gentamicin causes significant alterations in the lysosomal enzymes (viz proteinases, acid phosphatase, and cathepsin B and L) in the proximal tubule Gentamicin significantly inhibited the cathepsin activities but not the acid phosphatase. The urine excretion of cathepsin B and L was decreased after gentamicin. These alterations were not due to structural changes in the lysosomes or to urinary loss of cathepsin B or L. It was concluded that gentamicin reduces renal protein catabolism by decreasing the activity of key lysosomal enzymes (Olbricht *et al.*, 1991).

(7) Mitochondrial injury. It has been shown that mitochondrial injury plays a role in the pathogenesis of aminoglycoside nephrotoxicity (Klune and Hook, 1978; Simmons *et al.*, 1980). The drug inhibits phosphorylation and impairs cellular energy production.

(8) Vascular factors. In addition to tubular factors, gentamicin-induced renal failure was suggested to be due to vascular factors. Gentamicin activated renin-angiotensin system and increased renal endothelium content (Hishida *et al.*, 1994). Suppression of renin- angiotensin system significantly attenuates gentamicin nephropathy (Yamada *et al.*, 1992). Others have also found that gentamicin treatment increased glomerular renin content and decreased both basal and stimulated glomerular renin release. The former effect remained 1 week after gentamicin discontinuation (FernandezRepollat and Fantauzzi, 1994). *In vitro*, gentamicin calcium-dependently inhibited both basal and stimulated glomerular renin release. On the other hand at the nephrotoxic dose of 80 mg/kg for 6 days Ali and Bashir (1992b) found that gentamicin was without significant effect on the activity of serum angiotensin converting enzyme in the rat.

2.3.5.4 Modification of Gentamicin-induced Nephrotoxicity

Most of the attempts to reduce gentamicin nephrotoxicity without compromising the antibacterial action have been met with little success. Changes in the structure of the aminoglycosides cause loss of antibacterial effect (Ali, 1995). There is obviously a need for effective, safe and practical agents that can reduce gentamicin nephrotoxicity without adversely affecting the antibacterial effect.

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2.4 PLATINUM COORDINATION COMPLEXES AND NEPHROTOXICITY

2.4.1 CISPLATIN

Cisplatin [*cis*-diammine-dichloroplatinum (II), CDDP] (Figure 6) is a commonly used chemotherapeutic agent that was discovered in 1970 as an inhibitor of growth in *Escherichia coli* (Rosenberg *et al.*, 1969). The clinical benefits of cisplatin as an anti-cancer agent have been recognized for over 30 years (Rabik and Dolan, 2007) Cisplatin is considered to be curative treatment for testicular cancer, when combined with bleomycin and etoposide. Unfortunately cisplatin is also one of the most toxic anticancer drugs, its dose-limiting toxicity being nephrotoxicity (Madias and Hartington, 1978; Goldstein and Mayor, 1983; Safirstein *et al.*, 1986). Despite the use of optimal methods for administering cisplatin, such as the use of active hydration (Cvitkovic *et al.*, 1977) or sodium chloride as the vehicle (Ozols *et al.*, 1984), approximately 30% of patients will manifest nephrotoxicity.

Cisplatin is closely related to its second generation analog carboplatin; the two compounds share a mechanism of action and form identical lesions on DNA. Both agents are used for many other types of cancer, including ovarian, cervical, head and neck, non-small cell lung, and lymphoma. However, for many, particularly head and neck, lung, and relapsed lymphomas, cisplatin treatment is plagued by problems (Kartalou and Essigmann, 2001).

Early clinical trials of cisplatin in cancer patients showed a striking incidence of persistent azotaemia and acute renal failure (Rossof *et al.*, 1972; Lippman *et al.*, 1973). In later studies serum creatinine levels increased within 6-7 days of treatment, and then apparently returned to pre-treatment levels by approximately 3 weeks (Hayes *et al.*, 1977). Similar results were seen following the injection of cisplatin into rats (Ward and Fauvie, 1976; Chopra *et al.*, 1982). Thus cisplatin-induced nephrotoxicity initially appeared to be an acute reversible condition. However, more recent findings suggest that cisplatin causes a permanent reduction in GFR (Dentino *et al.*, 1978; Meijer *et al.*, 1983; Fjeldborg *et al.*, 1986), which may indeed be progressive in nature (Groth *et al.*, 1986; Jaffe *et al.*, 1987).

Hypomagnesaemia is frequently noted in patients receiving cisplatin (Buckley *et al.*, 1984; Vogelzang *et al.*, 1985), and is associated with inappropriately high levels of urinary excretion of magnesium. This deficiency in magnesium leads to hypokalaemia and hypocalcaemia. This selective renal loss of magnesium is not

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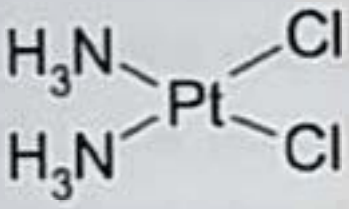
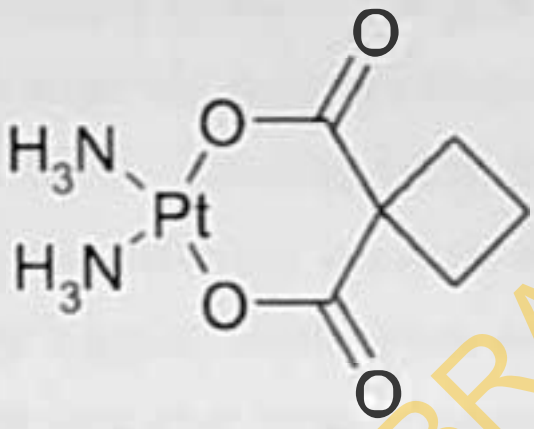
unusual and may be even more common than other renal abnormalities as an expression of cisplatin nephrotoxicity.

Light microscope studies of human kidneys have revealed focal acute tubular necrosis, affecting primarily the distal and collecting tubules, with dilatation of convoluted tubules and cast formation (Gonzalez-Vitale *et al.*, 1977). More recently, Tanaka *et al.* (1986) reported sporadic degenerative lesions, necrosis, and regenerative changes in the S2 and S3 regions of the proximal tubule and also in the distal tubule and collecting duct. The glomeruli and vasculature appeared uninvolved. These observations are somewhat different to those seen in the rat, where cisplatin-induced damage is largely confined to the S3 segment of the proximal tubule, located in the outer stripe of the outer medulla (Chopra *et al.*, 1982). With increasing time cystic tubules develop in this region (Dobyan, 1985). However, these cysts have not been reported clinically.

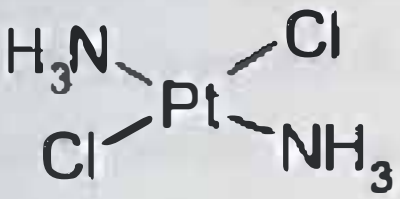
The activity of a number of urinary enzymes, including alanine aminopeptidase, *N*-acetyl-ß-D-glucosaminidase, leucine aminopeptidase and ß-glucurinidase, has been shown to be elevated as early as 36-48h after cisplatin treatment (Kulin *et al.*, 1978; Jones *et al.*, 1980). $ß_2$-micro-globulin excretion has also been shown to be transiently increased after cisplatin treatment (Daugaard *et al.*, 1988a,b). It is of interest to note that this proteinuria (involving proteins of low relative molecular mass), predominantly tubular in origin, was transient, whereas a persistent proteinuria consisting of proteins of high relative molecular mass, such as albumin and IGg, and glomerular in origin was seen after the completion of cisplatin treatment.



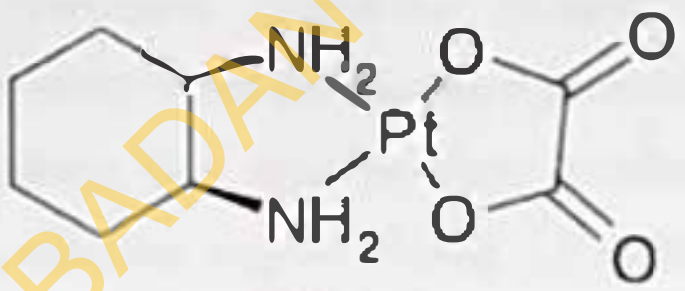
cis-DDP, cisplatin



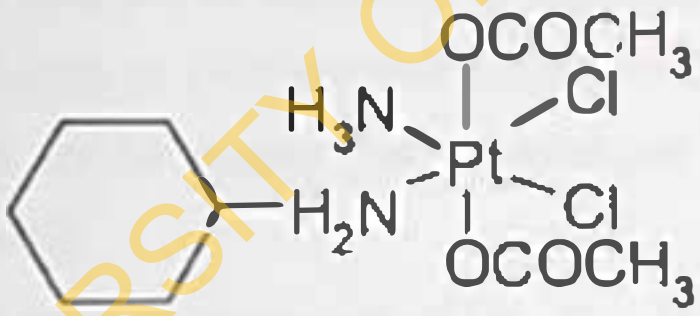
Carboplatin



trans-DDP, transplatin



Oxaliplatin



Satraplatin

Figure 6. The chemical structures of cisplatin, transplatin, carboplatin, oxaliplatin, and satraplatin.

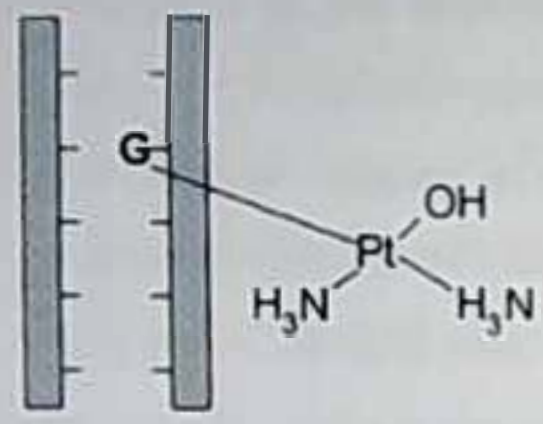
2.4.2 MOLECULAR MECHANISMS OF TOXICITY OF CISPLATIN AND OTHER PLATINATING AGENTS

Mechanism of Actian

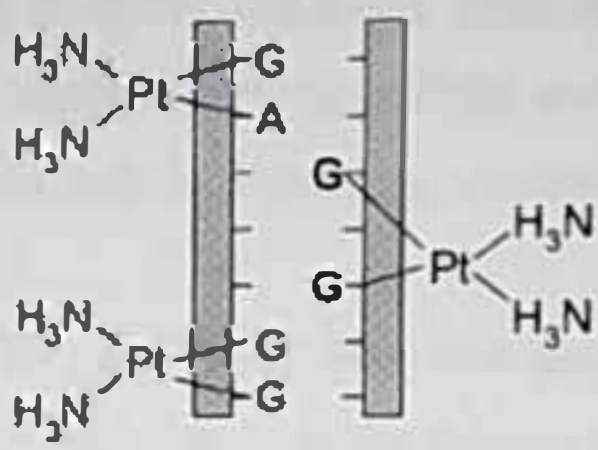
DNA Lesions

Upon entering a cell, all platinating agents become hydrated, losing chloride or oxalate ions, and gaining two water molecules. This positively charged molecule is then able to interact with nucleophilic molecules within the cell, including DNA, RNA, and proteins. It is generally agreed that DNA is the preferential and cytotoxic target for cisplatin and other platinating agents (Zorbas and Keppler, 2005). When binding to DNA, platinating agents favour the N7 atoms of the imidazole rings of guanosine and adenosine. Three different types of lesions can form on purine bases of DNA: monoadducts, intrastrand crosslinks, and ioterstrand crosslinks (Figure 7). Mooonadducts are first formed as one molecule of water is lost from aquated platinating agents; however, greater than 90% of monoadducts then react to form crosslinks. Almost all of these crosslinks are intrastrand, with the majority being 1,2-d(GpG) crosslinks. Additional DNA lesions include interstrand crosslinks. Oxaliplatin forms fewer crosslinks than cisplatin at equimolar concentrations; however, it is equally as potent at these concentrations (Woynarowski *et al.*, 1998; Woynarowski *et al.*, 2000) and is able to induce similar numbers of single-strand and double-strand breaks on DNA (Faivre *et al.*, 2003).

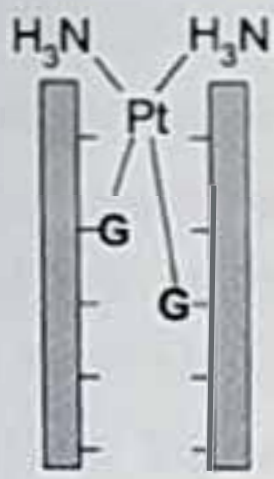
All crosslinks result in contortion of the DNA (Fuertes *et al.*, 2003). Cisplatin and carboplatin intrastrand crosslinks bend the double helix by 32-35° toward the major groove, whereas oxaliplatin treatment bends the helix even further (Di Francesco *et al.*, 2002). Both 1,2-d(GpG) and 1,2-d(ApG) intrastrand crosslinks unwind DNA by 13°, while the 1,3-d(GpXpG) intrastrand lesion unwinds DNA by 34°. Interstrand lesions induce even more steric changes in DNA, with extrusion of the cytosines at the crosslinked d(GpC)d(GpC) sites, bending of the double helix toward the minor groove by 20-40°, and extensive DNA unwinding of up to 80°. Oxaliplatin adducts are bulkier and more hydrophobic than those formed from cisplatin or carboplatin, leading to different effects in the cell (Misset *et al.*, 2000).



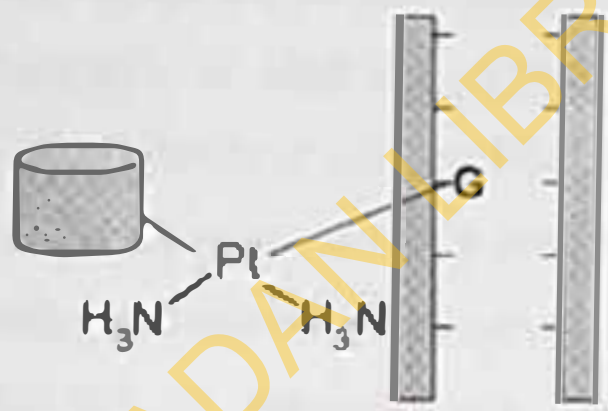
DNA Monoadduct



Intrastrand Crosslinks



Interstrand Crosslink



DNA-Protein Crosslink

Figure 7. Platinating agent adducts on DNA. Platinating agents are able to interact with DNA to form monoadducts, intrastrand crosslinks (1,2-d(GpG), 1,2-d(ApG), 1,3-d(GpXpGp)), interstrand crosslinks (G-G), and DNA-protein crosslinks. From Rabik and Dolan, 2007.

HMG Involvement

There are different theories as to which lesion is responsible for cytotoxicity. Some believe that the interstrand crosslink is cytotoxic because of the level of distortion in the DNA; however, most believe that the predominant 1,2-intrastrand crosslinks are the cytotoxic lesion because of comparisons with the biologically inactive *trans* isomer of cisplatin, *trans*-diaminedichloroplatinum (II) (*trans*-DDP) (Figure 1). *trans*-DDP is unable to form 1,2-intrastrand crosslinks, but is able to form 1,3-intra- and inter-strand linkages (Eastman and Barry, 1987). Additionally, high mobility group (HMG) proteins are able to recognize and bind to DNA at the 1,2-d(GpG) intrastrand crosslinks. HMG domains are basic domains of 80 amino acids which contain three α-helical domains. They are intimately associated with the curvature of chromatin. Their presence is thought to be crucial for sensitivity to cisplatin and carboplatin, partly because the testis, which is exquisitely sensitive to cisplatin, expresses several HMG domain

proteins. Members of the HMGB family, including HMGB1 (HMG-1), have been shown to bind to 1,2- d(GpG) crosslinks induced by cisplatin, but not to DNA treated with *trans*-DDP. The binding of HMGB1 to cisplatin aids in preventing replicative bypass (translesion synthesis) (Vaisman *et al.*, 1999).

Additionally, HMGB proteins such as SRY, UBF, and LEF-1 have been shown to block nucleotide excision repair (NER) components from repairing the lesion via a "shielding mechanism" (Reeves and Adair, 2005). The cisplatin-DNA-HMGB1 ternary complex is also able to block transcription factors, thus preventing both transcription and replication. This block in cellular processes may be responsible for sending out DNA damage signals that result in initiation of apoptosis (Siddik, 2003). In support of this theory, He *et al.*, found that overexpression of HMGB1 caused by estrogen exposure sensitized breast tumor cells to cisplatin (He *et al.*, 2000). HMGB1 generally functions to facilitate binding steroid hormone receptors to their promoter sites on DNA. In the MCF-7 breast cancer line, priming the cells with estrogen or progesterone resulted in increased transcription of HMGB1 by approximately two-fold. This increased HMGB1 expression is associated with a concomitant increase in sensitivity to cisplatin in this cell line. HMG has a much lower affinity for oxaliplatin crosslinks on DNA than it does for cisplatin or carboplatin adducts (Vaisman *et al.*, 1999). The molecular geometry of the oxaliplatin adduct, with a narrower major groove and correspondingly wider minor groove, is thought to be responsible for this observation.

Endoplasmic Reticulum Stress

Although cisplatin is known to induce apoptosis following DNA damage, it has also been shown to cause activation of apoptotic caspases through activation of the endoplasmic reticulum (ER) stress pathway (Figure 8) (Mandic *et al.*, 2003). The ER stress pathway is based on the cellular unfolded protein response (UPR). When the ER experiences stress such as starvation or treatment with inhibitors of N-glycosylation (e.g. tunicamycin), it cannot fold or transport proteins correctly, and the UPR is activated. The first step of the UPR is phosphorylation of eIF2α at Ser51, halting new protein synthesis, then regulatory components of the ER stress pathway, including ATF4, ATF6, XBP1, and BiP (Grp78), are upregulated (Breckenridge *et al.*, 2003). In some cases, these regulatory proteins are able to restore normal ER function. In other circumstances, the UPR initiates apoptosis. This ER stress-induced apoptosis is dependent upon the activation of caspase-12 (Breckenridge *et al.*, 2003). Caspase 12 is

located at the cytosolic face of the ER and is cleaved by the calpain protease (Nakagawa and Yuan, 2000). Inhibition of calpain by calpeptin prevents cisplatin-induced caspase-12 cleavage (Mandic *et al.*, 2003).

Treatment of enucleated melanoma 224 and colon carcinoma HCT 116 cell lines with cisplatin resulted in activation of caspase 12, followed by caspase 3 activation. Grp78 (BiP) was also upregulated in 224 cells (Mandic *et al.*, 2003). Additionally, it has been observed in a pancreatic cancer cell line that cisplatin is capable of activating ER stress pathways, including upregulation of chaperone proteins and caspase 12 cleavage (Nawrocki *et al.*, 2005). The stimulation of pro-apoptotic pathways in enucleated cells by cisplatin-induced ER stress was a novel finding and one that other groups are beginning to evaluate further as a secondary mechanism of cisplatin cytotoxicity. ER stress activation has not been shown as yet for either carboplatin or oxaliplatin.

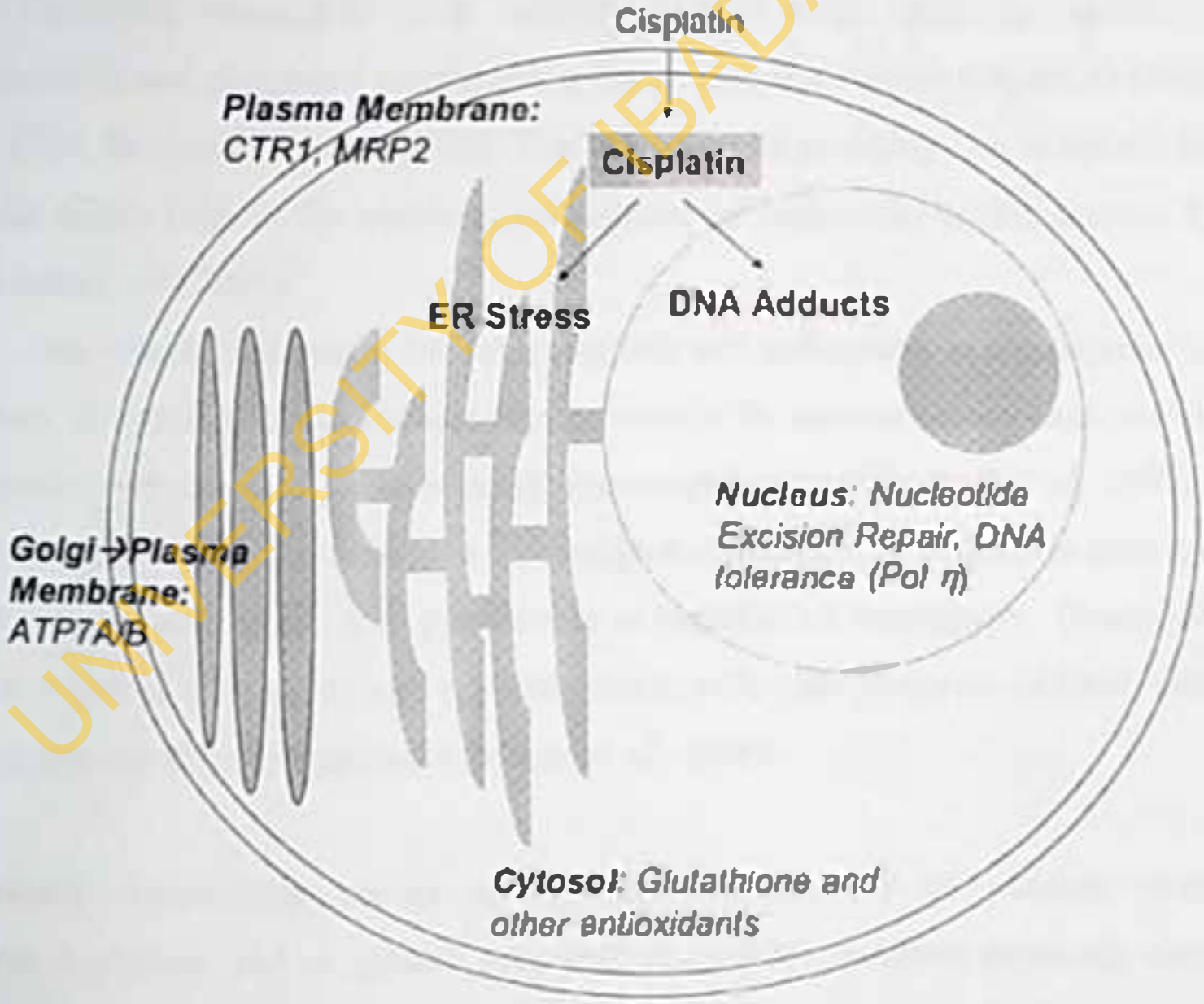


Figure 8. Mechanism of cisplatin activity and mechanisms of resistance to platinating agents, as exemplified here by cisplatin. Cisplatin can act in the cell either by causing DNA damage, or by activating the ER stress pathway, both of which can lead to cellular apoptosis. In addition, many mechanisms of resistance (italics) are present, including transport, cellular antioxidants, increased DNA damage repair, and DNA tolerance. From Rabik and Dolan, 2007.

Clinical Utility of Cisplatin and Commonly Used Platinating Agents

Cisplatin, carboplatin, and oxaliplatin are all commonly used intravenous platinating agents. Cisplatin is still used regularly for head and neck and germ cell tumors, while carboplatin has supplanted the use of cisplatin for most ovarian tumors and for the treatment of non-small cell lung carcinoma (Hartmann and Lipp, 2003). Oxaliplatin is currently approved for treatment in colorectal cancer, but has also been shown to have activity against breast and endometrial cancers and malignant melanoma in Phase I studies (Misset *et al.*, 2000). Additional Phase II trials show oxaliplatin to be active against non-small cell lung cancer, prostate cancer, germ-cell malignancies, ovarian carcinoma, non-Hodgkin's lymphoma, and malignant mesothelioma; minimal or no activity was observed in head and neck carcinoma and in malignant astrocytoma (Misset *et al.*, 2000).

Toxicities

Toxicities associated with cisplatin range from mild to severe, with nephrotoxicity and peripheral neurotoxicity being the most serious (Figure 9) (Donzelli *et al.*, 2004; Stachurska *et al.*, 2004). Nephrotoxicity is primarily due to uptake by the proximal tubule cells of the nephron, with uptake by other cells having a lesser effect (Stachurska *et al.*, 2004).

One notable distinction between cisplatin and carboplatin is a difference in the spectrum of toxicities. Carboplatin rarely results in nephrotoxicity and peripheral neuropathy, with its major toxicity being myelosuppression (Wagstaff *et al.*, 1989). The most common toxicity associated with oxaliplatin treatment is peripheral neuropathy, which ranges from acute and transient to a cumulative neuropathy. Oxaliplatin is generally free of ototoxicity and nephrotoxicity, with only moderate isolated cases of neutropenia and thrombocytopenia (Misset *et al.*, 2000).

Ototoxicity.—Ototoxicity occurs in approximately 23-54% of patients receiving cisplatin treatment, and in greater than half of pediatric patients receiving cisplatin (Figure 9) (Rybak and Whitworth, 2005). Bolus higher doses of cisplatin have been shown to be more ototoxic and nephrotoxic than repeated infusions at lower doses in adults (Reddel *et al.*, 1982). In children, however, prolonged infusions are less nephrotoxic than bolus doses but still result in considerable ototoxicity (Lanvers-Kaminsky *et al.*, 2006).

Platinum-based chemotherapeutic agents damage the outer hair cells of the cochlea (inner ear), resulting in functional deficits (Rybak and Whitworth, 2005). The mechanisms underlying these troublesome side effects most likely involve the production of reactive oxygen species (ROS) in the cochlea, which can trigger cell-death pathways. This is thought to be due to activation of the NADPH oxidase isoform NOX3, which is expressed only in the inner ear, as kidney cells transfected with the *nox3* gene exhibit enhanced superoxide formation upon treatment with cisplatin (Banfi *et al*., 2004). The superoxide radical can then be transformed by cellular enzymes into hydrogen peroxide and the hydroxyl radical, which has been hypothesized to play a major role in cisplatin-induced ototoxicity (Lee *et al*., 2004). In the outer hair cells of the cochlea, cell death pathways are triggered by the release of cytochrome *c* and activation of caspases 9 and 3 (Wang *et al*., 2004).

One strategy to protect the inner ear from ototoxicity is pretreatment with thiol-containing drugs that act as antioxidants, including sodium thiosulfate (STS), methionine, glutathione ester, and amifostine (Rybak and Whitworth, 2005). α-Tocopherol (Vitamin E) was shown to reduce cisplatin-induced ototoxicity in male rats as measured both by hearing threshold differences and cochlear morphology (Kalkanis *et al*., 2004). In Hartley albino guinea pigs, treatment with α-tocopherol alone was less effective, but when given in conjunction with the thiol-containing compound tiopronin, cisplatin-induced hearing loss was significantly slowed (Fetoni *et al*., 2004).

High serum concentrations of carboplatin have also been linked to oxotoxicity, although this phenomenon is relatively rare, occurring in ~1% of patients. Oxaliplatin has not been linked to ototoxicity (Hartmann and Lipp, 2003).

Nephrotoxicity—Nephrotoxicity is associated with cisplatin treatment, but is rare with therapies involving its later generation analogs carboplatin or oxaliplatin (Figure 9) (Cassidy and Misset, 2002; Wolfgang *et al*., 1994). Due to the renal excretion of cisplatin, the kidney accumulates a higher effective concentration of cisplatin than any other organ (Ikari *et al*., 2005). This accumulation preferentially affects the terminal proximal tubule and the distal nephron and can cause either apoptosis or necrosis, depending on exposure time and concentration (Ikari *et al*., 2005). Low, prolonged doses of cisplatin typically induce apoptosis, whereas necrosis is caused by short exposures to higher concentrations of cisplatin (Lieberthal *et al*., 1996). Similar to cisplatin-induced ototoxicity, nephrotoxicity related to cisplatin treatment is due to the

production of ROS. Notably, ROS are only thought to mediate the apoptosis pathway, and are not involved in the necrotic death pathway (Licberthal *et al.*, 1996). ROS damage is thought to be mitigated by bypoxia-inducible factor 1α (HIF1α). Immortalized rat renal tubular cells expressing dominant negative HIF1α were more susceptible to apoptosis following cisplatin treatment in hypoxic conditions than cells expressing wild-type HIF1α (Tanaka *et al.*, 2005). Future studies hope to use activation of HIF-1 as a target for further protecting patients from nephrotoxicity, possibly with siRNA or gene therapy.

The human organic cation transporter (hOCT) has been proposed to be involved in potentiating cisplatin-induced nephrotoxicity in the proximal tubule. This transporter is expressed primarily in the kidney (Gorboulev *et al.*, 1997). After treatment with a concentration of cisplatin known to induce apoptosis, the hOCT2 substrate cimetidine was able to suppress cisplatin-induced apoptosis (Ciarimboli *et al.*, 2005). Cotreatment of cisplatin with a hOCT2 inhibitor could lead to reduction in nephrotoxicity (Ciarimboli *et al.*, 2005). More evidence in favor of the OCT2 transporter as a target for cisplatin-induced nephrotoxicity was uncovered using HEK293 cells transfected with the rat OCT2 transporter. In these cells, cisplatin-induced cytotoxicity was increased by the presence of the rOCT2 transporter, as a direct result of increased platinum uptake. This indicates that rOCT2 expression was a definitive marker of cisplatin-induced nephrotoxicity (Yonezawa *et al.*, 2005).

Many antioxidant treatments, including tiopronin, *N*-acetylcysteine pre-treatment and sodium thiosulfate post-treatment have been evaluated against cisplatin nephrotoxicity. STS post-treatment was time-sensitive, with a 2h delay being protective against nephrotoxicity, and up to 4 h giving otoprotection (Dickey *et al.*, 2005; Viale *et al.*, 1999).

Irregular kidney function can result in toxicities in rare cases of carboplatin treatment, usually in patients with renal dysfunction; in contrast, patients with a high glomerular filtration rate can have subtherapeutic systemic concentrations of carboplatin (Hartmann and Lipp, 2003).

Neurotoxicity—The dorsal root ganglia of the spinal cord are the primary location of cisplatin damage in the central nervous system (Figure 9) (Meijer *et al.*, 1999). This explains the primary sensory neuropathy commonly observed in patients treated with cisplatin (Meijer *et al.*, 1999). Cisplatin-induced neuropathy is characterized by

decreased sensory nerve conduction velocity, possibly by acting as a calcium channel blocker (Hartmann and Lipp, 2003). Co-treatment of rats with acetyl-L-carnitine was able to protect animals from neurotoxicity while having no effect on the anti-neoplastic activity of cisplatin (Pisano *et al.*, 2003).

Vitamin E has been shown to be decreased in patients treated with cisplatin (Bove *et al.*, 2001), and vitamin E deficiency causes a sensory neuropathy very similar to that observed with cisplatin treatment (Halliwell and Gutteridge, 1993). Therefore, vitamin E was tested as a means to protect against cisplatin-induced neuropathy in a controlled clinical trial. One group of patients received vitamin E concomitantly with cisplatin, and for three months following the last cisplatin treatment; the other group received cisplatin as prescribed by dosing recommendations (Argyriou *et al.*, 2006). Neurotoxicity, as measured by a peripheral neuropathy score, was significantly decreased in patients treated with cisplatin plus vitamin E as compared with those treated with cisplatin alone.

Erythropoietin has also been associated with neuroprotection *in vivo*. In preclinical experiments in rats, erythropoietin was protective against cisplatin-induced neuropathy (Orhan *et al.*, 2004; Bianchi *et al.*, 2006). A carbamylated derivative of erythropoietin was also tested, to avoid the erythropoietic effects of the parent drug, and it was also shown to be effective as a neuroprotectant for cisplatin neurotoxicity in rats (Bianchi *et al.*, 2006). Carbamylated erythropoietin is currently undergoing further experimentation for long-term side effects, with future clinical trials planned.

Carboplatin is notably less neurotoxic than cisplatin at conventional doses, with a similar sensory neuropathy occurring in approximately 6% of patients (Canetta *et al.*, 1985). In rare cases, high doses of carboplatin have been shown to result in a sensory ataxia soon after treatment. These patients had all received cisplatin prior to carboplatin, and experienced a mild neuropathy from the first platinating agent (Heinzlef *et al.*, 1998). Among gynecologic carcinoma patients treated with a combination of carboplatin and paclitaxel, 25% of patients developed peripheral neurotoxicity (Markman *et al.*, 2001).

Oxaliplatin neuropathy has a wide spectrum, ranging from an acute sensory neuropathy immediately following treatment to a chronic, dose-limiting neuropathy that usually takes several weeks of treatment to appear. Acute neurotoxicity causes numbness and pain in the distal extremities, and worsens upon exposure to cold temperatures; this is thought to be due to inhibition of voltage-gated sodium currents by

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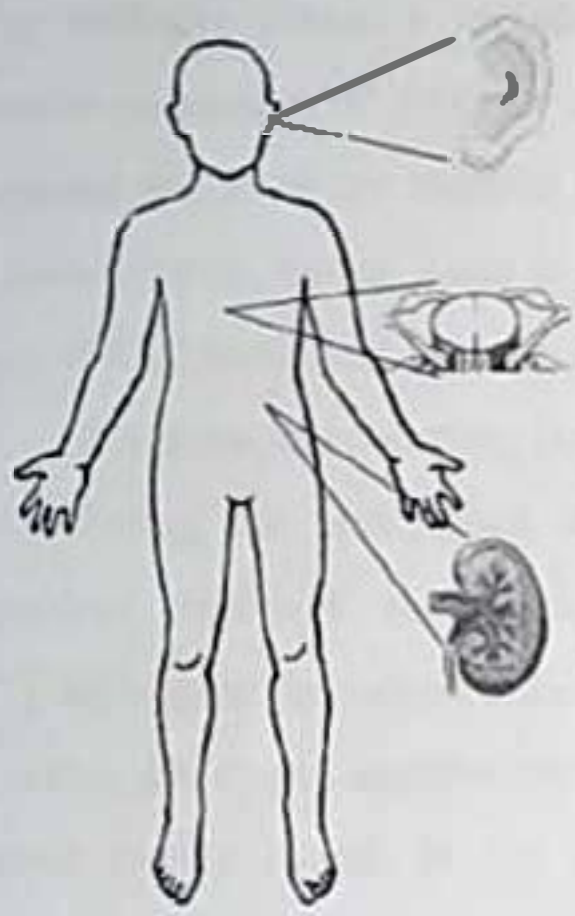
oxaliplatin, and may also be due to the presence of free oxalate ions acting as calcium chelators (Hartmann and Lipp, 2003). This neurotoxicity occurs in greater than 95% of patients and can be managed by treatment of calcium gluconate or magnesium sulfate preceding and following treatment (Gamelin *et al.*, 2002; Grolleau *et al.*, 2001; Gamelin *et al.*, 2002).

The cumulative neuropathy caused by oxaliplatin occurs in approximately 15% of patients, and its reversible symptoms include non-cold-related numbness and pain, sensory loss, and sensory ataxia. Amifostine and glutathione have been used to reduce the severity of this neuropathy, as have the anti-epileptic agents gabapentin and carbamazepine (Hartmann and Lipp, 2003; Grothey, 2005). Chronomodulating the delivery of oxaliplatin also helps to prevent this toxicity (Hartmann and Lipp, 2003).

Myelosuppression—The dose-limiting side effect of carboplatin is myelosuppression, specifically neutropenia and thrombocytopenia (Figure 9). While conventional carboplatin doses result in thrombocytopenia in 20-40% of patients and severe neutropenia in less than 20%, high doses can result in life-threatening toxicity, made more manageable by addition of granulocyte colony stimulating factor (GM CSF). In the majority of cases, neither cisplatin nor oxaliplatin is associated with severe myelosuppression.



Toxicity	Cisplatin	Carboplatin	Oxaliplatin
Ototoxicity	Adult: 23-50% Children: >50%	~ 1%	Rare
Peripheral Neuropathy	Adult: 30-86% (avg 62%) Children: ~10% lower	Conventional dose: 6% High dose: 25%	Acute: 95% Cumulative: 15%
Nephrotoxicity	~20-31%	Patients with irregular kidney function	Rare
Myelosuppression	<5%	Conventional dose: 20-40% High dose: >90%	Rare

Figure 9. Toxicities associated with treatment with platinating agents. A. The most common side effects associated with cisplatin treatment are ototoxicity, peripheral neuropathy, myelosuppression, and nephrotoxicity. Ototoxicity is notably higher in pediatric patients, while neuropathy is relatively more common in adult patients. B. The most common toxicity associated with carboplatin is myelosuppression, with rare cases of neurotoxicity and nephrotoxicity. Oxaliplatin most commonly causes neurotoxicity. From Rabik and Dolan, 2007.

2.5 OXIDATIVE STRESS AND PROTECTIVE EFFECTS OF POLYPHENOLS: COMPARATIVE STUDIES IN HUMAN AND RODENT KIDNEY

Considerable experimental evidence has contributed to support a key role of reactive oxygen species (ROS) in the numerous mechanisms of seemingly unrelated nephropathies (Rodrigo and Rivera, 2002). While enzymatic and nonenzymatic systems preserve the antioxidant/oxidant status, these defense systems become overwhelmed during oxidative stress, a metabolic derangement due to an imbalance caused by excessive generation of ROS or a diminished antioxidant capacity. It has long been recognized that ROS are harmful for cells, mainly because they injure lipids, proteins, and nucleic acids, which leads to structural and functional impairments (Freeman and Crapo, 1982; Mantle and Preedy, 1999).

Numerous interventions have been put forward to counteract the effects of ROS, by reinforcing the antioxidant defense systems. Dietary supplementation with the antioxidant vitamin E slowed the rate of progression of renal deterioration (Fryer, 1997), attenuated the nephrotoxicity caused by ferric nitrilotriacetate (Fe-NTA) (Iqbal and Athar, 1998) and ameliorated the glomerulosclerosis occurring in the nephrectomy remnant kidney model in the rat (Hahn *et al.*, 1999). Also, gentamicin-induced nephrotoxicity was ameliorated with garlic, known to be rich in polyphenols (Pedraza-Chaverri *et al.*, 2000). Recently, the possible advantage of a moderate wine consumption in patients with chronic renal failure was hypothesized (Caimi *et al.*, 2004). Therefore, it is expected that the naturally occurring nutritional sources of antioxidants, such as fruits, vegetables, tea or wine, would also attenuate the renal damage caused by oxidative challenges. Polyphenolic compounds, abundant in these nutritional sources, could play a major role in enhancing the antioxidant system, since they behave as ROS scavengers, metal chelators and enzyme modulators (Pietta *et al.*, 1998). In agreement with this view, it was demonstrated that resveratrol, a stilbene polyphenol found in grapes and red wine, suppresses the proteinuria, hypoalbuminemia and hyperlipidemia induced by anti-rat kidney antiserum (Nihei *et al.*, 2001). Also, renoprotective effects have been reported for other polyphenols such as quercetin (Ishikawa and Kitamura, 2000) and alpha-Grutin (Shimoi *et al.*, 1997). Although these studies have been performed in rodents, it was suggested that this protection may be useful to prevent or treat myoglobinuric acute renal failure in humans (Stefanovic *et al.*,

2000), two species with great similarity on the mechanism of renal injury in this setting. Although dietary supplements containing polyphenols have been used in humans, a safety assessment of the applied dose has been recommended due to the possibility of some adverse effect of this mode of consumption (Mennen *et al.*, 2005).

2.5.1 Renal alterations associated with oxidative stress

Oxidative stress mediates a wide range of renal impairments, ranging from acute renal failure (Paller *et al.*, 1998; Baliga *et al.*, 1999; Shah, 2001), rhabdomyolysis (Vanholder *et al.*, 2000), obstructive nephropathy (Klahr, 2001), hyperlipidemia (Wanner *et al.*, 1997; Sakatsume *et al.*, 2001) and glomerular damage (Kitamura and Ishikawa, 1999) to chronic renal failure and hemodialysis and associated inflammation (Handelman *et al.*, 2001). Thus, increased levels of malondialdehyde and F2-isoprostanes, two products of lipid peroxidation, have been reported in various clinical settings associated with renal damage (Martin-Mateo *et al.*, 1999), although most of these studies have been performed in rats or mice.

2.5.1.1 Glomerular alterations

Oxidative stress may alter the structure and function of the glomerulus because of the effect of ROS on mesangial and endothelial cells (Klahr, 1997). The glomerulus is considerably more sensitive to oxidative injuries than other nephron segments. Lipoprotein glomerulopathy has been characterized by a relatively rapid progression to renal impairment and the development of glomerulosclerosis (Sakatsume *et al.*, 2001). Both native and oxidized forms of LDL (LDL-ox) may be involved in the glomerular damage mediated by oxidative stress. Oxidative stress participates in the renal damage induced by hyperlipoproteinemia (Scheuer *et al.*, 2000), mainly associated with the glomerular accumulation of LDL (Lee and Kim, 1998). Subsequently, oxidation of LDL by mesangial cells could occur (Wheeler *et al.*, 1994), thereby activating the apoptosis pathway of endothelial and mesangial cells, as shown by studies of these cells of humans *in vitro*, an effect prevented by antioxidants (Galle *et al.*, 1999). Also, native LDL has shown a dose-dependent stimulation of proliferation of cultured mesangial cells (Nishida *et al.*, 1999), a response attributed to an enhancement of expression of c-jun and c-fos genes, involved in the cellular proliferation and DNA synthesis in mesangial cells during LDL exposure (Gröne *et al.*, 1996). Antioxidant enzymes, such as catalase (CAT) and superoxide dismutase (SOD), but not glutathione peroxidase

(GSH-Px), may partially inhibit the effect of LDL on DNA synthesis (Greiber *et al.*, 1996). Native LDL was found to induce the generation of ROS in rat glomerular cells (Wanner *et al.*, 1997), although other studies found no effect of LDL in the production of superoxide anion by mesangial and endothelial cells *in vitro* (Galle *et al.*, 1999). This controversy may be due to the different conditions of the experimental models that were used. Although the presence of an excessive amount of LDL is recognized as a factor of glomerular damage, its role in the production of oxidative stress has yet to be fully elucidated. This damage could be direct or indirect, because oxidation of LDL is induced by infiltrating leukocytes resulting in increased glomerular damage. In addition, native LDL can stimulate fibronectin secretion by mesangial cells. LDL-ox may stimulate the genic expression of fibronectin through the autocrine secretion of transforming growth factor-β (TGF-β) in cultured human glomerular epithelial cells (Ding *et al.*, 1997). These data support a role of oxidative stress and dyslipoproteinemia in the pathogenesis of glomerulosclerosis associated with renal diseases. Studies in rats demonstrated that long-term wine exposure reduced LDL-cholesterol through its non-alcoholic components, thereby protecting the kidney against the deleterious effects of LDL and their oxidized derivatives on the glomerulus (Cascon *et al.*, 2001); this effect could be reinforced by a preservation of polyunsaturated fatty acids of kidney phospholipids also attributed to polyphenols (Araya *et al.*, 2001).

Oxidative stress could also be involved in other inflammatory lesions caused by a series of mediators, including cytokines and chemokines leading to leukocyte activation, production of ROS and increased glomerular damage (Takemura *et al.*, 1994). Also, the molecules causing inflammation could be produced by the resident renal cells, such as glomerular mesangial and endothelial cells, proximal tubular epithelial cells, and interstitial fibroblasts (Rovin and Phan, 1998). The nuclear factorkappaB (NF-κB) is one of the most important regulators of proinflammatory gene expression (Tak and Firestein, 2001), and it has been demonstrated that ROS can stimulate its activation in mesangial cells (Massy *et al.*, 1999). The antioxidants may play a key role against the glomerular inflammatory processes, through a diminution of the activity of inflammatory enzymes (Ozaki *et al.*, 1999) and cytokine secretion, or by inhibiting the activity of NF-κB (Massy *et al.*, 1999), as shown for the wine polyphenol quercetin (Ishikawa and Kitamura, 2000). Also, resveratrol, a stilbene polyphenol found in grapes and wine, is a potent antiglomerulonephritic factor capable of suppressing proteinuria, hypoalbuminemia, and hyperlipidemia induced by anti-rat

kidney antiserum (Nihei *et al.*, 2001). In addition, it has been documented that superoxide anion participates in tumor necrosis factor-α (TNF-α)-induced mesangial cell apoptosis (Moreno-Manzano *et al.*, 2000). Polyphenols may counteract this mechanism through a cytoprotective action of the glomerular mesangial cells, exerted by a restriction on apoptosis (Kitamura and Ishikawa, 1999). The mechanisms whereby antioxidants exert such effects are still unknown, but their renoprotective effects could be expected in renal pathologies, such as glomerulosclerosis. It should be noted that, in the case of wine, ethanol could also be involved in glomerular protection. In addition to the evidence that acute ethanol consumption reduces glomerular damage (Cecchin and De Marchi, 1996), its renoprotection against oxidative injury may also be postulated on the basis of data found in experimental models of chronic ethanol consumption (Scott *et al.*, 2000; Orellana *et al.*, 1998), but the physiological relevance of these findings has not been yet established.

2.5.1.2 Tubulointerstitial alterations

The renal tubular epithelia could be exposed to injurious chemical species when molecules appear in the urinary space because of the loss of glomerular permeability and selectivity occurring in chronic renal diseases, or because of their increased plasma levels. Among these macromolecules are LDL-ox (Chen *et al.*, 2000), transition metals (Shah, 2001; Barrouillet *et al.*, 1999), hemoglobin and myoglobin (Zager and Burkhart, 1997) or potentially nephrotoxic drugs (Baliga *et al.*, 1999). LDL-ox may induce a pro-oxidant environment (Agarwal *et al.*, 1996). In turn, this oxidative stimulus may activate heme-oxygenase, an enzyme that catalyzes the degradation of the heme groups of hemoglobin and myoglobin (Zager and Burkhart, 1997), two hemopigments found in the urinary space in numerous glomerulopathies in which the glomerular barrier is impaired. Subsequently, iron liberation results in tubular production of hydroxyl radicals and lipid peroxidation. This cytotoxic effect is attenuated by the administration of iron chelators, or by hydroxyl radical scavengers (Shah and Walker, 1988). During myoglobinuria, tubular cells show an increased production of hydrogen peroxide (Zager and Burkhart, 1997) and a dramatic drop of reduced glutathione (GSH) (Abul-Ezz *et al.*, 1991). The accumulation of macrophages within the interstitial space of the renal cortex plays a pathogenic role in the development of tubular injury and interstitial fibrosis in progressive chronic renal diseases (Vielhauer *et al.*, 2001). Proximal tubular epithelial cells are thought to mediate the interstitial macrophage infiltration because of

their anatomic position and their ability to produce chemotactic cytokines, chemokines, and other inflammatory mediators. It was reported that ROS may induce gene expression of these mediators in the tubular epithelial cells, resulting in the recruitment of leukocytes. Thus, in renal tubular cells, the expression of chemokines, such as monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage inflammatory protein1 (MIP-1), and T cell activation gene 3 (TCA3), precedes the production of infiltrates containing monocytes, macrophages and T lymphocytes, in experimental acute tubulointerstitial nephritis (Ou *et al.*, 1999). In contrast, the effect of NF-κB, an important modulator of inflammatory responses occurring in tubular epithelial cells, can be inhibited by various antioxidants. In this context, the quercetin-mediated inhibition of NF-κB was associated with the reduction of both pro-[interleukin-1β (IL-1β), TNF-α, MCP-1, and MCP-2] and anti-inflammatory (TGF-β) and IL-10 cytokine transcription in proximal tubular cells (Kuhlmann *et al.*, 1998). *In vitro* quercetin prevented cisplatin-induced cellular injury and upregulation of chemokines in the renal cortex (Jones and Shoskes, 2000). Also, pretreatment with quercetin or curcumin resulted in preservation of histological integrity, with a decrease in tubular damage and interstitial inflammation. These wine polyphenols were associated with a strong attenuation of the expression of MCP-1 and regulated upon activation normal T-cell expressed and secreted (RANTES), two chemokines activated by ischemia–reperfusion (Shoskes, 1998). However, further studies are needed to determine whether the administration of quercetin could attenuate the chronic upregulation of NF-κB in tubulointerstitial diseases *in vivo* (Rangan *et al.*, 1999a,b). Ureteral obstruction is another renal alteration where generation of renal damage is closely related with the occurrence of oxidative stress. ROS may play an important role in the tubulointerstitial inflammation associated with obstructive nephropathy (Klahr, 2001). The mechanical disturbance due to a complete ureteral obstruction causes tubular injury resulting in a pro-inflammatory and tubulointertitial fibrosis (Ricardo and Diamond, 1998). The antioxidant enzymes CAT and SOD from tubular cells from the obstructed kidney show a downregulation, which causes increased susceptibility of the kidney to oxidative damage (Cvetkovic *et al.*, 1998), a process exacerbated by sodium depletion (Kinter *et al.*, 1999). Alternatively, angiotensin II (Ang II) also plays a pivotal role in the progression of renal diseases, including obstructive nephropathy (Klahr, 1998). Ang II mediates the activation of membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and, subsequently, the generation of superoxide anion

that, in turn, leads to hypertrophy of renal tubular cells (Hannken *et al.*, 1998). In addition, upregulation of the expression of TGF-β and TNF-α between other cytokines by Ang II was also reported in obstructive nephropathy. TGF-β is a major cytokine involved in the process of expansion and fibrosis of the tubulointertitial space, but its expression is inhibited by polyphenols (Shi *et al.*, 2004). TNF-α, secreted by renal tubular cells, has a role in the recruitment of inflammatory cells to the renal interstitium (Klahr, 2001). Calcium oxalate urolithiasis constitutes a frequent example of obstructive nephropathy. Together with the pathogenic role of oxalate in the formation of stones, it was reported early that it has the ability to generate free radicals, causing lipid peroxidation. Studies in animal models are in agreement with these data, as shown by the association of hyperoxaluria-induced lipid peroxidation (Thamilselvan *et al.*, 1997) accompanied by a diminution of GSH levels (Muthukumar and Selvam, 1998). The possibility that the effects of antioxidants ameliorate tubulointertitial damage has been studied in rats and mice using two experimental models. The nephrotoxicity caused by ferric nitrilotriacetate (Fe-NTA) was attenuated by antioxidants such as α-tocopherol (Iqbal and Athar, 1998), 2-mercaptoethanesulphonate, and N-acetylcysteine (Umemura *et al.*, 1996). Also, gentamicin-induced nephrotoxicity was ameliorated with garlic, rich in polyphenols (Pedraza-Chaverri *et al.*, 2000).

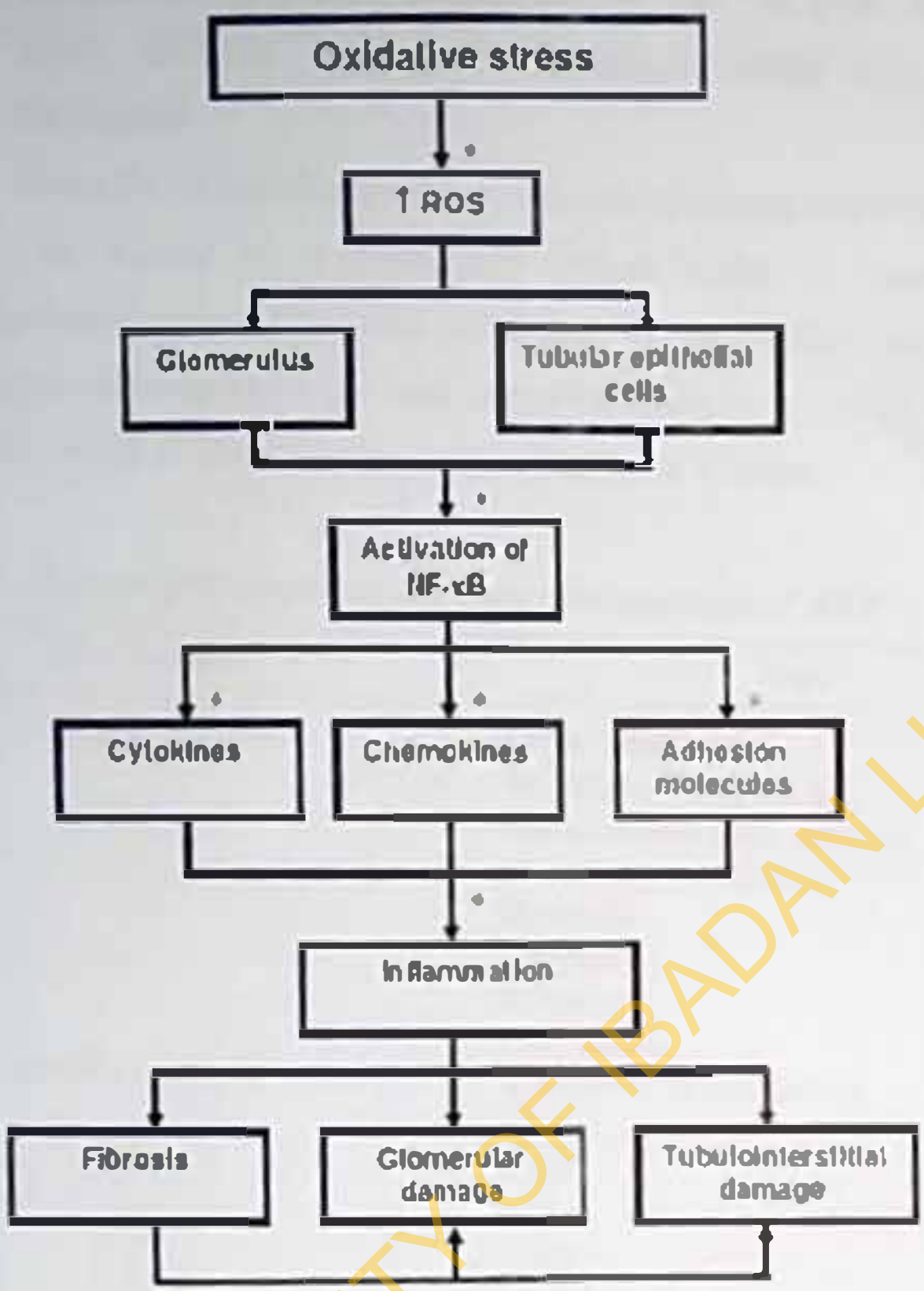


Figure 10. Hypothesis to explain the glomerular and tubulointerstitial damage caused by oxidative stress and the levels of renoprotective effects of polyphenols. * sites for potential effects of polyphenols

Alpha G-rutin, a wine polyphenol that works as an antioxidant *in vivo* either by scavenging ROS or by chelating ferric ions, served to prevent oxidative renal damage in mice treated with Fe-NTA (Shimoi *et al*., 1997). A hypothesis to explain the renoprotective effects of polyphenols against oxidative stress is depicted in Figure 10.

2.6 PHARMACOLOGIC TREATMENT OF ACUTE KIDNEY INJURY: WHY DRUGS HAVEN'T WORKED AND WHAT IS ON THE HORIZON

Given the failure of multiple pharmaceutical agents in the therapy of AKI, novel agents are needed in well-designed clinical trials. A number of drugs and investigational compounds seem promising in preclinical studies, and promising investigational compounds are used in clinical trials for a variety of indications (Jo *et al.*, 2007). Some of these agents are shown in Table 1 below.

Table 1. Emerging Pharmacological agents for treatment of AKI[a]

Action/Mechanism	Drugs
Antiapoptosis/necrosis	Caspase inhibitors
	Nonselective caspase inhibitors
	Selective caspases 3 and 7 inhibitors
	Selective caspase 1 inhibitors
	Minocycline
	Guanosine
	Pifithrin-α
	PARP inhibitor
Anti-inflammatory	Sphingosine 1 phosphate analog
	Adenosine 2A agonist
	α-MSH
	IL-10
	Fibrate
	PPAR-γ agonist
	Minocycline
	Activated protein C
	iNOS inhibitor
Antisepsis	Insulin
	Activated protein C
	Ethyl pyruvate
Growth factor	Recombinant erythropoietin
	Hepatocyte growth factor
Vasodilator	Carbon monoxide release compound and bilirubin
	Endothelin antagonist
	Fenoldopam
	ANP

[a]ANP, atrial natriuretic peptide; iNOS, inducible nitric oxide synthase; α-MSH, alpha-melanocyte–stimulating hormone; PARP, poly ADP-ribose polymerase; PPAR, peroxisome proliferator–activated receptor.

Antiapoptosis/Necrosis Agents

Caspase Inhibitors. Caspases are a family of proteases that are involved in the initiation and execution phase of apoptosis. Nonselective and selective caspase inhibitors are effective in attenuating renal injury in ischemia- or endotoxemia-induced AKI when administered before or at the time of injury (Daemen *et al.*, 1999; Tiwari *et al.*, 2005; Melnikov *et al.*, 2002). Pancaspase inhibitors are in early clinical trials (Valentino *et al.*, 2003), and early targets include hepatitis C and orthotopic liver transplantation.

Minocycline. Minocyclines are second-generation tetracycline antibiotics with proven human safety data. Minocycline is known to have antiapoptotic and anti-inflammatory effects. When administered 36 h before renal ischemia, minocycline reduced tubular cell apoptosis and mitochondrial release of cytochrome c, p53, and bax (Kelly *et al.*, 2004). Furthermore, minocycline reduced kidney inflammation and also microvascular permeability (Sutton *et al.*, 2004). Minocycline has been used in clinical trials for rheumatoid arthritis (Tilley *et al.*, 1995) and is undergoing testing in phase I/II clinical trials for amyotrophic lateral sclerosis (Gordon *et al.*, 2004).

Guanosine and Pifithrin-α (p53 Inhibitor). GTP salvage by exogenous administration of guanosine reduced renal tubular cell apoptosis, an effect that was associated with inhibition of p53 expression (Kelly *et al.*, 2001). Pifithrin-α, a novel p53 inhibitor, also led to decreased tubule cell apoptosis and preserved renal function (Kelly *et al.*, 2003). This agent is nearing clinical trials in cancer therapy.

Poly ADP-Ribose Polymerase Inhibitor. Poly ADP-ribose polymerase (PARP) is a ubiquitous nuclear enzyme that participates in DNA repair (Padanilam, 2003; Chiarugi, 2002). Paradoxically, excessive activation of PARP from cellular injury leads to intracellular NAD^+ and to ATP depletion, ultimately resulting in cell death. PARP overactivation has been known to play a role in the pathogenesis of ischemia/reperfusion injury to kidney, heart, and brain (Chatterjee *et al.*, 2004; Thiemermann *et al.*, 1997; Zingarelli *et al.*, 1997). Inhibition of PARP immediately at reperfusion reduced injury. PARP inhibitors are in clinical trials for breast cancer (phase I) and cardiac reperfusion injury (phase II).

Free Radical Scavengers

Deferoxamine. A key early feature of AKI is the generation of reactive oxygen species. The iron chelator deferoxamine is a widely known free radical scavenger. In several models of AKI, deferoxamine was proved effective (Walker and Shah, 1988; Walker and Shah, 1990; Baliga *et al.*, 1998; Baliga *et al.*, 1999). The protective effect of deferoxamine in various models suggests the central role of free radicals in AKI. Studies in AKI are planned to test the efficacy of iron chelation.

Antisepsis

Ethyl Pyruvate. Pyruvate has been known as a potent endogenous antioxidant and free radical scavenger, and its derivative, ethyl pyruvate, proved to be effective in reducing mortality in animal models of lethal hemorrhagic shock and systemic inflammation caused by endotoxemia or sepsis (Ulloa *et al.*, 2002). In addition to an effect on mortality, ethyl pyruvate reduced kidney injury using the technique cecal ligation puncture as a model of sepsis (Miyaji *et al.*, 2003). Ethyl pyruvate is a widely used food additive and has been shown to be safe in phase I clinical trials. It now is being tested in a phase II trial in patients who undergo cardiopulmonary bypass surgery.

Activated Protein C. Activated protein C (APC) is a physiologic anticoagulant that is generated by thrombin-thrombomodulin complex in endothelial cells. In addition to its effect on coagulation, APC has been shown to have anti-inflammatory, antiapoptotic effects (Grey *et al.*, 1994; Grey *et al.*, 1993). APC also attenuated renal IRI by inhibiting leukocyte activation (Mizutani *et al.*, 2000). APC is approved by the Food and Drug Administration for treating patients who have severe sepsis and an Acute Physiology, Age, Chronic Health Evaluation (APACHE) score of 25 or higher.

Insulin. Insulin resistance and hyperglycemia are common in critically ill patients, and intensive insulin therapy that targeted blood glucose level between 80 and 110 mg/dl reduced the incidence of AKI that required dialysis or hemofiltration (van den Berghe *et al.*, 2001). The relationship of hyperglycemia and adverse outcome in critically ill patients with AKI also was observed recently in a subgroup analysis of the PICARD study (Basi *et al.*, 2005). The mechanism for clinical benefit may relate to the dosage of insulin as opposed to glycemic control (Langouche *et al.*, 2005). Endothelial dysfunction and subsequent hypercoagulation and dyslipidemia, commonly observed in

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critically ill patients, are corrected partially by insulin independent of its blood glucose–lowering effect (Langouche *et al.*, 2005; Mesotten *et al.*, 2004). However, despite these promising results, the effect of intensive insulin treatment in the setting of AKI has not been tested and needs to be confirmed in appropriately powered, randomized, clinical trials.

Growth Factors

Recombinant Erythropoietin. Erythropoietin has been shown to have anti-inflammatory and antiapoptotic effects in ischemic brain damage, spinal cord injury, and retinal damage (Celik *et al.*, 2002). Exogenously administered erythropoietin before or at the time of reperfusion reduced kidney injury by reducing tubular necrosis and apoptosis (Yang *et al.*, 2003; Vesey *et al.*, 2004; Sharples *et al.*, 2004). It enhanced tubular proliferation in cisplatin-induced AKI (Vaziri *et al.*, 1994) and also mediated mobilization and proliferation of endothelial progenitor cells from the bone marrow that has been shown to participate in tissue repair (Patschan *et al.*, 2006; Bahlmann *et al.*, 2003). Clinical use of recombinant erythropoietin should facilitate translation to human PKI.

Hepatocyte Growth Factor. Hepatocyte growth factor (HGF) can promote cell growth, motility, and morphogenesis of various types of cells (Nakamura *et al.*, 1989; Gohda *et al.*, 1988). Renal expression of HGF and its receptor, c-met, increases after IRI, and exogenous administration of HGF reduces renal injury and accelerates renal regeneration in a murine model of AKI (Liu *et al.*, 1999; Rabkin *et al.*, 2001; Kawaida *et al.*, 1994). The mechanism of protection is thought to involve a decrease in leukocyte-endothelial interaction with reduced inflammation and also a decrease in tubular cell apoptosis (Mizuno and Nakamura, 2005). Currently, phase I/II study of recombinant human HGF in fulminant hepatic failure patients and another phase II study of HGF *via* plasmid vector in patients with critical limb ischemia and peripheral ischemic ulcer are under way. Experience in these clinical trials may shed light on human AKI.

Carbon Monoxide Release Compounds and Bilirubin. In a seminal study, Nath *et al.* (Nath *et al.*, 1992) found that heme oxygenase (HO) induction played a central role in limiting the extent of myoglobin-induced AKI. HO activity leads to the production of carbon monoxide (CO) and a potent antioxidant, bilirubin, and it is thought that the protective effect of HO activation is through these factors (Nath *et al.*, 1992; Sikorski *et al.*, 2004). In renal ischemia/reperfusion injury (IRI) administration of CO donor compounds tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$) or tricarbonylchloro(glycinato)ruthenium(II) ([Ru(CO)3Cl(glycinate)] 1 hour before the onset of ischemia significantly decreased the levels of plasma creatinine 24 hours after reperfusion as compared with vehicle-treated mice (Vera *et al.*, 2005). This suggests that CO itself may be protective and limit renal damage in ischemia-induced AKI (Vera *et al.*, 2005). Bilirubin also has been shown to reduce kidney injury from IRI (Adin *et al.*, 2005), and when biliverdin and CO are used in combination, they are synergistic in improving heart allograft survival (Nakao *et al.*, 2005).

Endothelin Antagonist. A potent vasoconstrictor, endothelin-1 (ET-1), has been implicated to play important roles in animal models of AKI or radiocontrast nephropathy (Birck *et al.*, 1998; Heyman *et al.*, 1992). ET-1 mediates its biologic effects by binding to ETA or ETB receptors. In rat kidney, ETA receptor stimulation is known to mediate vasoconstriction, whereas ETB receptor activation also can mediate vasodilation by generation of nitric oxide and prostacyclin (Goto *et al.*, 1996; Edwards *et al.*, 1993). In addition, ET-1 can stimulate the expression of adhesion molecules and the production of cytokines from monocytes and neutrophils, suggesting the possible role of ET-1 in inflammation in AKI (Hayasaki *et al.*, 1996). Several studies demonstrated the beneficial effect of selective ETA or nonselective endothelin receptor antagonist in ischemic AKI, but the major limitation of those studies is that endothelin receptor antagonist was administered before injury. Administration of the drug at later time point during the reperfusion was ineffective. However, Wilhelm *et al.* (2001) recently showed that tezosertan, a dual ET-1 receptor antagonist, attenuated renal injury even when administered after ischemia.

Inflammatory cells, including polymorphonuclear cells, monocytes, macrophages, and T cells, have received considerable attention as important contributors to ischemic acute renal failure. Several new compounds seem to be effective in reducing injury from ischemia-reperfusion through direct action on leukocytes.

Sphingosine 1 Phosphate Analogs. Sphingosine 1 phosphate (S1P) is a specific ligand for a family of G protein–coupled endothelial differentiation gene receptors (S1PR 1 through 5) that evoke diverse cellular signaling responses. S1PR regulate different biologic processes depending on their pattern of expression and the diverse G proteins present. S1P binds to receptors or acts as a second messenger to stimulate cell survival, inhibit cell apoptosis, and inhibit cell adhesion and movement (Spiegel and Milstien, 2003). An S1P analog, FTY720, acts as an agonist at four S1PR, which lead to sequestration of lymphocytes in secondary lymphatic tissue (Brinkmann *et al.*, 2002). In studies of kidney IRI, FTY720 or similar compounds produced lymphopenia and renal tissue protection (Awad *et al.*, 2006; Lien *et al.*, 2006). With discovery of new S1P analogs, more potent and selective agents will be available for preclinical and clinical studies (Foss *et al.*, 2005). Recently, in a phase II study, FTY720 reduced the number of lesions that were detected on magnetic resonance imaging and clinical disease activity in patients with multiple sclerosis (Kappos *et al.*, 2006).

A2A Agonists and Other Adenosine Analogs. Adenosine binds to receptors, which are members of the G protein–coupled receptor family that includes four subtypes: A1, A2A, A2B, and A3Rs (Linden, 2001). Selective activation of A2ARs reduces parenchymal injury in nonrenal tissue, including heart, liver, spinal cord, lung, and brain (Jordan *et al.*, 1997; Lasley and Mentzer, 2001; Day *et al.*, 2004). The selective A2AR agonist ATL146e is highly protective against IRI of kidney and reduces injury by 70 to 80% (Day *et al.*, 2003; Okusa *et al.*, 1999; Li and Okusa, 2006). After administration either before or immediately at the onset of reperfusion, ATL146e alone or in combination with a phosphodiesterase inhibitor reduced renal injury (Okusa *et al.*, 2001). ATL146e is in human clinical studies for cardiac imaging, and current efforts are directed toward human clinical studies in AKI. Additional studies demonstrate that strategies that use A1 agonists or A3 blockers may be effective in AKI (Lee *et al.*, 2003; Lee *et al.*, 2004).

Inducible Nitric Oxide Synthase Inhibitors. The role of nitric oxide (NO) and nitric oxide synthases (NOS) has been studied extensively. Both *in vivo* and *in vitro* studies point toward the important role of inducible NOS in mediating injury to proximal tubules (Noiri *et al.*, 1996). A nonselective NOS inhibitor (N^{ω}-methyl-L-arginine) has been studied in a phase III clinical trial in septic patients (Cobb, 1999). This study was stopped prematurely by the data safety monitoring board because of an excess of mortality in the N^{ω}-methyl-L-arginine group. More selective inducible NOS inhibitors are currently used in human investigation for a variety of indications.

Fibrates. Peroxisome proliferator–activated receptors (PPAR) are transcription factors that regulate glucose and lipid metabolism. Recent studies indicated that PPAR play an important role in inflammation and immunity (Daynes and Jones, 2002). Pretreatment of animals with fibrates (PPAR-α ligand) ameliorated cisplatin-induced renal dysfunction, and this was accompanied by suppression of NF-κB activation, cytokine/chemokine expression, and neutrophil infiltration, suggesting that the protective effect of fibrates is mediated through its anti-inflammatory effect (Li *et al.*, 2005).

Other Compounds. Neutrophil gelatinase–associated lipocalin (Mishra *et al.*, 2004), IL-6 and C5a antagonists (Riedemann *et al.* 2003), IL-10 (Deng *et al.*, 2001), and α-melanocyte–stimulating hormone (Chiao *et al.*, 1997) are other potential compounds that have multiple mechanisms of tissue protection and may be beneficial in human AKI.

Flavonoids continue to draw wide attention as possible very useful therapeutic agents for combating "free radical pathologies" i.e. the pathologic states associated with free radical overproduction (inflammation, ischemia/reperfusion, environment-associated disease, etc.) (Afanas'ev *et al.*, 1995). The role of dietary flavonoids in the prevention of several chronic diseases is the subject of intense research interest and the soy isoflavones have been the focus of particular attention (Messina *et al.*, 1994; Knight and Eden, 1996; Hertog *et al.*, 1993).

Until about 15 years ago, there was very little soy in the western diet (Gobloitz, 1995). Now, science continues to prove how and why the properties of soy are so essential for preventing causes and conditions including: LDL cholesterol and atherosclerosis; tumor growth and cancer; and osteoporosis.

Approximately ten percent of the Japanese diet consists of soy protein (Hawrylewicz *et al.*, 1995). Along with the Chinese, the Japanese have significantly lower rates of breast, colon and prostate cancer (Persky and Von Horn, 1995). In the eastern world, much more soy is utilized for medicinal and food properties and these statistics show what an amazing difference it makes in overall health. Soy is low in saturated fats, is cholesterol-free and, unlike animal proteins, soy does not cause any stress on the kidneys (Messina, 1995).

What makes this bean so amazingly powerful? Protein composes 36% of soybean. Soy protein contains amino acids, isoflavones, saponins, phytic acid, trypsin inhibitors and fiber, which work synergistically. Soybeans are a rich source of saponins, which reveal in clinical studies chemopreventive properties and depression of tumor cells. The saponins and trypsin inhibitors bind with bile acids (tumor promoters) and also affect the growth of tumor cells by deforming them. The fiber regulates lipids. Some cancer cells contain cholesterol, which soy binds to and destroys. Phytic acid is a natural chelator of iron, calcium, zinc and magnesium in the gut. Compounded with Bowman-Birk Inhibitor (BBI) a protease inhibitor, and Beta-sitosterol, phytic acid has shown suppression of benign and malignant tumors in colon, liver, oral and lung cancer. Soy proteins have been tested in a variety of studies, inhibiting tumor growth in animal subjects.

Genistein has been in the spotlight of recent research since its discovery in 1987. It was originally considered a "phytoestrogen" until other non-estrogenic

properties were identified. There have been no side effects or toxicity reported with high doses of genistein *in vivo*. One of the identification of genistein is as a tyrosine kinase inhibitor (TKI). Researchers of this natural TKI attribute the dramatically lower incidence of breast and prostate cancer in Southeast Asian populations to its anti-cancer properties. (Barnes *et al.*, 1995). Independent studies have shown that the isoflavones genistein and daidzein inhibited the growth of tumors in breast cancer cells (Kennedy, 1995). Genistein also has biological antioxidant properties. In a recent study, laboratory mice were fed genistein for 30 days and there was an increase in antioxidant enzymes in their organs. This increase of antioxidant enzyme activity may contribute to the chemopreventive properties of genistein (Cai and Wei, 1996). There are a number of studies still being conducted on the role genistein plays in cancer prevention and inhibition.

When diets use soy protein over animal, the results are amazing on how the soy significantly lower LDL cholesterol readings. Historically, this comparison was done in laboratory rabbits, then on humans and now in larger, controlled studies. The most recent findings show that adding soy protein to a high cholesterol diet greatly reduced LDL cholesterol levels (Carroll *et al.*, 1995). In studies conducted on children with high cholesterol, soy reduced cholesterol level by 20% (Sirtori *et al.*, 1995). Researchers believe that soy protein could possibly act in a few different ways to lower cholesterol. One is "hepatic metabolism", which is when soy assists the receptor cells in the liver that binds LDL cholesterol (Potter, 1995). Soy alters hormone concentrations of insulin, glucagon and thyroxine, which are also responsible for lowering LDL (Forsythe, 1995).

The cholesterol lowering properties of soy also protect against atherosclerosis. LDL cholesterol is responsible for thrombosis, a condition in which plaque forms in a damaged artery or other pathway (Wilcox and Blumenthal, 1995). Documentation has shown that genistein depresses the reproduction of cells that adhere to artery walls and cause lesion formation (Raines and Ross, 1995). Evidence is consistent that soy has the potential to reform atherosclerosis by reduction of cholesterol levels, thrombosis and tyrosine kinase activity (Wilcox and Blumenthal, 1995).

There are many advantages to supplementing soy to the human diet. International diet trends and soy research continually show the remarkable benefits of its properties. Further studies are being conducted about its effect on renal disease to show how soy can protect the body from common threats of degenerative conditions.

2.7.1 Isoflavone content of soy foods

Not all soy foods are necessarily equivalent and investigators are warned to carefully determine the isoflavone content of the product they may be using in an animal experiment or in a human clinical trial. This may be an additional source of the variation of response to soy in the animal experiments.

In studies carried out by Coward *et al.* (1993), it was shown that soy flours contain all the isoflavones that were present in the whole soybean. The total isoflavone content is the same irrespective of the heating used in the manufacture of the soy flour. Full-fat soy milk and tofu have isoflavone concentrations (expressed as dry weight or relative to protein content) similar to that in soy flour. The isoflavone content of soy protein concentrates depends on the washing medium used to prepare them from soy flour. Water or acid-washing does not alter the isoflavone content; however, alcohol-water mixtures substantially reduce it. Isolated soy proteins have generally lower isoflavone content; however, some newer preparations have an isoflavone content that approaches that of soy flour or soy protein concentrate. "Lite" versions of soy milk and tofu, which are based on isolated soy protein, have lower isoflavone concenuations than their full-fat counterparts.

Although most investigators have reported that isoflavones are largely β-glucoside conjugates in soy foods, new evidence indicates that the conjugated form of each isoflavone in the hypocotyl and cotyledon is the 6"-O-malonylglucoside (Kudou *et al.*, 1991). Coward *et al.* (1993) also examined a wide range of unfermented soy foods and showed that the 6"-O-malonylglucosides are the principal form in each type, with the exception of full-fat soy milk and tofu that contain only the β-glucosides. Heating causes soy flours to contain increasing amounts of 6"-O-acetylglucoside conjugates (Barnes *et al.*, 1994). The relative bioavailability and the sites of absorption of the 6"-O-acetylglucosides, the 6"-O-malonylglucosides, the β-glucosides and the free aglycones are unknown at the present time. It is likely that the aglycones are absorbed in the upper small bowel, as are many drug compounds of a similar structure. The β-glucosides are excellent substrates for β-glucosidases and would be expected to be absorbed in the small bowel. The modified acetyl- and malonylglucosides may be sufficiently poor substrates for enzyme hydrolysis (Farmakalidis and Murphy, 1985) that they would be absorbed from the large bowel after hydrolysis induced by bacterial enzymes.

2.7.2 Concentration of isoflavones at cellulnr targets

It is important for the evaluation of the significance of the cell culture studies to know the concentrations of isoflavones that normal cells and tumor cells are exposed to in individuals consuming a soy-containing diet. Adlercreutz *et al.* (1993) were able to report plasma total genistein concentrations as high as 0.1 μmol/L in some vegetarian women by using reverse isotope dilution gas chromatography-mass spectrometry. Adlercreutz *et al.* (1995) and Setchell (unpublished observations, 1994) have suggested that the plasma level of genistein in people on a high-soy-containing diet was 1-4 μmol/L. Limitations in the existing analytical methods remain to be resolved. These include not only the measurement technique, but also the protocols for the extraction of genistein (and other isoflavones) from plasma.

Nonetheless, reasonable estimates for the plasma level of genistein can be inferred from consideration of dietary intake and rates of metabolism and excretion. A person consuming 35 g/d of soybeans (the average amount consumed by Taiwanese) (Soyatech Survey and Estimates, 1991) has an intake of approximately 50 μg (185 μmol) of genistein (mostly in the conjugated form). Maximum plasma levels (23 μmol/l) would be attained if genistein were fully absorbed from the diet, confined to the blood compartment (approximately 8 L) and not metabolized or excreted. In reality, none of these conditions apply. Genistein, being relatively hydrophobic, will be taken up by cells. Previous studies on the tissue distribution of daidzein (Yuch and Chu, 1977) showed that in most tissues it is in similar concentrations to those in blood. Therefore, if one assumes that genistein equilibrates with total body water (56 L), the equilibrium plasma concentration would be 3.3 μmol/l. Because there is no evidence that genistein has an extended half-life in the body, the effects of carryover from day to day are expected to be minimal. Based on these above estimates, genistein would have less than a 20% inhibitory effect on cellular processes with IC_{50} values of greater than 13.2 μmol/l (5 μg/mL).

2.7.3 Soybean constituents known to have anticarcinogenic activity

There are only a few soybean constituents that have been studied in a pure form and have been shown to significantly suppress carcinogenesis in animals. The few soybean-derived agents that have been studied in a pure form in animal carcinogenesis assay systems include Bowman-Birk inhibitor (BBI) (a protease inhibitor), inositol hexaphosphate (phytic acid), β-sitosterol (a soybean-derived sterol),

It has been observed that the protease inhibitor, the Bowman-Birk inhibitor (BBI), is the compound in soybeans that has shown the greatest suppression of carcinogenesis in animal carcinogenesis assays. Experiments have shown that BBI can completely prevent colon carcinogenesis (100% suppression) and suppress carcinogenesis in the liver by 71%, in the oral epithelium by 86%, and in the lung by 48% (St Clair *et al.*, 1990a; Kennedy *et al.*, 1993a and Witschi and Kennedy, 1989). The ability of BBI to suppress carcinogenesis in the various systems studied far exceeds the ability of other soybean-derived compounds to affect carcnogenesis. Two other compounds in soybeans have also been shown to suppress carcinogenesis in animals: phytic acid and β-sitosterol. Phytic acid (inositol hexophosphate) was observed in one experiment to suppress colon tumorigenesis by 2.5% (Shamsuddin *et al.*, 1989). In other experiment in which the suppression of colon carcinogenesis by phytic acid was studied, all of the animals in the experiments developed colon tumors and phytic acid reduced the number of tumors per animal by 27-36% (Shamsuddin *et al.*, 1988; Shamsuddin and Ullah, 1989).

In the single experiment in which phytic acid reduced the percentage of animals bearing tumors, the suppression of carcinogenesis was accompanied by weight loss (Shamsuddin *et al.*, 1989). In the single experiment in which the soybean sterol, β-sitosterol, was assayed for its ability to suppress colon carcinogenesis, it was able to reduce the total number of benign and malignant tumors by 39% (Raicht *et al.*, 1980). In this same experiment, however, β-sitosterol led to more animal (a 50% increase) bearing malignant colon tumors.

2.7.4 Potential adverse effect from dietary soybean-derived protease inhibitors

There has been concern that high levels of soybean protease inhibitors in the diet could cause adverse health effects (Kennedy, 1993a). One potential deleterious effect widely attributed to the soybean protease inhibitors has been a suppressive effect on the growth and weight of young animal (Birk, 1993; Kennedy, 1993a). Although the soybean protease inhibitors do apparently contribute a bit to growth depression in young animals (Kakade *et al.*, 1973), the major soybean-produced growth-suppressive effects cannot be attributed to the protease inhibitors (Birk, 1993). The major health concern, however, has been that the soybean protease inhibitors could contribute to pancreatic cancer development while suppressing carcinogenesis in other organ systems (Kennedy, 1993a). High levels of soybean products in the diet of rats are associated

with pancreatic hyperplasia and hypertrophy, with a few rats fed such high level of soybean products for long periods of time developing pancreatic cancer (McGuincss *et al* 1984; Morgan. 1987). Although some investigators have attributed this effect to the protease inhibitors in soybeans, the evidence for this is not clear (Kennedy, 1993a).

It is likely that much of the enhancing effect of soybeans on pancreatic carcinogenesis is due to soybean fat, because most studies attributing effect on the pancreas to protease inhibitors have had significant levels of soybean fat present in the preparations of soybean products used. Much of the evidence implicating protease inhibitors in the observed effects comes from studies using Wistar rats and full fat soy flour with approximately 20g/100g polyunsaturated soybean fat (McGuiness *et al.*, 1980; McGuiness *et al.*, 1984). Conclusions about the effect of protease inhibitors on the pancreas cannot be drawn from such studies. Although the soybean-derived protease inhibitors were present in the diets, the evidence implicating them is circumstantial and the effects could have been produced by other soybean-derived compounds The high levels of polyunsaturated fat are a reasonable candidate for this because unsaturated fat promotes carcinogenesis in the rat pancreas (Roebuck *et al.*, 1987). The evidence provided by the USDA trypsin inhibitor (TI) study is also problematic because protease inhibitors were not studied in a pure form (Gumbmann *et al.* 1985). Further, the data from this study do not support the notion the TIs cause pancreatic cancer in Wistar rats for the following reasons:

1. No cancer was observed in the study- only pancreatic adenoma, a benign, proliferative lesion.
2. There is no dose-response relationship between TI intake and the incidence of adenomas. In the control group (0 TI), 1/104 (adenomas) occurred, which is not statistically different from 0/107, 3/109 or 2/102 (results representing increasing TI dietary levels up to approximately 300mg/100g of the diet) by standard statistical analyses. If there is a clear dose-response relationship, one should observe statistically significant differences between these treatment group in the study, at approximately 600mg/100 g of the diet (577 mg TI per 100 g diet), appear to represent a statistically significant result, with 13/105 rats developing pancreatic adenomas in this treatment group with TI at approximately 600 mg/100 g of the diet.
3. Even if there was a clear dose-response relationship between TI intake and incidence of adenomas, this is not convincing evidence that protease inhibitors

cause pancreatic cancer. Protease inhibitors have been demonstrated to be clearly associated with increased growth in the rat pancreas, leading to hypertrophy and hyperplasia (Kennedy, 1993a), but it is not at all clear whether hypertrophy and hyperplasia are related to cancer development in the pancreas. There are species that have these pancreatic lesions and do not get cancer and other species that have reduced cancer rates in the pancreas even though they have pancreatic hypertrophy and hyperplasia. Thus, hypertrophy and hyperplasia are clearly separable phenomena from the development of cancer in the pancreas (Birk, 1993; Kennedy, 1993a). As discussed above, there is only one treatment group in the USDA 'Tl study that shows a statistically significant difference in the pancreatic adenoma incidence from the control group; and this group represents a dietary addition of Tl at approximately 600mg/100 g of the diet, an exceptionally high level. It is expected that a dietary concentration of 100-10 mg/100 g of BBI will be effective for prevention of human cancer (Kennedy *et al.*, 1993a, Kennedy, 1993c; Kennedy, 1993d). At a level of 100 mg/100 g in the diet, there is clearly not a significant effect of Tl in the USDA Tl study. In fact, if there is an effect at all, it is in a downward direction (i.e., with the number of animals bearing pancreatic adenomas decreasing with Tl dietary supplementation). The results for pancreatic adenoma incidence at 100 mg/ 100g of the diet (93 mg Tl/100 g diet) are 0/107 vs. the control incidence of 1/104.

There are a few other studies suggesting that at very high level, preparations of soybean protease inhibitors studied along with the soybean fat may contribute to the development of rat pancreatic adenoma. All of these studies have used a particular model system: azaserine-induced pancreatic carcinogenesis in rats. These studies have suggested that soybean concentrates enriched in protease inhibitor activity can act as a promoter of azaserine-induced rat pancreatic carcinogenesis (Myers *et al.*, 1991; Roebuck *et al.*, 1987). This effect can be observed only at high concentrations of soy Tl (e.g., diets containing 3.7% soybean Tl concentrate) (Myers *et al.*, 1991). Even such exceptionally high concentrations of soybean Tl concentrate did not initiate pancreatic carcinogenesis (Myers *et al.*, 1991). Similarly, soybean Tl cannot initiate pancreatic carcinogenesis but can only promote azaserine-induced pancreatic carcinogenesis in rats (Roebuck *et al.*, 1987). Other investigators concluded that whole soybean products

do not initiate pancreatic carcinogenesis but instead can act as a promoter of multistage rat pancreatic carcinogenesis (McGuiness *et al.*, 1984).

The azaserine-induced rat pancreatic carcinogenesis system is an unusual system leading to a type of pancreatic cancer, acinar cell neoplasms, which rarely occurs in human pancreatic cancer development. This type of pancreatic cancer is observed in 4% of human pancreatic cancer (Pour, 1991). The common type of human pancreatic cancer is of ductal or ductular origin; ductal neoplasm account for 90% of human pancreatic cancer (Pour, 1991). Many modifying agents for carcinogenesis have opposite effect on the two types of pancreatic cancer development (i.e., azaserine-induced acinar cell pancreatic neoplasms in rats and nitrosamine-induced neoplasms of ductal origin in hamsters) (Pour, 1991). One example of such opposing effect involves soyflour treatment. Raw soyflour dietary additions containing a high level of soy TI activity lead to highly significant reductions in the levels of experimentally induced pancreatic carcinogenesis in hamsters (Hasdai and Liener, 1985; Liener and Hasdai 1986; Pernert, 1993; Pour, 1991).

In studies such as those cited above, the soybean fat has been studied along with the soybean protease inhibitors, so that all conclusions about the effects of protease inhibitors in the rat model system are problematic. There appears to be only one study in which the soybean products have been defatted (Gumbmann *et al.*, 1985). Although it was still an impure soybean preparation being studied, the TI preparation used was defatted and low-molecular-weight compounds were removed. This is believed to be important because low-molecular-weight compounds in soybeans are thought to mask the anticarcinogenic activity of BBI (Kennedy *et al.*, 1993b; Yavelow *et al.*, 1985). Even in this study reported by Gumbmann *et al.* (1985) on the effects of soy TI preparations, there were no significant differences in the incidence of pancreatic cancer; only acinar adenomas were observed in rats, and not even adenomas (or other pathologic alteration) were observed in mice. Thus pancreatic hyperplasia and hypertrophy are separable from adenoma development (given the differences observed between the rat and mouse studies) and there is no evidence suggesting that the adenomas occurring in rats lead to cancer. The incidence of acinar adenoma in rats attributable to protease inhibitors is very low in this study. At 100 mg/100 g of the soyflour addition, there is not a statistically significant difference in the incidence of pancreatic adenomas compared with the no-addition treatment group. The results from this treatment group are comparable with the results from the soy TI treatment group at

100 mg/100 g of the diet, but the soy TI treatment was significantly different from controls. Both the dietary additions of soy flour and soy TI led to approximately a doubling of the spontaneous incidence of pancreatic adenoma, with 10% to 20% of the rats having evidence of this histopathologic lesion. Even though this is a relatively small effect, any effect of this sort is of concern if results from studies such as these can be extrapolated to humans.

Although an adverse effect of soy TI may exist in the rat pancreas, a similar effect on the human pancreas is highly unlikely (Kennedy, 1993a). The rat pancreas is quite unusual in its response to soy TI, the human pancreas is not expected to respond to soy TI with hyperplasia, hypertrophy, adenoma or cancer development, for many reasons that have been discussed previously (Kennedy, 1993a). Of particular relevance to the question of whether the use of high dietary levels of soybean products could lead to the development of human pancreatic cancer is the epidemiologic evidence. The populations having high levels of soybean products and TI activity in their diets do not have elevated risks of pancreatic cancer (Correa 1981; Kennedy, 1993a), in fact, some of these populations have significantly reduced rates of pancreatic cancer development (Mills *et al.*, 1988). Thus the available epidemiologic evidence suggests that the human pancreas is likely to respond to soybean products with a reduced cancer rate. This would not be surprising because 90% of human pancreatic cancer is ductal in origin (Pour, 1991) and nitrosamine-induced pancreatic carcinogenesis of ductal origin in animals is reduced with soybean flour dietary additions (Hasdai and Liener, 1985; Liener and Hasdai, 1986; Permert *et al.*, 1993; Pour, 1991). Whether there will be effects in the human pancreas associated with the administration of soybean protease inhibitors, or soybean products in general, will never be completely determined with epidemiologic studies, however. Thus, it will be important to monitor the response of the human pancreas to the administration of soybean products as cancer preventive agents in human trials.

Gentamicin and Cisplatin (cis-diamminedichloroplatinum II, CDDP) are in no doubt among the most effective chemotherapeutic agents in clinical practice. In spite of their obvious clinical benefits, nephrotoxicity and induction of ARF remain a major challenge (Cassidy and Misset, 2002; Wolfgang *et al.*, 1994) and account for nearly 10-15% of drug toxicity (Kumar *et al.*, 2000). In fact, they are one of the five principal causes of hospital acquired acute renal failure.

It is obvious from literature that interest in gentamicin and cisplatin nephrotoxicity is still thriving. Despite extensive studies, the mechanisms of the nephrotoxicity of these important therapeutic drugs can not be clearly explained. Furthermore, several clinical and experimental strategies have been employed in order to ameliorate or abolish the signs of gentamicin and cisplatin nephrotoxicity. Most of these have been unsuccessful, impractical or unsafe. Thus, there is a pressing need for ways to protect the kidneys while administering effective doses of chemotherapeutic agents such as cisplatin and gentamicin in patients at risk.

Considerable experimental evidence has contributed to support a key role of reactive oxygen species (ROS) in the numerous mechanisms of seemingly unrelated nephropathies (Rodrigo and Rivera, 2002). Also, flavonoids continue to draw attention as possible, very useful therapeutic agents for combating pathologic states associated with free radical production (Lopez-Velez *et al.*, 2003). The role of dietary flavonoids in the prevention of several chronic diseases has been the subject of intense research interest and the soy phenolics have been the focus of particular attention (Omoni and Aluko, 2005). There is increasing evidence that dietary phytoestrogens present primarily in soybeans as isoflavones have a beneficial role in chronic renal disease (Ranich *et al.*, 2001). Nutritional intervention studies have shown that consumption of soy-based protein reduces proteinuria and attenuates renal functional or structural damage in animals and humans with various forms of chronic renal disease (Ranich *et al.*, 2001). Also several studies have demonstrated the efficacy of dietary soy protein in delaying the progression of polycystic kidney disease in experimental animal models (Aukema *et al.*, 1999; Ogborn *et al.*, 1998; Tomobe *et al.*, 1998).

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i. Examine the possible protective effect of concurrent administration of soybean, using the methanolic extract (MESB), in a rat model of cisplatin- and gentamicin- mediated renal dysfunction and injury. This is an attempt to further identify safe, practical and effective agents to ameliorate the nephrotoxicity in patients at risk.

ii. Assess the extent of disruption of antioxidant defense system and activities of membrane-bound enzymes following treatment with gentamicin and cisplatin *in vivo*.

iii. Further elucidate the cellular and molecular mechanisms involved in cisplatin- and gentamicin- mediated renal toxicity.

iv. Correlate the biochemical alterations induced by gentamicin and cisplatin both in blood and renal tissues with overrall renal function and morphology. This should give some insight into the mechanism(s) of amelioration or protection against this adverse effect.

v. Explain the mechanism(s) by which soybean offer protection against these experimental models of nephrotoxicity.

vi. Evaluate the free radical scavenging and antioxidant activities of MESB *in vitro*. This would further give some insight into the mechanisms of amelioration or protection observed by this extract against the renal toxicity of gentamicin and cisplatin *in vivo*.

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CHAPTER THREE

MATERIALS AND METHODS

3.1 CHEMICALS

Gentamicin sulphate was obtained from Formulations Plc, (England). Cisplatin, Phenylmethanesulfonyl fluoride, alpha, alpha-Azodiisobutyramidine dihydrochloride (AAPH), 2-Deoxy-D-ribose, DL-Dithiothreitol, 2,4,6-Tri(2-pyridyl)-S-triazine (TPTZ), Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\cdot+}$), Phenazine methosulfate (PMS) were purchased from Sigma-Aldrich (Germany). 1-Chloro-2,4-dinitrobenzene (CDNB), 5,5- Dithiobis-2-nitrobenzoic acid (DTNB), Reduced Glutathione (GSH), Adenosine monophosphate (AMP), Hydrogen peroxide (H_2O_2), Xanthine (2,6-Dihydroxypurine), Tris(hydroxymethyl)aminomethane, Nitrobluc tetrazolium (NBT), 1,1-Dipheny-2-picrylhydrazyl (DPPH) radical, Thiobarbituric acid (TBA), Glucose-6-phosphate (G6P), Nicotinamide adenine dinucleotide, reduced (NADH), Nicotinamide Adenine Dinucleotide (NAD), Glycine, Catechin were obtained from Sigma Chemical Company (USA). Metaphosphoric acid was obtained from MRS (U.K.) Adrenaline, Sulphanilic acid (4-Aminobenzenesulphonic acid) was obtained from Surechem products Ltd (England). N-(1-Naphthyl) ethylenediamine dihydrochloride, Xylenol orange sodium salt, Nitro Blue Tetrazolium (NBT), Tetramethylbenzidine, Triphenylphosphine and Cadmium were obtained from Alfa Aesar, Heyshan (Lancaster). N-acetyl-β-D-glucosaminidase (NAG) assay kit was purchased from PPR diagnostics Ltd, (London). Y-Glutamyltransferase (Y-GT) and Aspanate aminotransferase (AST) assay kits were obtained from Randox Laboratories (Crumlin, U.K.). Sodium acetate, Magnesium chloride, Trichloroacetic acid (TCA), Ammonium molybdate, Ferrous sulphate, Potassium dichromate, Glacial acetic acid, Ethylenediamine tetraacetic acid (EDTA), Sodium chloride (NaCl), Ascorbic acid, Sodium hydroxide, Zinc sulphate, Hydrochloric acid, Sulphuric acid were obtained from BDH (Poole, U.K.) and Hopkins and Williams.

3.2 ANIMALS

Albino rats of the Wistar strain were obtained from the Pre-clinical animal house of the Faculty of Basic Medical Sciences of the University of Ibadan, and fed with commercially available standard pelleted feed and water *ad libitum*.

3.3 METHANOLIC EXTRACTION OF SOYBEAN

Soybean seeds were obtained from Bodija market in Ibadan. They were blended into powder and packed into the soxhlet extractor and defatted with petroleum ether (b.p. 40-60°C). The defatted soybean was then extracted in absolute methanol and the methanolic fraction evaporated in a water bath to obtain the solid extract from which stock solution was prepared and administered to rats.

3.4 COLLECTION OF BLOOD SAMPLES AND PREPARATION OF POSTMITOCHONDRIAL FRACTION (PMF) OF KIDNEY SAMPLES

Rats were sacrificed by cervical dislocation. Blood samples were collected by cardiopuncture into heparinized tubes and kidneys immediately removed, rinsed in ice-cold 1.15% KCl, bloned and weighed. The kidneys were then minced with scissors in 3 volumes of ice-cold 100 mM potassium phosphate buffer, pH 7.4 and homogenized in a teflon homogenizer. The homogenates were later centrifuged at 12,500 g for 15 minutes at 4°C and the supernatants, termed the postmitochondrial fractions (PMF), were aliquoted and used for the enzyme assays.

3.5 EXPERIMENTAL DESIGN FOR PROTECTION AGAINST GENTAMICIN–INDUCED NEPHROTOXICITY

Rats weighing between 110 and 275g were randomly divided into five groups of five animals per group. Group I (control) received normal saline (0.9% NaCl) subcutaneously (s.c). Group II received gentamicin (80 mg/kg/day, s.c). Groups III and IV received 500- and 1000- mg/kg/day of methanolic extract of soybean (MESB) orally respectively and after one hour, gentamicin (80 mg/kg, s.c.) was administered. Group V was treated with 1000 mg/kg/day of MESB only. All treatments were given for 12 days. On the 12th day after treatment, blood and kidney samples were collected for determination of renal function, histology and other biochemical assays.

3.6 EXPERIMENTAL DESIGN FOR PROTECTION AGAINST CISPLATIN–INDUCED NEPHROTOXICITY

Six groups of six rats per group weighing between 204 and 265g were studied in this experiment. Group I (control) received 0.9% NaCl; Group II was treated with CDDP (2 mg/kg/day intraperitoneally for 5 days); Groups III, IV and V were treated with 250-, 500- and 1000- mg/kg/day oral doses of MESB respectively one hour before

CDDP injection; Group VI was treated with 1 g/kg/day MESB only. All treatments were given for five days. Rats were sacrificed by cervical dislocation 24 hours after the last injection of CDDP (i.e. on the sixth day). Blood and kidney samples were collected and processed for histology and biochemical evaluation.

3.7 *IN VIVO* EVALUATION OF THE NEPHROPROTECTIVE EFFECTS OF EXTRACT FROM SOYBEAN

3.7.1 RENAL FUNCTION TEST

3.7.1.1 PLASMA CREATININE ESTIMATION

PRINCIPLE

Creatinine yields a definite colour reaction in the presence of picric acid in alkaline solution due to the formation of a red tautomer of creatinine picrate (Jaffe *et al*., 1987).

REAGENTS

1. **Saturated picric acid.**

   Picric acid was dissolved in distilled water until no more could dissolve.

2. **Sodium hydroxide (4 N).**

   This was prepared by dissolving 16 g of sodium hydroxide pellet in distilled water and the volume made up to 100 ml with same.

3. **Creatinine standard (2 mg/100ml).**

   Creatinine (2 mg) was dissolved in distilled water and the volume made up to 100 ml with same.

PROCEDURE

To already pipetted 3.5 ml of picric acid was added 0.5 ml of plasma sample. The mixture was centrifuged for 5 minutes. 3 ml of the supernatant was taken and to it was added 0.2 ml of 4N NaOH. This was incubated for 10 minutes. The absorbance of the suspension was read at 520 nm and the concentration of creatinine determined

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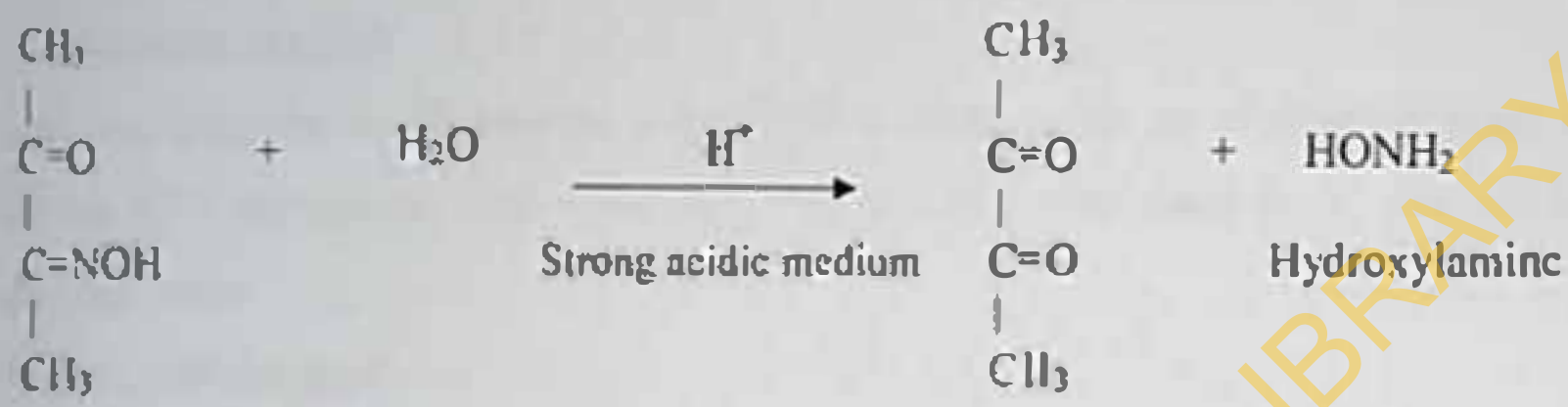
3.7.1.2 BLOOD UREA NITROGEN ESTIMATION

PRINCIPLE

THE FEARON REACTION

The principle of this reaction is based on the condensation of diacetyl with urea to form the chromogen, diazine. Since diacetyl is unstable, it is usually generated in the reaction system from diacetyl monoxime.



Diacetylmonoxime

Diacetyl

The reaction of diacetyl and urea gives diazine

Urea

Diazine

which absorbs strongly at 520 nm. Thiosemicarbazide and Fe (III) are added to the system to enhance and stabilize the colour. Urea must be used as standard for a direct method.

REAGENTS

1. Urea colour reagent

(a) Diacetylmonoxime (5 g) was dissolved in distilled water and made up to 1 litre with the same to prepare a concentration of 5 g/L diacetyl monoxime.

(b) Thiosemicarbazide (5 g) was dissolved in distilled water and made up to 1 litre with the same to obtain a 5 g/L concentration. Equal volumes of (a) and (b) were mixed to prepare the urea colour reagent.

2. Acid stock reagent

This was prepared by dissolving 5 g of $FeCl_3.6H_2O$ in 20 ml of distilled water and adding 85% phosphoric acid (100 ml). The solution was made up to 250 ml with distilled water.

3. Urea acid reagent

Concentrated sulphuric acid (200 ml) was diluted to 1 litre with distilled water plus 0.5 ml acid stock reagent.

PROCEDURE

Sample (0.1 ml) was added into a universal bottle containing 19.9 ml of distilled water and the suspension was shaken very well. 1ml of the suspension was transferred into a test tube and to it was added 1ml of colour reagent (5 g/l diacetylmonoxime + 5 g/l thiosemicarbizide) followed by 1 ml of acid reagent (200 ml of conc. H_2SO_4 diluted to 1 litre with distilled water plus 0.5 ml acid stock reagent). The mixture was heated in boiling water bath for 20 minutes. It was then cooled and the absorbance read at 520 nm against blank. The concentration of urea in mg/100ml was then calculated (See Appendix).

3.7.1.3 DETERMINATION OF SODIUM (Na^+) AND POTASSIUM (K^+) CONCENTRATIONS

PRINCIPLE

FLAME EMISSION SPECTROPHOTOMETRY

Sample was diluted with a solution containing an internal standard (Lithium or Caesium) and aspirated into a prepared air flame. Na^+, K^+, Li^+ and Cs^+, when excited, emit spectra with sharp bright lines at 589, 768, 671 and 852 nm respectively.

As the thermally excited ions return to the ground state, the internal standard signal is taken as a reference against which the analytical signal (Sodium, Potassium or Lithium) is compared.

The system is standardized relative to low and high concentration of each analyte and the relation of signal to concentration is computed by an associated microprocessor upon aspiration of controls and sample into the standardized instrument. Results are prepared in mmol/l on a digital read-out devices.

PROCEDURE

Sample was diluted by adding 0.1 ml of the sample into 19.9 ml of distilled water (1 in 200 dilution) and shaken. This was then aspirated into the flame photometer and the value read after setting instrument to zero with glass distilled water and calibrated with respective standards (140 mEq/L for Na^+ and 5 mEq/L for K^+).

3.7.2 DETERMINATION OF CATALASE ACTIVITY

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

PRINCIPLE

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured colorimetrically at 570 - 610 nm. Since dichromate has no absorbency in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

REAGENTS

1. Dichromate Solution (5%)

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

2. Hydrogen peroxide (0.2M)

H_2O_2 (0.67 g) was mixed with distilled water in a 100 ml volumetric flask and the solution made up to the mark with same.

3. Dichromate/acetic acid

This reagent was prepared by mixing 5% solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3 by volume) and it is stable for about one month.

4. Phosphate buffer (0.01M, pH 7.0)

$Na_2HPO_4.12H_2O$ (3.5814 g) and 1.19 g $NaH_2PO_4.2H_2O$ dissolved in 900 ml of distilled water. The pH adjusted to 7.0 and distilled water added to make up to 1 litre.

PROCEDURE

Colorimetric determination of H_2O_2

Different amounts of H_2O_2, ranging from 10 to 100 µmoles were taken in small test tubes and 2 ml of dichromate/acetic acid was added to each. Addition of the reagent instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10 minutes in a boiling water bath changed the colour of the solution to stable green due to formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made to 3 ml and the optical density measured with a spectrophotometer at 570 nm. The concentrations of the standard were plotted against absorbance.

Test tube	*1*	*2*	*3*	*4*	*5*	*6*	*7*
H_2O_2 (ml)	0.00	0.10	0.20	0.30	0.40	0.50	0.60
Dichromate/acetic acid (ml)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Distilled water (ml)	1.00	0.90	0.80	0.70	0.60	0.50	0.40

Determination of catalase activity of samples

Sample (1 ml) was mixed with 49 ml distilled H_2O to give 1 in 50 dilution of the sample. The assay mixture contained 4 ml of H_2O_2 solution (800 µmoles) and 5 ml of phosphate buffer in a 10 ml flat bottom flask. 1ml of properly diluted enzyme preparation was rapidly mixed with the reaction mixture by a gentle swirling motion. The reaction was run at room temperature. A 1 ml portion of the reaction mixture was withdrawn and blown into 2 ml dichromate/acetic acid reagent at 60 seconds intervals. The hydrogen peroxide contents of the withdrawn sample were determined by the method described above.

3.7.3 DETERMINATION OF SUPEROXIDE DISMUTASE ACTIVITY.

The level of superoxide dismutase (SOD) activity was determined by the method of Misra and Fridovich (1972).

PRINCIPLE

The ability of superoxide dismutase to inhibit the autoxidation of adrenaline (epinephrine) at pH 10.2 makes this reaction a basis for a simple assay for this dismutase.

Superoxide ($O^{\cdot}_2$) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per $O^{\cdot}_2$ introduced increased with increasing pH (Valentino and McCormack, 1971) and also increased with increasing concentration of epinephrine. These results led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide ($O^{\cdot}_2$) radical and hence inhibitable by SOD.

REAGENTS

1. Carbonate buffer (0.05 M, pH 10.2)

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

2. Adrenaline (0.3 mM)

Adrenaline (0.0137 g) was dissolved in 200 ml distilled water and then made up to 250 ml. This solution was prepared just after the experiment.

PROCEDURE

Sample (1 ml) was diluted in 9 ml of distilled water to make a 1 in 10 dilution. An aliquot (0.2 ml) of the diluted sample was added to 2.5 ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 ml buffer, 0.3 ml of substrate (adrenaline) and 0.2 ml of water. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

3.7.4 DETERMINATION OF REDUCED GLUTATHIONE LEVEL

The method of Beutler *et al.* (1963) was followed in estimating the level of reduced glutathione (GSH).

PRINCIPLE

The reduced form of glutathione comprises in most instances the bulk of cellular nonprotein sulfhydryl groups. This method is therefore based upon the development of a relatively stable (yellow) colour when 5',5' – dithiobis - (2-nitrobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman's reagent with the reduced glutathione, 2 – nitro5-thiobenzoic acid possesses a molar absorption at 412 nm.

This method has the following advantages over the earlier modification of the nitroprusside method.

i. The precipitation process is carried out with a single easily prepared reagent. It does not require addition of solid sodium chloride or prolonged shaking.
ii. The determination may be carried out at any temperature likely to be encountered in the laboratory.
iii. The colour formed is relatively stable
iv. The reagent for colour development is stable for many weeks
v. The sensitivity of the method is so great that it may readily be adapted to a micro-procedure.

REAGENTS

1. GSH working standard

GSH (40 mg) was dissolved in 0.1M phosphate buffer, pH 7.4, and made up to 100 ml with the same.

2. Phosphate buffer (0.1 M, pH 7.4)

a. First 0.1M $Na_2HPO_4.12H_2O$ was prepared by dissolving 7.1628 g in 200 ml of distilled water.

b. 0.1M $NaH_2PO_4.2H_2O$ was prepared by dissolving 1.5603 g in 100 ml of distilled water.

Finally 0.1M phosphate buffer was prepared by adding 200 ml of (a) to 100 ml of (b) and the pH adjusted to 7.4 with drops of concentrated HCl or NaOH as the case

may be. This is stable indefinitely unless mold forms. If crystals develop during storage at 4°C, heating may dissolve these.

3. Ellman's Reagent [5', 5'-Dithiobis- (2-nitrobenzoate) DTNB]

This was prepared by dissolving 40 mg of Ellman's reagent in 0.1M Phosphate buffer and made up to 100 ml.

4. Precipitating Solution

Prepared from 1.67 g metaphosphoric acid, 0.2 g disodium or dipotassium ethylenediamine tetraacetic acid (EDTA) and 30 g NaCl per 100 ml of distilled water and stored at 4°C. It is stable for 3 weeks. The EDTA was added to prevent difficulties that might arise where water supply contains appreciable concentration of metallic ions.

CALIBRATION OF GSH STANDARD CURVE

PROCEDURE

Serial dilutions of the GSH working standard were prepared as shown in the table below.

Preparation of GSH Standard Curve

Stock GSH (ml)	*Phosphate buffer (ml)*	*Ellman's reagent (ml)*	*GSH conc (µg)*
0.025	0.475	4.5	10
0.05	0.45	4.5	20
0.10	0.40	4.5	40
0.20	0.30	4.5	80
0.30	0.20	4.5	120
0.40	0.10	4.5	160
Blank	0.5	4.5	-

To each test tube was added 4.5 ml of Ellman's reagent. GSH concentration was proportional to the absorbance at 412 nm. The readings were taken before five minutes. This is because the colour is stable for at least 5 minutes after the addition of Ellman's reagent. After 10 minutes of standing, there is frequently a loss of 1 to 2% of the colour. However, an additional delay of 5 – 15 minutes will result in only a small error. Each sample was prepared in duplicate. A graph of optical density against concentration was plotted.

Estimation of GSH level

Sample (0.2 ml) was added to 1.8 ml of distilled water and 3 ml of the precipitating solution was mixed with sample. The rate of addition was not critical. The mixture was then allowed to stand for approximately 5 minutes and then filtered. At the end of the fifth minute, 1ml of filtrate was added to 4 ml of 0.1M phosphate buffer. Finally 0.5 ml of the Ellman's reagent was added.

A blank was prepared with 4 ml of the 0.1M phosphate buffer, 1 ml of diluted precipitating solution (3 parts to 2 parts of distilled water) and 0.5 ml of the Ellman's reagent. The optical density was measured at 412 nm. GSH was proportional to the absorbance at that wavelength and the estimate was obtained from the GSH standard.

3.7.5 DETERMINATION OF GLUTATHIONE-S-TRANSFERASE ACTIVITY

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

PRINCIPLE

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

REAGENTS

1. 1-Chloro- 2, 4, - dinitrobenzene (20 mM)

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

2. Reduced Glutathione (0.1 M)

Reduced glutathione (GSH, 30.73 mg) was dissolved in 1 ml of 0.1 M phosphate buffer (pH 6.5).

3. Phosphate buffer (0.1M, pH 6.5)

This was prepared by dissolving 4.96 g of dipotassium hydrogen phosphate (K_2HPO_4) and 9.73 g of potassium dihydrogen phosphate (KH_2PO_4) in distilled water. The pH was adjusted to pH 6.5 and the volume made up to 1000 ml.

PROCEDURE

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

GLUTATHIONE-S-TRANSFERASE ASSAY MEDIUM

Reagent	*Blank*	*Test*
Reduced glutathione (0.1M)	30 µl	30 µl
CDNB (20 mM)	150 µl	150 µl
0.1 M Phosphate buffer, pH 6.5	2.82 ml	2.79 ml
PMF	-	30 µl

3.7.6 DETERMINATION OF LIPID PEROXIDATION

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Varshney and Kale (1990).

PRINCIPLE

Under acidic condition, malondialdehyde (MDA) produced from the peroxidation of membrane fatty acid and food products react with the chromogenic reagent, 2-thiobarbituric acid (TBA), to yield a pink coloured complex with maximum absorbance at 532 nm and fluorescence at 553 nm. The pink chromophore is readily extractable into organic solvents such as butanol.

REAGENTS

1. Trichloroacetic acid (TCA, 30%)

TCA (9 g) was dissolved in distilled water and made up to 30 ml with same.

2. Thiobarbituric acid (0.75%)

This was prepared by dissolving 0.225 g of thiobarbituric acid (TBA) in 0.1 M HCl and made up to 30 ml with same.

3. Tris-KCl buffer (0.15 M, pH 7.4)

KCl (1.12 g) and 2.36 g of Tris base were dissolved separately in distilled water and made up to 100 ml with same. The pH was then adjusted to 7.4.

PROCEDURE

An aliquot (0.4 ml) of the kidney PMF was mixed with 1.6 ml of Tris-KCl buffer to which 0.5 ml of 30% TCA was added. Then 0.5 ml of 0.75% TBA was added and placed in a water bath for 45 minutes at 80°C. This was then cooled in ice and centrifuged at 3000 g. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi (1982). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$.

3.7.7 DETERMINATION OF PLASMA LIPID HYDROPEROXIDE LEVELS (MODIFIED FOX-2 METHOD)

Plasma lipid hydroperoxide levels were determined spectrophotometrically according to the modified ferrous oxidation with xylenol orange (FOX-2) method (Nourooz-Zadeh *et al.*, 1994)

PRINCIPLE

The basic principle of the ferrous oxidation-xylenol orange (FOX) assay is based on the ability of hydroperoxides to oxidize ferrous ions to ferric ions selectively in dilute acid. The resulting ferric ions are complexed by xylenol orange to produce a coloured (blue-purple) complex with an extinction coefficient of $1.5 \times 10^4 M^{-1} cm^{-1}$, which can be measured spectrophotometrically. The classical FOX-2 method measured any substance that was converting Fe^{2+} to Fe^{3+}, irrespective of their chemical nature. In the modified FOX-2 method, triphenylphosphine which replaced methanol reduces plasma hydroperoxide and this modification allows measurement of plasma hydroperoxide concentration more accurately.

REAGENTS

1. Triphenylphosphine (10 mM)

This was prepared by dissolving 262.3 mg of triphenylphosphine in distilled water and made up to 100 ml with same.

2. Ammonium Ferrous Sulphate hexahydrate (2.5 mM)

Ammonium ferrous sulphate hexahydrate (98.04 mg) was dissolved in 50 ml of 250 mM H_2SO_4 and made up to 100 ml with same.

3. Sulphuric acid ((250 mM))

Concentrated H_2SO_4 (2.45 g) was dissolved in 80 ml of distilled water and made up to 100 ml with same.

4. Xylenol orange (38% w/v)

Xylenol orange (19 mg) was dissolved in methanol and made up to 50 ml.

5. Preparation of FOX-2 reagent

This was carried out according to the method of Wolff (1994) as described by Eymard and Genot (2003). Briefly, ammonium ferrous hexahydrate (2.5 mM) was dissolved in H_2SO_4 (250 mM). An aliquot (25 ml) of this solution was then mixed with 19 mg of xylenol orange previously dissolved in 50 ml of methanol. The volume of the mixture

was finally made up to 250 ml with methanol. Concentrations of xylenol orange, ammonium ferrous sulphate hexahydrate and H_2SO_4 were 100 µM, 0.25 mM and 25 mM, respectively. FOX-2 reagent was prepared just before use and can be stored for 24 hour at -20°C.

PROCEDURE

Triphenylphosphine (10 µl, 10 mM) was added to 90 µl of serum in a test tube and immediately mixed by gently shaking the test tube. Finally, 900 µl of FOX-2 reagent (xylenol orange reagent) was added. Each tube was incubated at room temperature for 30 minutes, at which time colour development was virtually complete. The absorbance was read at 560 nm after removal of any flocculated material by centrifugation. The signal was read against a H_2O_2 standard curve.

3.7.8 DETERMINATION OF URINARY N-ACETYL-β-D-GLUCOSAMINIDASE ACTIVITY

N-acetyl-β-D-glucosaminidase (NAG) activity was estimated in the urine of experimental rats using PPR diagnostic kit (PPR diagnostics Ltd. London).

PRINCIPLE

2-methoxy-4-(2'-nitrovinyl)-phenyl-2-deoxy-β-D-glucopyranoside (MNP-GlcNAc) is hydrolysed by N-acetyl-β-D-glucosaminidase (NAG) with the release of 2-methoxy-4-(2'-nitrovinyl)phenol, which on the addition of an alkaline buffer produces a colour which can be measured colorimetrically at 505 nm. The intensity of the colour produced is proportional to the concentration of the enzyme and can be calculated by comparison with the calibrating solution provided.

PROTOCOL TABLE

Test tube	Reagent blank	Calibrant	Sample
Distilled H_2O	50 µl	-	-
NAG Calibrant	-	50 µl	-
Urine sample	-	-	50 µl
Solution 1	750 µl	750 µl	750 µl

The above mixture was incubated for 30 minutes, followed by the addition of

Solution 2	750 µl	750 µl	750 µl

Test tube content was mixed and absorbance (OD) read at 505 nm after 10 minutes using water as blank.

3.7.9 DETERMINATION OF MYELOPEROXIDASE ACTIVITY

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

PRINCIPLE

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

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

(tetramethylbenzidine) (oxidized tetramethylbenzidine)

REAGENTS

1. NaH_2PO_4 buffer (43 mM, pH 5.4)

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

2. Tetramethylbenzidine (1.2 mM)

Tetramethylbenzidine (288.42 mg) was dissolved in distilled water and made up to 100 ml mark with same in a standard volumetric flask.

3. H_2O_2 (100 mM)

H_2O_2 (3.4 mg) was dissolved in distilled water and made up to 100 ml mark with same in a standard volumetric flask.

PROCEDURE

An aliquot (0.2 ml) of the post-mitochondrial supernatant of the kidney homogenate was allowed to react with a solution of tetramethylbenzidine (1.2 mM) and 100 mM H_2O_2 in 43 mM NaH_2PO_4 (pH 5.4) in a final volume of 3 ml. Absorbance kinetics was monitored spectrophotometrically at 450 nm. MPO activity was defined as the quantity of enzyme degrading 1 µmol peroxide min^{-1} and was expressed in units per mg protein.

3.7.10 DETERMINATION OF XANTHINE DEHYDROGENASE AND XANTHINE OXIDASE ACTIVITY

Xanthine oxidase activity was measured using the spectrophotometric technique of Parks *et al.* (1988) as described by Prichard *et al.* (1991).

PRINCIPLE

The assay is based on the spectrophotometric measurement of uric acid production. The reaction which includes xanthine, EDTA and potassium phosphate buffer leads to the measurements of combined xanthine dehydrogenase (XDH) plus xanthine oxidase (XO) activity. With the addition of nicotinamide adenine nucleotide, conversion of XDH to XO is facilitated, allowing measurement of XO activity alone. The formation of uric acid from xanthine by XO results in an increase in absorbency at 295 nm. XDH activity was obtained by subtracting XO activity from combined XDH plus XO activity.

REAGENTS

1. Xanthine (50 µM)

Xanthine (0.761 mg) was dissolved in distilled water and made up to 100 ml with same.

2. EDTA (100 µM)

This was prepared by dissolving 2.923 mg of EDTA in distilled water and made up to 100 ml with same.

3. Potassium Phosphate Buffer (50 mM, pH 7.8)

Potassium dihydrogen phosphate (KH_2PO_4, 4.865 g) and dipotassium hydrogen phosphate (K_2HPO_4, 2.58 g) in 500 ml of distilled water and made up to 1000 ml with same.

4. Nicotinamide Adenine Dinucleotide (500 µM)

This was prepared by dissolving 35.47 mg of NAD in distilled water and made up to 100 ml with same.

PROCEDURE

Xanthine (50 µM) was added to potassium phosphate buffer (50 mM, pH 7.8) containing 100 µM of EDTA. The reaction was initiated by adding an aliquot (0.2 ml) of enzyme preparation (final volume of reaction mixture was 3.0 ml), leading to the measurements of combined xanthine dehydrogenase plus xanthine oxidase activity. With the addition of nicotinamide adenine dinucleotide (500 µM), xanthine oxidase activity was measured. Xanthine dehydrogenase activity was calculated by subtracting xanthine oxidase activity from the combined enzyme activity (XDH + XO). Production of uric acid was determined at 295 nm over a 5 minutes period at 25°C (by monitoring the increase in absorbance). Blank was prepared by replacing the enzyme solution with 0.2 ml of distilled water.

3.7.11 DETERMINATION OF NITRATE/NITRITE (NITRIC OXIDE) LEVEL

Serum nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as index of nitric oxide (NO) production. Quantitation was based on the Griess reaction (Cortas and Wakid, 1990) as described by Navarro-Gonzalvez *et al.* (1998).

PRINCIPLE

As NO rapidly recombines into its stable oxidative metabolites (NO_3^- and NO_2^-) in aqueous solution (Palmer *et al.*, 1987), serum concentrations of NO_3^- and NO_2^- were estimated as an index of $NO^\cdot$ production. The $NO^\cdot$ radical plays an important role as a physiological messenger (Moncada *et al.*, 1991). NO is formed from L-arginine (Palmer *et al.*, 1988) by NO synthase, which exists in several isoforms (Griffith and Stuehr, 1995). Constitutive calcium-dependent isoforms (cNOS) modulate the control of vascular tone in endothelial cells or the neurotransmission in neurons, whereas inducible calcium-independent isoforms (iNOS) are located in macrophages, chondrocytes and hepatocytes and are induced by cytokines and endotoxin (Bredt and Snyder, 1994; Nathan, 1992). Pathological conditions associated with increased release of cytokines and endotoxin, e.g. inflammation or sepsis (Curzen *et al.*, 1994) can therefore increase NO production.

Upon coming into the blood stream, nitrite reacts immediately with ox)haemoglobin to form methaemoglobin. Consequently, most NO produced is detected in serum as the remaining product, nitrate (Wennmalm *et al.*, 1993). The method is based on the reduction of nitrate to nitrite by cadmium, and the nitrite produced was determined by Griess reaction at 545 nm. This involves the formation of a chromophore during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride (Moshage *et al.*, 1995; Green *et al.*, 1982; Cortas and Wakid, 1990).

REAGENTS

1. Zinc Sulphate (75 mmol/L)

This was prepared by dissolving 2.157 g of zinc sulphate ($ZnSO_4$) in distilled water and made up to 100 ml with same.

2. Sodium Hydroxide (55 mmol/L)

This was prepared by dissolving 220 mg of sodium hydroxide (NaOH) in distilled water and made up to 100 ml with same.

3. Glycine buffer (45 g/L, pH 9.7)

Glycine (4.5 g) was dissolved in distilled in about 90 ml water. The pH was adjusted to 9.7 with drops of HCl or NaOH and then made up to 100 ml with same.

4. Glycine – NaOH buffer (15 g/L, pH 9.7)

This was prepared by dissolving 1.5 g of glycine and 1.5 g of NaOH in distilled water. The pH was adjusted to 9.7 with HCl or NaOH and then made up to 100 ml with same.

5. Copper Sulphate (5 mmol/L)

Copper (II) sulphate ($CuSO_4.5H_2O$, 124.84 mg) was dissolved in glycine - NaOH buffer (15 g/L, pH 9.7) and made up to 100 ml with same.

6. Hydrochloric acid (3 mol/L)

This was prepared by dissolving 11.0 g of concentrated hydrochloric acid HCl (Density 1.18) in distilled water and made up to 100 ml with same.

7. Reagent 1

Reagent 1 was prepared by dissolving 50 mg of N-naphthylethylenediamine in about 200 ml of distilled water and made up to 250 ml with same. This is stable for at least a year at 4°C.

8. Reagent 2

Reagent 2 was prepared by dissolving 5 g of sulfanilic acid (4-aminobenzenesulphonic acid, $NH_2C_6H_4SO_3H$) in about 400 ml of 3 mol/L HCl and made up to 500 ml with same. This solution is stable for at least a year at 4°C.

9. Sulphuric acid (100 mmol/L)

Concentrated Sulphuric acid (H_2SO_4) (980 mg, Density 1.36) was dissolved in distilled water and made up to 100 ml with same.

10. Sodium nitrite (20 mmol/L)

Sodium nitrite ($NaNO_2$) (13.8 mg) was dissolved in distilled water and made up to 100 ml with same.

PROCEDURE

Serum (300 µL) was deproteinized by adding 250 µL of 75 mmol/L $ZnSO_4$ solution, stirring and centrifuging at 10,000 g for at least 1 minute at room temperature, after which 350 µL of 55 mmol/L NaOH was added. Again, the solution was stirred and centrifuged at 10,000 g for 3 minutes and the supernatant was recovered (the supernatant must be free of turbidity for measuring nitrate concentrations). Then, 750 µL of supernatant was diluted with 250 µL of glycine buffer (45 g/L, pH 9.7).

Cadmium granules (2 - 2.5 g) were rinsed three times with distilled water and swirled in a 5 mmol/L $CuSO_4$ solution in glycine – NaOH buffer (15 g/L, pH 9.7) for 5 minutes. The copper-coated granules were rinsed and stored in 100 mmol/L H_2SO_4 solution. They were regenerated by repeating these steps.

Freshly activated cadmium granules (2 - 2.5 g) were added to 1 ml of pre-treated deproteinized serum. After continuous stirring for 10 minutes, the samples were transferred to appropriately labelled test tubes for nitrite determination.

Finally, 20 µL of sample was mixed with 75 µL of reagent 1 and 80 µL of reagent 2. Final concentrations were 1.47 mmol/L (0.43 g/L) N-naphthylethylenediamine, 26.4 mmol/L (4.57 g/L) sulfanilic acid, and 1.37 mmol/L HCl. The reaction mixture was incubated at room temperature and absorbance read after 10 minutes at 545 nm.

CALIBRATION CURVE

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

3.7.12 DETERMINATION OF RENAL GLUCOSE-6-PHOSPHATASE ACTIVITY

This was carried out according to the method of Swanson (1950).

PRINCIPLE

Glucose-6-phosphatase, a multifunctional enzyme acts as a phosphohydrolase and phosphotransferase. The mechanism of reaction involves the formation of covalently bound enzyme-inorganic phosphate (Pi) intermediate, which reacts with a variety of Pi acceptors such as water (Phosphohydrolase function) and glucose (Phosphotransferase function). The inorganic phosphate liberated is complexed with ammonium molybdate, which is reduced by iron (II) sulphate to give a blue coloured product. The inorganic phosphate released is measured colorimetrically at 700 nm.

REAGENTS

1. Sodium acetate buffer (1.0 M, pH 5.8)

(a) Acetic acid (0.1 M) was prepared by dissolving 6.0 g acetic acid in distilled water and the volume made up to 100 ml with the same.

(b) Sodium acetate (0.1 M) was prepared by dissolving 8.2 g of sodium acetate (anhydrous) in distilled water and made up to 100 ml.

4.8 ml of solution (a) was mixed with 45.2 ml of solution (b) and the solution was adjusted with 0.1 M of NaOH or HCl solution.

2. Trichloroacetic acid (10% w/v)

Trichloroacetic acid (10 g) was dissolved in 100 ml of distilled water and stored at 4°C.

3. Sulphuric acid (1 N)

Concentrated H_2SO_4 (18 ml) was diluted to 108 ml with distilled water to make 6 N H_2SO_4. 16.7 ml of this solution was diluted to 100 ml with distilled water.

4. Ammonium molybdate (1.6% w/v) in 1 N H_2SO_4

Ammonium molybdate (1.6 g) was dissolved in 1 N H_2SO_4 acid solution and made up to 100 ml with same.

5. Sulphuric acid (0.15 M)

H_2SO_4 (2.5 ml, 6N) was dissolved in distilled water and made up to 100 ml with same.

6. Ferrous Sulphate (10% w/v) in 0.15 N H_2SO_4

Ferrous sulphate ($FeSO_4$, 2.5 g) was dissolved in 25 ml of 0.15N H_2SO_4 (This solution was prepared fresh each day).

7. Glucose-6-Phosphate (0.4 M, pH 5.75)

Glucose-6-phosphate (56.41 mg) was dissolved in 5 ml of distilled water and thoroughly shaken.

8. Stock standard solution (30 mmol/L)

Anbydrous potassium dihydrogen phosphate (4.083 g) was dissolved and made up to 1 litre with distilled water containing 2 ml concentrated sulphuric acid.

9. Working Standard (0.12 mmol/L)

This was prepared by diluting stock solution 1 to 250 with distilled water (1 ml = 4 µg phosphorus).

PROCEDURE

Glucose-6-phosphate (0.15 ml, 0.04 M) was incubated with 0.2 ml of 1.0 M sodium acetate buffer (pH 5.8) and 0.15 ml sample and 0.5 ml distilled water (final volume 1.0 ml) for 30 minutes at 37°C. The reaction was terminated by adding 0.5 ml TCA. Protein precipitate was removed by centrifugation. 0.5 ml of supernatant was added to 5.0 ml of ammonium molybdate, then 0.2 ml of sodium acetate and 0.8 ml of ferrous sulphate solution. The optical density was measured at 700 nm after shaking.

Glucose-6-Phosphatase assay Medium

	Blank	*Test*	*Standard*
G-6-P substrate	0.15 ml	0.15 ml	-
Sodium acetate	0.20 ml	0.20 ml	0.20 ml
Enzyme	0.15 ml	0.15 ml	0.15 ml
Product	-	-	0.15 ml
Total	0.5 ml	0.5 ml	0.5 ml

Substrate was added to the blank after stopping the reaction and product to the standard after stopping the reaction.

3.7.13 DETERMINATION OF RENAL 5'-NUCLEOTIDASE ACTIVITY

This was done following the method described by George *et al.* (1982).

PRINCIPLE

5'-Nucleotidase, a suitable marker enzyme for plasma membranes, cleaves phosphate group (PO_4^{3-}) from purine nucleotides adenosine monophosphate (AMP) and guanosine monophosphate (GMP) respectively. The inorganic phosphate (Pi) released was measured colorimetrically. The Pi is complexed with ammonium molybdate, which is reduced by iron (II) sulphate to give a blue coloured product. Results are expressed in micromoles of Pi released per minute per mg of protein.

REAGENTS

1. Magnesium Chloride (10 mM)

$MgCl_2$ (0.095 g) was dissolved in 50 ml distilled water and this solution was made up to 100 ml with same.

2. Adenosine Monophosphate (AMP, 10 mM)

AMP (0.0694 g) was dissolved in 10 ml of distilled water and then made up to 20 ml level with distilled water. The solution was put in hot water and shaken to dissolve.

3. Tris buffer (5 mM)

Tris (0.788 g) and potassium chloride (1.2 g) were dissolved in distilled water. The pH was adjusted to 7.6 with HCl of NaOH and made up to 1 litre with the same.

4. Sulphuric acid (1 N)

Concentrated H_2SO_4 (18 ml) was diluted to 108 ml with distilled water to make 6 N H_2SO_4. 16.7 ml of this solution was then diluted to 100 ml with distilled water.

5. Ammonium molybdate (1.6% w/v) in 1 N H_2SO_4

Ammonium molybdate (1.6 g) was dissolved in 1 N H_2SO_4 acid solution and made up to 100 ml.

6. Sulphuric acid (0.15 N)

H_2SO_4 (2.5 ml, 6N) was dissolved in distilled water and made up to100 ml.

7. Ferrous Sulphate (10% w/v) in 0.15 N H_2SO_4

Ferrous Sulphate ($FeSO_4$, 2.5 g) was dissolved in 25 ml of 0.15 N H_2SO_4. This solution must be prepared fresh each day.

8. Trichloroacetic acid (10% w/v)

Trichloroacetic acid (10 g) was dissolved in distilled water and made up to 100 ml and stored at 4°C.

9. Stock Standard Solution (30 mmol/L)

Anhydrous potassium dihydrogen phosphate (4.083 g) was dissolved and made up to 1 litre with distilled water containing 2 ml concentrated sulphuric acid.

10. Working Standard (0.12 mmol/L)

This was made by diluting stock solution 1 to 250 with distilled water (1 ml = 4 µg phosphorus).

PROCEDURE

AMP (0.15 ml, 10 mM) was incubated with 0.1 ml of 5 mM Tris KCl (pH 7.6) followed by 0.1 ml of 10 mM $MgCl_2$ and 0.15 ml aliquot of Kidney homogenate (PMF) at 37°C for 20 minutes. The reaction was stopped by addition of 0.5 ml 10% ($^{w}/_{v}$) trichloroacetic acid and the protein precipitate was removed by centrifugation. 0.5 ml of supernatant was added to 5.0 ml of ammonium molybdate, then 0.2 ml of sodium acetate and 0.8 ml of ferrous sulphate solution. The optical density was measured at 700 nm after shaking.

5' – Nucleotidase assay medium

	Blank	Test	Standard
AMP Substrate	0.15 ml	0.15 ml	-
$MgCl_2$	0.10 ml	0.10 ml	0.10 ml
Tris buffer	0.10 ml	0.10 ml	0.10 ml
Enzyme	0.15 ml	0.15 ml	0.15 ml
Product	-	-	0.15 ml
Total	0.50 ml	0.50 ml	0.50 ml

Substrate was added to blank after stopping the reaction and product was added to the standard after stopping the reaction.

3.7.14 DETERMINATION OF ASPARTATE AMINOTRANSFERASE ACTIVITY

Aspartate aminotransferase (AST) activity was determined following the principle described by Reitman and Frankel (1957).

PRINCIPLE

α-Oxoglutarate + L- aspartate $\xrightarrow{\text{AST}}$ L - glutamate + Oxaloacetate

Aspartate aminotransferase was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine.

REAGENTS

1. Buffer	
Phosphate buffer	100 mmol/L, pH 7.4
L-aspartate	100 mmol/L
α-oxoglutarate	2 mmol/L
2. 2,4-dinitrophenylhydrazine	2 mmol/L
3. Sodium hydroxide	0.4 mol/L

PROCEDURE

Briefly, 0.1 ml of diluted sample was mixed with phosphate buffer (100 mmol/L, pH 7.4), L-aspartate (100 mmol/L), and α-oxoglutarate (2 mmol/L) and the mixture incubated for exactly 30 minutes at 37°C. 0.5 ml of 2,4-dinitrophenylhydrazine (2 mmol/L) was added to the reaction mixture and allowed to stand for exactly 20 minutes at 25°C. Then 5.0 ml of NaOH (0.4 mol/L) was added and the absorbance read against the reagent blank after 5 minutes at 546 nm.

AST ASSAY MEDIUM

Wavelength:	546 nm (530 – 550 nm)
Cuvette:	1 cm light path
Incubation Temperature:	37°C

MEASUREMENT AGAINST REAGENT BLANK

The following were pipetted into test tubes:

	Reagent Blank	sample
Sample	-	0.1 ml
Solution 1	0.5 ml	0.1 ml
Distilled water	0.1 ml	-

The above was mixed and incubated for exactly 30 minutes at 37°C

Solution 2	0.5 ml	0.5 ml

The reaction mixture was mixed on addition of solution 2 and allowed to stand for exactly 20 minutes at 20 to 25°C

Sodium Hydroxide	5.0 ml	5.0 ml

The reaction mixture was mixed thoroughly on addition of sodium hydroxide and absorbance of samples read against the reagent blank after 5 minutes.

CALIBRATION OF AST STANDARD CURVE

Absorbance	U/l	Absorbance	U/l
0.02	7	0.10	36
0.03	10	0.11	41
0.04	13	0.12	47
0.05	16	0.13	52
0.06	19	0.14	59
0.07	23	0.15	67
0.08	27	0.16	76
0.09	31	0.17	89

3.7.15 DETERMINATION OF γ-GLUTAMYLTRANSFERASE ACTIVITY

γ-Glutamyltransferase (γ-GT) activity was determined following the principle described by Szasz (1969).

PRINCIPLE

L-γ-glutamyl-*p*-nitroanilide + glycylglycine $\xrightarrow{\gamma\text{-GT}}$ L-γ-glutamylglycylglycine + *p*-nitroaniline

REAGENT

Contents	Concentrations in the Test
Substrate	
Glycylglycine	71.5 mmol/l. pH 8.25
L- γ-glutamyl – p- nitroanilide	126 mmol/l
surfactants	4 mmol/l

PROCEDURE

Briefly, 0.2 ml of diluted sample was mixed with 71.5 mmol/l Tris buffer (pH 8.25), 126 mmol/l glycylglycine and 4 mmol/l L-γ-glutamyl-*p*-nitroanilide (final concentrations) at 25°C. The initial absorbance was read and timer started simultaneously. The absorbance was read again after 1, 2 and 3 minutes at 405 nm.

γ-GT ASSAY MEDIUM

Wavelength:	405 nm (400–420)	
Cuvette:	1 cm light path	
Temperature:	37°C	
Measurement:	Against air	
Pipette into cuvette:	Macro	Semi-micro
Sample	0.20 ml	0.10 ml
Reagent (37°C)	2.0 ml	1.0 ml

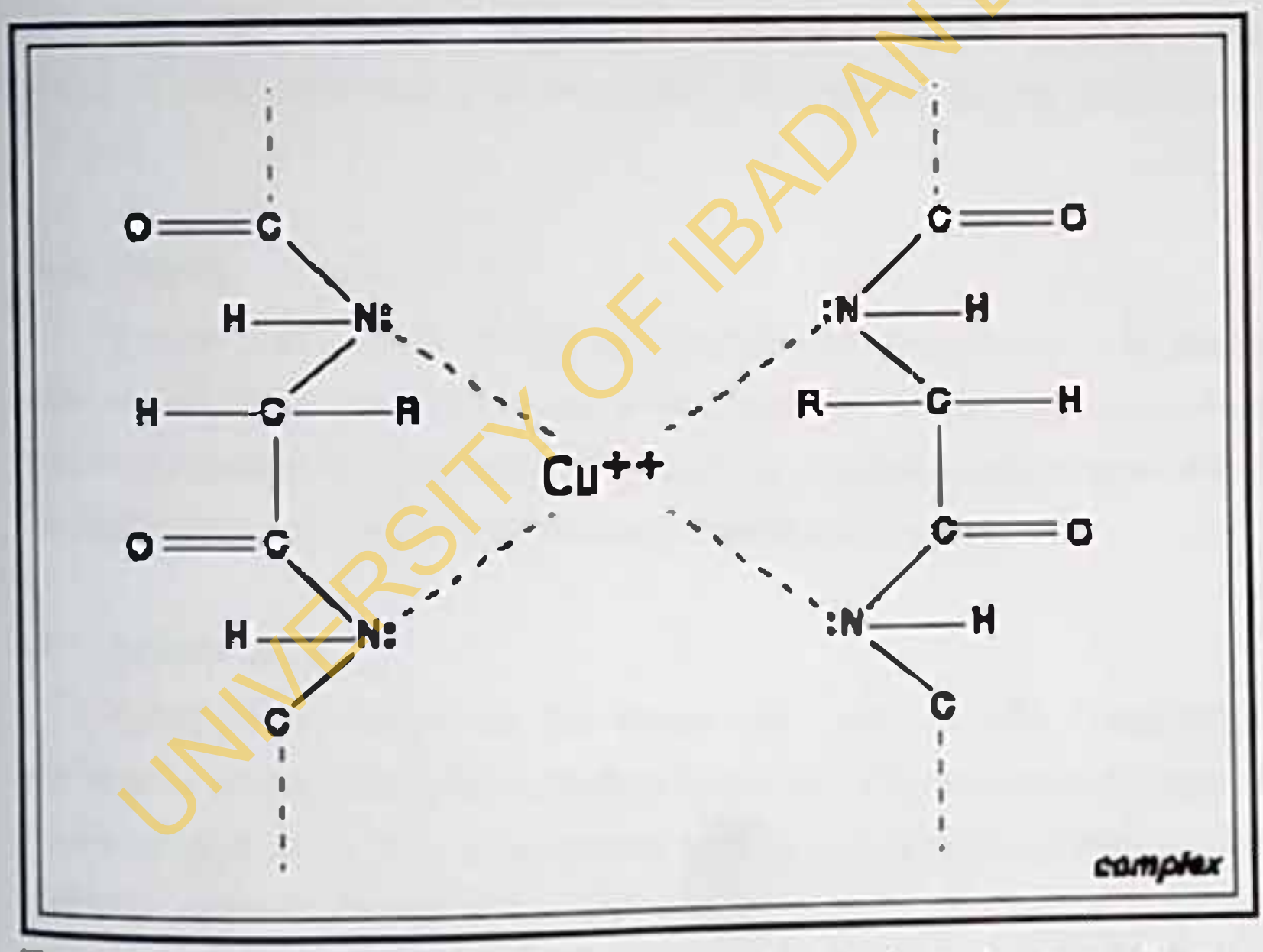
Sample was mixed with the reagent. Immediately, the initial absorbance was read and the timer started simultaneously. Absorbance was read again after 1, 2 and 3 minutes.

3.7.16 PROTEIN DETERMINATION

The principle was based on Biuret reaction as described by Gornall *et al.* (1949).

PRINCIPLE

The Biuret reaction involves a reagent containing copper (cupric) ions in alkaline solution. Molecules containing 2 or more peptide bonds associate with the cupric ions to form a coordination complex that imparts a purple colour to the solution with λmax = 540 nm. The purple colour of the complex can be measured independently of the blue colour of the reagent itself with a spectrophotometer or colorimeter.



The Tetravalent Coordination Complex Formed by Proteins in the Presence of Biuret Reagent

REAGENTS

1. Normal Saline (0.9% w/v NaCl)

NaCl (2.7 g) was dissolved in distilled water and made up to 300 ml with the same. This was stored at 4°C.

2. Sodium Hydroxide (0.2 M)

NaOH (8.0 g) dissolved in distilled water and the solution made up to a litre.

3. Stock Bovine Serum Albumin (BSA, standard)

BSA (7.4 mg) dissolved in 0.9% NaCl and made up to 100 ml so that the final concentration gives 7.4 mg/100 ml.

4. Biuret reagent.

$CuSO_4.5H_2O$ (3 g) and sodium potassium tartarate (5 g) were dissolved in 500 ml of 0.2M NaOH. Potassium iodide (100 ml) was added and the solution made up to a litre with 0.2M NaOH. Potassium iodide was added to the reagent to prevent precipitation of Cu^{2+} ions.

PROCEDURE

Sample (1 ml) was dissolved in 39 ml of 0.9% saline to give a 1 in 40 dilution. Biuret reagent (3 ml) was added to 2 ml of diluted sample. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read at 540 nm. The protein content of sample was calculated using BSA as standard.

3.8 HISTOLOGY

Kidneys from rats of all the groups were fixed in 10% formaldehyde, dehydrated in graded alcohol and embedded in paraffin. Fine sections were obtained, mounted on glass slides and counter-stained with hematoxylin-eosin (H&E) for light microscopic analyses. The slides were coded and examined by a histopathologist who was ignorant about the treatment groups. Renal histological damage (tubular necrosis) was assessed on a score previously described (Teixeria *et al*., 1982) as follows: zero (0) = no cell necrosis; 1= mild usually single-cell necrosis in sparse tubules; 2= moderate, more than one cell involved in sparse tubules; 3= marked tubules exhibiting total necrosis in almost every power field; 4= massive total necrosis.

3.9 *IN VITRO* ANTIOXIDANT ACTIVITIES OF METHANOLIC EXTRACT OF SOYBEAN

3.9.1 DETERMINATION OF TROLOX EQUIVALENT ANTIOXIDANT CAPACITY

This was carried out using an improved 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) decolourization assay (Re *et al.*, 1999) as described by Neerghcen *et al.* (2006).

PRINCIPLE

2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\cdot+}$) decolourization test is a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. The method is based on scavenging of the long-lived radical, $ABTS^{\cdot+}$, (generated by oxidation of ABTS with potassium persulfate) by potential antioxidants. Trolox is used as reference standard and the antioxidant properties of these substances are expressed as trolox equivalent antioxidant capacity (TEAC). The TEAC assay is also used for measurement of the total antioxidant capacity of biological matrices such as plasma and serum, such that all compounds, present in the serum or plasma that are able to scavenge $ABTS^{\cdot+}$ are detected as potential antioxidants.

REAGENTS

1. ABTS (0.5 mM)

This was prepared by dissolving 27.4 mg of ABTS (Mwt. 548.68) in 0.1 M phosphate buffer and made up to 100 ml with same.

2. Potassium Persulfate (1 mM)

Potassium persulfate ($K_2S_2O_6$) (27. 03 mg) was dissolved in 0.1 M phosphate buffer and made up to 100 ml with same.

3. Phosphate buffer (0.1M)

K_2HPO_4 (4.96 g) and 9.73 g of KH_2PO_4 were dissolved in distilled water and made up to 1000 ml with same.

4. Trolox working standard (1.0 mg/ml)

Trolox (10 mg) was dissolved in distilled water and made up to 10 ml with same.

PROCEDURE

The $ABTS^{\bullet}$ radical was generated by a reaction between ABTS (0.5 mM) and 1 mM potassium persulfate in 0.1 M phosphate buffer. To 3 ml of the $ABTS^{\bullet}$ solution, 0.5 ml of the extract was added and the decay in absorbance was followed for 6 minutes at 734 nm. Trolox was used as a reference standard and TEAC values were expressed as µmol trolox equivalent.

TROLOX CALIBRATION CURVE FOR TEAC

This was prepared by reacting 40-640 µmol of trolox with $ABTS^{\bullet}$ solution as shown in the table below:

Test tube	Trolox (µl)	Trolox (µmol)	Buffer (ml)	$ABTS^{\bullet}$ (ml)
1	-	-	0.5	3
2	10	40	0.49	3
3	20	80	0.48	3
4	40	160	0.46	3
5	80	320	0.42	3
6	160	640	0.34	3

3.9.2 DETERMINATION OF FERRIC REDUCING ANTIOXIDANT POWER

The method of determination of ferric reducing antioxidant power (FRAP) was adapted from Benzie and Strain (1996).

PRINCIPLE

The FRAP assay is a simple, automated test measuring the ferric reducing ability of compounds. This assay has been presented as a novel method for assessing 'antioxidant power' (Benzie and Strain, 1996). Ferric to ferrous ion reduction at low pH causes the formation of a coloured ferrous-tripyridyltriazine complex. FRAP values are obtained by comparing the change in absorbance. This assay is simple and inexpensive but does not measure the sulphydryl group-containing antioxidants (Cao and Prior, 1998). The antioxidant capacity of an antioxidant against a free radical does not necessarily match its ability to reduce Fe^{3+} to Fe^{2+}.

REAGENTS

1. Acetate buffer (0.25 M, pH 3.6)

This was prepared by dissolving 4.10 mg of sodium acetate and 3.0 mg of acetic acid in 100 ml of distilled water. The solution was then made up to 200 ml mark with distilled water. pH was adjusted to 3.6 ml with NaOH or HCl.

2. 2,4,6-Tripyridyltriazine (TPTZ) (10 mM)

TPTZ (624.66 mg) was dissolved in about 100 ml of 0.25 M acetate buffer and made up to 200 ml with same.

3. Ferric chloride (20 mM)

This was prepared by dissolving 1.0812 g of ferric chloride ($FeCl_3.6H_2O$) in 0.25 M acetate buffer and made up to 200 ml with same.

4. Preparation of FRAP reagent

FRAP reagent was prepared by mixing 10 mM TPTZ and 20 mM ferric chloride in 0.25M acetate buffer (pH 3.6). This reagent was prepared fresh just before the assay.

5. Ferrous sulphate solution (1 mM) (working standard)

This was prepared by dissolving 27.8 mg of $FeSO_4.7H_2O$ in distilled water and made up to 100 ml with same.

3.9.2 DETERMINATION OF FERRIC REDUCING ANTIOXIDANT POWER

The method of determination of ferric reducing antioxidant power (FRAP) was adapted from Benzie and Strain (1996).

PRINCIPLE

The FRAP assay is a simple, automated test measuring the ferric reducing ability of compounds. This assay has been presented as a novel method for assessing 'antioxidant power' (Benzie and Strain, 1996). Ferric to ferrous ion reduction at low pH causes the formation of a coloured ferrous-tripyridyltriazine complex. FRAP values are obtained by comparing the change in absorbance. This assay is simple and inexpensive but does not measure the sulphydryl group-containing antioxidants (Cao and Prior, 1998). The antioxidant capacity of an antioxidant against a free radical does not necessarily match its ability to reduce Fe^{3+} to Fe^{2+}.

REAGENTS

1. Acetate buffer (0.25 M, pH 3.6)

This was prepared by dissolving 4.10 mg of sodium acetate and 3.0 mg of acetic acid in 100 ml of distilled water. The solution was then made up to 200 ml mark with distilled water. pH was adjusted to 3.6 ml with NaOH or HCl.

2. 2,4,6-Tripyridyltriazine (TPTZ) (10 mM)

TPTZ (624.66 mg) was dissolved in about 100 ml of 0.25 M acetate buffer and made up to 200 ml with same.

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This was prepared by dissolving 1.0812 g of ferric chloride ($FeCl_3.6H_2O$) in 0.25 M acetate buffer and made up to 200 ml with same.

4. Preparation of FRAP reagent

FRAP reagent was prepared by mixing 10 mM TPTZ and 20 mM ferric chloride in 0.25M acetate buffer (pH 3.6). This reagent was prepared fresh just before the assay.

5. Ferrous sulphate solution (1 mM) (working standard)

This was prepared by dissolving 27.8 mg of $FeSO_4.7H_2O$ in distilled water and made up to 100 ml with same.

PROCEDURE

The extract (100-300 µg) was added to 300 µl of distilled water followed by 3 ml of FRAP reagent. The absorbance was read at 593 nm after 4 minutes incubation at ambient temperature against a blank of distilled water. Results were expressed in µmol Fe(II).

Standard Curve for FRAP assay

This was prepared by mixing various concentrations of Fe (II) (100-500 µg) with FRAP reagent as carried out with the extract. ImM ferrous solution contains 5.6 x 10^{-5} Fe^{2+}/ ml. µmol Fe^{2+} was calculated using the formula; mole = mass/molar mass.

3.9.3 DETERMINATION OF HYDROXYL RADICAL SCAVENGING ACTIVITY BY INHIBITION OF DEOXYRIBOSE DEGRADATION

The hydroxyl radical (OH·) scavenging potential of the extract was determined using the deoxyribose assay (Halliwell *et al.* 1987; Aruoma, 1994a, 1994b) as described by Neerghcen *et al.* (2006).

PRINCIPLE

The deoxyribose assay allows determination of the rate constant for reaction between antioxidants and hydroxyl radicals. This assay has also been adapted to assess pro-oxidant actions.

In the 'deoxyribose assay', scavenging activity can be assessed by measuring competition between the test compound with deoxyribose for hydroxyl radicals (Halliwell *et al.*, 1987). OH· radicals are generated in a reaction mixture containg ascorbate, H_2O_2 and Fe^{3+} - EDTA at pH 7.4 (equations 1 and 2). The ascorbic acid greatly increases the rate of OH· radicals generation by reducing iron and maintaining a supply of Fe^{2+} - EDTA (equation 1). However, artefacts may occur with this test as some substances can rapidly react with hydrogen peroxide. Also powerful iron-chelators cannot be used in this assay as the compounds interfere with the measurement products.

$$Fe^{3+}\text{--EDTA} + \text{ascorbate} \longrightarrow Fe^{2+}\text{—EDTA} + \text{oxidised ascorbate} \quad \text{—} \quad (1)$$

$$Fe^{2+}\text{—EDTA} + H_2O_2 \longrightarrow Fe^{3+}\text{—EDTA} + OH^{\cdot} + OH^{-} \quad \text{—} \quad (2)$$

REAGENTS

1. KH_2PO_4 – KOH (100 mM)

This was prepared by dissolving 1.361 g of KH_2PO_4 (Mwt. 136.09) and 5.6 mg of KOH in distilled water. The solution was then made up to 100 ml with distilled water.

2. Deoxyribose (15 mM)

Deoxyribose (201.2 mg) was dissolved in distilled water and made up to 100 ml with same.

3. $FeCl_3$ (500 µM)

$FeCl_3$ (13.5 mg) was dissolved in distilled water and made up to 100 ml with same.

4. EDTA (1 mM)

EDTA (292.3 mg) was dissolved in distilled water and made up to 100 ml with same.

5. Thiobarbituric acid (1% w/v)

TBA (1.0 g) was dissolved in 0.1 M HCl and made up to 100 ml with same.

6. Trichloroacetic acid (TCA) (2.8% w/v)

TCA (2.8 g) was dissolved in distilled water and made up to 100 ml with same.

7. Hydrogen peroxide (10 mM)

This was prepared by dissolving 34.01 mg of H_2O_2 solution in distilled water and made up to 100 ml with same.

PROCEDURE

Sample (200-1000 µg) in 100 µl of distilled water was added to a solution containing 200 µl KH_2PO_4 – KOH (100 mM), 200 µl deoxyribose (15 mM), 200 µl $FeCl_3$ (500 µM) and 100 µl EDTA (1 mM) in a test tube and allowed to mix. Then, 100 µl H_2O_2 (10 mM) and 100 µl ascorbic acid (1 mM) to initiate the reaction. The reaction mixture was incubated at 37°C for 1 hour. At the end of the incubation period, 1 ml of 1% w/v TBA was added to each mixture followed by the addition of 1 ml of 2.8% w/v TCA. The solution was heated in a water bath at 80°C for 20 minutes to develop the pink coloured MDA-$(TBA)_2$ adduct. After cooling, the solution was centrifuged and the absorbance of the supernatant measured at 532 nm against distilled water as blank. Results were expressed as the percentage inhibition of deoxyribose degradation.

3.9.4 DETERMINATION OF 1,1-DIPHENYL-2-PICRYLHYDRAZYL RADICAL SCAVENGING ACTIVITY

The effect of the methanolic extract of soybean (MESB) on 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical was estimated according to the method of Hatano *et al.* (1988).

PRINCIPLE

The relatively stable 1,1-diphenyl-2-picrylhydrazyl (DPPH·) free radical is used for antioxidant activity measurement of lipid soluble compounds. It is known that a freshly prepared DPPH· solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades/disappears when an antioxidant molecule can quench DPPH· (by providing hydrogen atoms or by electron donation, conceivably via a free radical attack on the DPPH· molecule) and convert them to a bleached product (i.e. 1,1-diphenyl-2-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance (Yamaguchi *et al.*, 1998). The disappearance of the radical can be followed spectrophotometrically and expressed as radical scavenging ability (Bondet *et al.*, 1997). Interpretation of the assay could be complicated if the absorption spectra of the test compounds overlap with the DPPH spectrum (517 nm) as carotenoids (Noruma *et al.*, 1997).

REAGENT

1. DPPH (1 mM)

DPPH (11.83 mg) was dissolved in methanol and made up to 30 ml with same.

PROCEDURE

MESB (25-500 µg) in 4 ml of distilled water was added to a methanolic solution of DPPH (1 mM, 1 ml). The mixture was shaken and left to stand at room temperature for 30 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. Catechin (50 µg) was used as standard. The radical scavenging activity (RSA) was calculated as percentage of DPPH· discolouration using the equation:

3.9.5 DETERMINATION OF INHIBITORY ACTIVITY AGAINST 2,2'-AZOBIS (2-AMIDINOPROPANE) HYDROCHLORIDE - INDUCED LIPID PEROXIDATION

This was carried out according to the method described by Neergheen *et al.* (2006).

PRINCIPLE

Since 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) is water-soluble and the rate of free radical generation from AAPH can be easily controlled and measured, it has been extensively used as a free radical initiator for biological studies. Thermal decomposition of AAPH at physiological temperature generates alkyl radicals which can react with oxygen and give alkylperoxyl radicals ($ROO^{\cdot}$). Alkylperoxyl radicals ($ROO^{\cdot}$) then attack polyunsaturated lipids initiating peroxidation (Equations 1 - 3). Lipid peroxidation products are measured by the TBARS method described by Neergheen *et al.* (2006).

$$AAPH \longrightarrow 2R^{\cdot} + N_2 \quad (1)$$

$$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot} + N_2 \quad (2)$$

$$ROO + L\text{-}H \longrightarrow L^{\cdot} + ROOH \quad (3)$$

REAGENTS

1. Phosphate buffer (0.1 M, pH 7.5)

This was prepared by dissolving 4.96 g of K_2HPO_4 and 9.73 g of KH_2PO_4 in 900 ml of distilled water and made up to 1000 ml with same. The pH was adjusted with concentrated HCl or NaOH to 7.5.

2. 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) (20 mM)

This was prepared by dissolving 54.24 mg of AAPH, Mwt. 271.20 in distilled water and made up to 10 ml with same.

3. TCA-TBA-HCl Stock

This was prepared by dissolving 15.0 g of TCA and 0.375 g of TBA in 0.25 N HCl and solution made up to 100 ml with same.

4. HCl (0.25 N)

The solution was prepared by dissolving 0.9125 g of concentrated HCl (density 1.18) in distilled water and made up to 100 ml with same.

PROCEDURE

Post-mitochondrial fraction (200 µl) of liver homogenate was diluted in 0.1 M potassium phosphate buffer, pH 7.5 (1 in 10 dilution). Then, 400 µl of extract (100-1000 µg) was added followed by 200 µl of AAPH (20 mM) to initiate peroxidation. The mixture was incubated at 37°C for 1 hour and the solution gently shaken at 10 minutes interval. After incubation, 1.6 ml TCA-TBA-HCl stock solution (15% w/v TCA, 0.375% w/v TBA, 0.25 N HCl) was added. The solution was heated in a boiling water bath for 15 minutes. After cooling, the precipitate was removed by centrifugation and the absorbance of the resulting supernatant measured at 532 nm. Results were expressed as percentage inhibition of peroxidation with catechin used as standard.

3.9.6 DETERMINATION OF INHIBITORY ACTIVITY AGAINST Fe^{2+}/ASCORBATE – INDUCED LIPID PEROXIDATION

This was carried out according to the method of Biyashee and Balasubramanian (1971) and thiobarbituric acid reactive substance (TBARS) estimated as described by Varshney and Kale (1990).

PRINCIPLE

It has been established that liver homogenate contains large quantity of polyunsaturated fatty acids (PUFA), which are susceptible to lipid peroxidation by free radicals and oxidants, usually by hydrogen abstraction. The Fe^{2+}/ascorbate system generates OH radicals and other non-specific free radicals. Aldehydes like malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are produced as a result of lipid peroxidation. These aldehydes especially MDA are capable of reacting with thiobarbituric acid (TBA) to form pink coloured complex that can absorb light at 532 nm. The method of estimation of TBARS was as described by Varshney and Kale (1990)

REAGENTS

1. Tris buffer (30 mM, pH 7.4)

Tris (181.65 mg) was dissolved in 50 ml of distilled water and pH was adjusted to 7.4 using concentrated HCl or NaOH.

2. Ferrous ammonium sulphate (0.16 mM)

$Fe(NH_4)_2(SO_4)_2$ (3.14 mg) was dissolved in distilled water and made up to 50 ml with same.

3. Ascorbic acid (0.06 mM)

Ascorbic acid (0.55 mg) was dissolved in distilled water and made up to 50 ml with same.

4. TBA (0.75% w/v)

TBA (0.75 g) was dissolved in 0.1 M HCl and made up to 100 ml with same. Dissolution was facilitated by stirring in a boiling water bath.

5. TCA (30% w/v)

TCA (30 g) was dissolved in distilled water and made up to 100 ml with same.

PROCEDURE

Liver homogenate from rat was prepared by removing liver immediately after sacrifice. The liver was rinsed in icecold 1.15% KCl to remove blood stain, blotted and weighed. The weighed tissue was then homogenized in 4 volumes of ice-cold 0.1 M phosphate buffer, pH 7.4. The reaction mixture containing 0.1 ml of liver homogenate in 30 mM tris buffer, 0.16 mM ferrous ammonium sulphate, 0.06 mM ascorbic acid and different amount of the extract (10-1000 µg), was incubated for 1 hour at 37°C. The resulting TBARS was measured by the method of Varshney and Kale (1990). An aliquot (0.4 ml) of the reaction mixture was mixed with 1.6 ml of 0.15 M tris-KCl buffer to which 0.5 ml of 30% TCA was added. Then 0.5 ml of 0.75% TBA was added and placed in a water bath for 30 minutes at 85°C, after which it was cooled in ice and centrifuged at room temperature for 3 minutes at 3000 g. The absorbance of the clear supernatant was measured against reference blank of distilled water at 532 nm.

3.9.7 DETERMINATION OF REDUCING POWER

The reducing power of MESB was determined according to the method of Oyaizu (1986).

PRINCIPLE

Radical chain reactions could be terminated when substances exhibiting high reducing tendencies donate electrons which can react with free radicals converting them to more stable products in the process. The principle of this assay therefore was based on the reduction of Fe^{3+}/ferricyanide complex to the ferrous form, and the Fe^{2+} was monitored by measuring the formation of Perl's Prussion blue at 700 nm (Chung *et al.*, 2002).

REAGENTS

1. Phosphate buffer (0.2 M, pH 6.6)

This was prepared by dissolving 3.48 g of dipotassium hydrogen phosphate (K_2HPO_4) and 2.72 g of potassium dihydrogen phosphate, (KH_2PO_4) in distilled water and the volume made up to 100 ml. The pH was adjusted to pH 6.6 with NaOH or HCl.

2. Potassium ferricyanide (1%)

$K_3Fe(CN)_6$ (1.0 g) was dissolved in distilled water and made up to 100 ml with same.

3. Ferric chloride (0.1% w/v)

$FeCl_3$ (0.1 g) was dissolved in distilled water and made up to 100 ml with same.

4. Trichloroacetic acid (10% w/v)

TCA (10 g) was dissolved in distilled water and made up to 100 ml with same.

PROCEDURE

Varying amounts of the extract (10-800 µg) in 1ml of distilled water were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 ml) of 10% TCA was added to the mixture which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

3.9.8 DETERMINATION OF NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

The scavenging effect of MESB on nitric oxide ($NO^{\cdot}$) radical was measured according to the method of Marcocci *et al.* (1994).

PRINCIPLE

The nitric oxide ($NO^{\cdot}$) plays an important role as a physiological messenger (Moncada *et al.*, 1991). NO is a very unstable, short half-life gas that is rapidly converted into the stable products nitrate and nitrite (Palmer *et al.*, 1987). In this assay, nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with molecular oxygen to produce nitrite ions. The resulting nitrite ions produced were measured by Griess reaction at 545 nm (Marcocci *et al.*, 1994). This involves the formation of a chromophore during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride.

REAGENTS

1. Sodium nitroprusside (25 mM)

This was prepared by dissolving 372.5 mg of sodium nitroprusside, Mwt 298.0 (Hopkins & Williams) in distilled water and made up to 50 ml.

2. Griess reagent

This was prepared by dissolving 1 g of sulphanilamide (Surechem, England) in 50 ml of distilled water containing 0.1 g of naphthylethylenediamine dihydrochloride (Alfa Aesar, Lancs.) and 5.0 ml of H_3PO_4 and finally made up to 100 ml with distilled water. This brings the final concentration of sulphanilamide, naphthylethylenediamine and H_3PO_4 to 1%, 0.1% and 5% respectively.

PROCEDURE

MESB (10-400 µg) was added in the test tubes to 1ml of sodium nitroprusside solution (25 mM) and the tubes incubated at 37°C for 2 hours. An aliquot (0.5 ml) of the incubation solution was removed and diluted with 0.3 ml of Griess reagent (1% sulphanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed was immediately read at 570 nm against

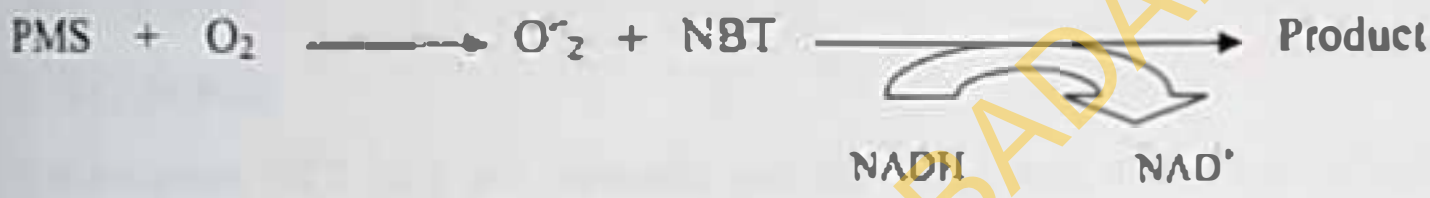
distilled water as blank with catechin (50 µg) used as standard. Results were expressed as percentage radical scavenging activity (RSA).

3.9.9 DETERMINATION OF SUPEROXIDE RADICAL SCAVENGING ACTIVITY

The effect of methanolic extract of soybean (MESB) on the superoxide radical ($O^{\cdot}_2$) production was evaluated according to the method of Nishikimi *et al.* (1972).

PRINCIPLE

In this assay, superoxide radical ($O^{\cdot}_2$) was generated in a nonenzymic system in the reaction of reduced phenazine methosulfate (PMS) and molecular oxygen. The generation of $O^{\cdot}_2$ was measured by means of spectrophotometric measurement of the product on reduction of nitro blue tetrazolium (NBT).



REAGENTS

1. Phosphate buffer (0.1 M)

This was prepared by dissolving 4.96 g of dipotassium hydrogen phosphate (K_2HPO_4) and 9.73 g of potassium dihydrogen phosphate (KH_2PO_4) in distilled water and the volume made up to 1000 ml and the pH was adjusted to 6.5.

2. Phenazine methosulfate (PMS) (60 µM)

PMS (0.54 mg) was dissolved in 0.1 M phosphate buffer and made up to 30 ml with same.

3. Nicotinamide Adenine Dinucleotide, Reduced (468 µM)

NADH (25 mg) was dissolved in 0.1M phosphate buffer (pH 7.4) and made up to 75.8 ml with same.

4. Nitroblue tetrazolium (NBT) (150 µM)

NBT (10 mg) was dissolved in 0.1 M phosphate buffer (pH 7.4) and made up to 81.3 ml with same.

PROCEDURE

Varying amount of MESB (10-200 µg) was mixed in a test tube with a solution containing 1.0 ml of 60 µM PMS and NADH (468 µM) in phosphate buffer (0.1M, pH 7.4). Finally, 1.0 ml of 150 µM NBT in phosphate buffer was added and the reaction mixture incubated at ambient temperature for 5 minutes. The colour was read at 560 nm against blank samples. Results were expressed as percentage radical scavenging activity (RSA).

3.9.10 DETERMINATION OF TOTAL PHENOLIC CONTENT OF METHANOLIC EXTRACT OF SOYBEAN

The total phenolic content of MESB was determined according to the method of Taga *et al* (1984) as described elsewhere by Yen *et al.* (2001).

REAGENTS

1. HCl (0.3%)

Concentrated HCl (0.3 ml, specific gravity 1.18) was dissolved in methanol/water (60:40 v/v) and made up to 100 ml with same.

2. Na_2CO_3 (2%)

This was prepared by dissolving 2 g of Na_2CO_3 in distilled water and made up to 100 ml with same.

3. Folin-Ciocalteau reagent (50%)

Folin-Ciocalteau reagent (10 ml) was dissolved in distilled water and made up to 20 ml with same.

4. Catechin (2 mg/ml)

This was prepared by dissolving 0.02 g of catechin in distilled water and made up to 10 ml with same. The solution was gently heated to facilitate dissolution.

PROCEDURE

0.1 g of the extract was dissolved in 5 ml of 0.3% HCl in methanol/water (60:40, v/v). The resulting solution (100 µl) was added to 2 ml of 2% Na_2CO_3. After 2 minutes, 50% Folin-Ciocalteau reagent (100 µl) was added to the mixture, which was then left for 30 minutes. Absorbance was measured at 750 nm using a

spectrophotometer. A standard curve was prepared using catechin as standard. Total phenolic content of the extract was therefore expressed as (+)-catechin equivalents.

3.10 STATISTICS

Results are expressed as mean ± standard error of mean (SEM). Differences between groups were determined by one-way analysis of variance (ANOVA) using SPSS software package for windows. Post hoc testing was performed for inter-group comparisons using the Least Significant Difference (LSD) test and p-value < 0.05 was considered significant.

3.11 PHYTOCHEMICAL ANALYSIS OF SOYBEAN

3.11.1 General Experimental Procedures

Solvents used for extraction and chromatography were redistilled before use. Adsorption column chromatography (CC) was performed with Kieselgel 60 (ASTM 70-230 mesh). Gel permeation was achieved using Sephadex LH-20. Reversed phase liquid chromatography (LC) was accomplished with RP-18 (size B, 40-63 mm, E. merck). Thin Layer Chromatography (TLC) analysis was done using analytical silica gel 60 $GF_{254+366}$ pre-coated aluminium backed plates (Merck, 0.25 mm thick). The resulting spots on TLC plates were visualized under UV light (254 nm) and detected by the use of vanillin/H_2SO_4, H_2SO_4, ferric chloride and DPPH spray reagents.

3.11.2 Antioxidant Test

3.11.2.1 TLC autographic analysis (Qualitative)

A small amount of the sample was dissolved in little amount of methanol and spotted on silica gel sheet and developed using a suitable solvent system. This was sprayed with 0.2% methanolic solution of the stable radical dipbenyl picryl hydrazyl hydrate (DPPH). Only zones where the colour turned from purple to yellow withio the first fifteen minutes after spraying were positive results established (i.e. possess antioxidant activity).

3.11.3 Phenolic Test

A methanolic solution of ferric chloride (5 g in 100 ml) was prepared. This was used to spray the developed TLC plate. Only spots or zones that turned blue-black after spraying were regarded as phenolic compounds.

3.11.4 Extraction and Solvent Partitioning of Soybean *(Glycine max)*

The dried powdered seeds (1 kg) were extracted with 3 litres of absolute methanol (MeOH) (x3) and the pooled extract was concentrated to dryness *in vacuo* on a rotary evaporator. The crude methanolic extract was coded SB. About 80 g of this was suspended in distilled water and partitioned with *n*-hexane, ethyl acetate and *n*-butanol successively which were in turn concentrated to dryness *in vacuo* on a rotary evaporator. The *n*-hexane, ethyl acetate and *n*-butanolic fractions were coded SBHF, SBEF and SBBF respectively.

3.11.5 Fractionation of SBEF on open column Chromatography

The ethyl acetate fraction SBEF (5 g) was adsorbed unto silica and allowed to dry before packing on to column. The column was wetted with *n*-hexane and gradient elution effected with the following solvent/ solvent mixtures:

Hexane	10 0%	300 ml
Hexane - ethyl acetate	(70:30)	500 ml
Hexane - ethyl acetate	(6 0:4 0)	400 ml
Hexane - ethyl acetate	(50:50)	600ml
Hexane - ethyl acetate	(40:60)	200 ml
Hexane - ethyl acetate	(20: 80)	100 ml
Ethyl acetate	100%	100 ml
Ethyl acetate – MeOH	(90:10)	200 ml
Ethyl acetate – MeOH	(80:20)	100 ml
Ethyl acetate – MeOH	(70:30)	100 ml

Fractions collected were analysed by TLC in 100% ethyl acetate, ethyl acetate: methanol (8.5:1.5 v/v) and dichloromethane: ethanol (7:3 v/v). The resulting spots on TLC plates were visualized under UV light (254 nm) and detected by the use of vanillin/H_2SO_4 and the active compound(s) were detected using DPPH spray reagents. Fractions having the same TLC patterns were bulked together, concentrated *in vacuo* to dryness and weighed resulting in seven different fractions coded SBEF1, SBEF2, SBEF3, SBEF4, SBEF5, SBEF6 and SBEF7.

3.11.6 Fractionation of SBEF5 on Sephadex LH-20

Fraction SBEF5 (450 mg) was dissolved in a minimum amount of dichloromethane-methanol (70:30 v/v) and loaded on a Sephadex LH-20 column previously equilibrated with the same solvent mixture. Elution was carried out using the following solvent mixtures:

Dichloromethane – methanol	(70:30)	400 ml
Dichloromethane – methanol	(60:40)	300 ml

Fractions collected were analysed by TLC in dichloromethane : ethanol (7:3 v/v), active compound(s) were detected using DPPH spray reagents and fractions having the same TLC patterns were bulked together, concentrated *in vacuo* to dryness and weighed resulting in four different fractions coded SBEF5a, SBEF5b, SBEF5c, SBEF5d and SBEF5e

3.11.7 Fractionation of SBBF on open column Chromatography

Fraction SBBF (5 g) was adsorbed unto silica and allowed to dry before packing on to column and fractionated on silica using the following solvent / solvent mixtures:

Hexane	100%	200 ml
Hexane - ethyl acetate	(80: 20)	200 ml
Hexane - ethyl acetate	(60:40)	200 ml
Hexane - ethyl acetate	(40:60)	200 ml
Hexane - ethyl acetate	(20:80)	400 ml
Ethyl acetate	100%	400 ml
Ethyl acetate – MeOH	(90: 10)	400 ml
Ethyl acetate – MeOH	(80:20)	800 ml
Ethyl acetate – MeOH	(70:30)	200 ml
Ethyl acetate – MeOH	(50:50)	200 ml
Ethyl acetate – MeOH	(30:70)	200 ml

Fractions collected were analysed by TLC in ethyl acetate: methanol (8.5: 1.5 v/v), active compound(s) were detected using DPPH spray reagents and fractions having the same TLC patterns were bulked together, concentrated in vacuo to dryness and weighed resulting in five different fractions coded SBBFa, SBBFb, SBBFc, SBBFd, and SBBFe.

3.11.8 Fractionation of SBBF on Reversed phase Liquid Chromatography (LC)

Fraction SBBFd (700 mg) was adsorbed unto RP-18 silica and allowed to dry before packing on to column. The column was wetted with 75% H_2O in ethanol (EtOH) and gradient elution effected with the following solvent / solvent mixtures. Elution was carried out using the following solvent mixtures:

H_2O – EtOH	(75:25)	100 ml
H_2O – EtOH	(60:40)	100 ml
H_2O – EtOH	(40:60)	100 ml
H_2O – EtOH	(25:75)	100 ml
H_2O – EtOH	(10:90)	100 ml
EtOH	100%	150 ml

Fractions collected were analysed by RP-18 TLC using the solvent system water - EtOH (7:4 v/v) and the active compound(s) were detected using DPPH spray reagents and fractions having the same TLC patterns were bulked together, concentrated *in vacuo* to dryness and weighed resulting in four different fractions coded SBBFd1, SBBFd2, SBBFd3 and SBBFd4.

3.11.9 Fractionation of SBBFd2 on Sephadex LH-20

Fraction SBBFd2 (270 mg) was dissolved in a minimum amount of dichloromethane : methanol (70:30 v/v) and loaded on a Sephadex LH-20 column previously equilibrated with the same solvent mixture. Elution was carried out using the following solvent mixtures:

Dichloromethane – methanol	(70:30)	400ml
Dichloromethane – methanol	(60:40)	300 ml

Fractions collected were analysed by TLC in dichloromethane : ethanol (7:3 v/v), active compound(s) were detected using DPPH spray reagents and fractions having the same TLC patterns were bulked together, concentrated *in vacuo* to dryness and weighed resulting in four different fractions coded SBBFd2a, SBBFd2b, SBBFd2c, SBBFd2d and SBBFd2e

3.11.10 Fractionation of SBBFb on Reversed Phase Liquid Chromatography (LC)

Fraction SBBFb (520 mg) was adsorbed unto RP-18 silica and allowed to dry before packing on to column. The column was wetted with 80% H_2O in EtOH and gradient elution effected with the following solvent / solvent mixtures. Elution was carried out using the following solvent mixtures:

H_2O – EtOH	(80:20)	100 ml
H_2O – EtOH	(60:40)	100 ml
H_2O – EtOH	(40:60)	100 ml
H_2O – EtOH	(30:70)	100 ml
H_2O – EtOH	(10:90)	100 ml
EtOH	100%	100 ml

Fractions collected were analysed by RP-18 TLC using the solvent system water : EtOH (7:4 v/v) and the active compound(s) were detected using DPPH spray reagents and fractions having the same TLC patterns were bulked together, concentrated *in vacuo* to dryness and weighed resulting in four different fractions coded SBBFb1, SBBFb2, SBBFb3 and SBBFb4.

3.11.11 Fractionation of SBBFb1 on Sephadex LH-20

Fraction SBBFb1 (210 mg) was dissolved in a minimum amount of dichloromethane : methanol (70:30 v/v) and loaded on a Sephadex LH-20 column previously equilibrated with the same solvent mixture. Elution was carried out using the following solvent mixtures:

Dichloromethane – methanol	(70:30)	300 ml
Dichloromethane – methanol	(60:40)	200 ml

Fractions collected were analysed by TLC in dichloromethane : ethanol (7:3 v/v), active compound(s) were detected using DPPH spray reagents and fractions having the same TLC patterns were bulked together, concentrated *in vacuo* to dryness and weighed resulting in four different fractions coded SBBFb2a, SBBFb2b, SBBFb2c and SBBFb2d.

CHAPTER FOUR

EXPERIMENTS AND RESULTS

4.1 EXPERIMENT 1: EFFECT OF THE METHANOLIC EXTRACTS OF SOYBEAN ON GENTAMICIN-INDUCED RENAL DYSFUNCTION AND INJURY

INTRODUCTION

Aminoglycosides have long been one of the commonest causes of drug-induced nephrotoxicity and account for nearly 10–15% of drug toxicity (Kumar *et al*., 2000). In fact, they are one of the principal causes of hospital-acquired acute renal failure. Although a clear recognition of the patient and treatment-related risk factors, combined with once-a-day schedule and effective monitoring procedures, have improved the situation over what prevailed in the early 1980s, the safety of the aminoglycosides still compares less to that of the other broad-spectrum antibiotics.

Gentamicin is an aminoglycoside antibiotic that is still commonly used in the treatment of life-threatening infections due to gram-negative organisms (Ali *et al*., 2005). However, about 30% of patients treated with this drug for more than 7 days show some signs of nephrotoxicity (Atessahin *et al*., 2003), and this side effect and other complications that arise from the use of this drug limit its clinical usefulness.

Several clinical and experimental strategies have been employed to reduce or protect against gentamicin-induced renal dysfunction and injury. Most of the attempts to reduce gentamicin nephrotoxicity without compromising the antibacterial action have been met with little success. Changes in the structure of the aminoglycosides cause loss of antibacterial effect (Ali *et al*., 2005). There is therefore a need for search for effective, safe and practical agents that can reduce gentamicin nephrotoxicity without compromising its efficacy as an antibacterial agent.

Based on the findings that gentamicin forms complexes with mitochondrial Fe^{2+} to catalyze the formation of free oxygen radicals, iron chelators were tested and were proved to be effective in the prevention of aminoglycoside-induced ototoxicity (Song *et al*., 1997). Extension of this finding to nephrotoxicity appears to be possible (Paralkpinar *et al*., 2005), but biophysical and biochemical considerations (Priuska and Schacht, 1997), suggest that the protective effect of deferroxamine may be critically dependent on the dosage of gentamicin. Other compounds were also used on account of

their antioxidant effects, but the mechanisms have not always been unambiguously established. Means of protection based on a correlation of the functional abnormalities or on an increase in cell regeneration capabilities have also been attempted, but no clinical application has so far been made (Mingeot-Leclercq and Tulkens, 1999).

Flavonoids continue to draw attention as possible, very useful therapeutic agents for combating pathologic states associated with free radical production (Lopez-Velez *et al.*, 2003). The role of dietary flavonoids in the prevention of several chronic diseases has been the subject of intense research interest and the soy phenolics have been the focus of particular attention (Omoni and Aluko, 2005). Furthermore, there is increasing evidence that dietary phytoestrogens present primarily in soybeans as isoflavones have a beneficial role in chronic renal disease (Ranich *et al.*, 2001). Nutritional intervention studies have shown that consumption of soy-based protein reduces proteinuria and attenuates renal functional or structural damage in animals and humans with various forms of chronic renal disease (Ranich *et al.*, 2001).

To the best of our knowledge, the influence of soybean and its products on gentamicin toxicity have not been investigated. In this study, the possible protective effect of the methanolic extracts of soybean (MESB) in a rat model of gentamicin-mediated nephropathy was examined, an attempt aimed at identifying agents with antioxidant property that could protect the kidney from undesirable side effects and improving the therapeutic indices of the aminoglycoside antibiotics.

PROCEDURE

Albino rats of the Wistar strain weighing between 110 and 275 g were used in this study. Rats were randomly divided into five groups of five animals each. Group I (control) received normal saline (0.9% NaCl) subcutaneously (s.c). Rats in group II received gentamicin (80 mg/kg/ day, s.c). Groups III and IV received 500 and 1000 mg/kg/day MESB orally, respectively, and after 1 hour, gentamicin (80 mg/kg, s.c.) was administered. Group V was treated with MESB alone (1000 mg/kg/day, orally). All treatments were given for 12 days. On the 12th day, after treatment, rats were sacrificed by cervical dislocation and dissected. Blood samples were collected by cardiopuncture into heparinized tubes for determination of plasma concentrations of creatinine, blood urea nitrogen (BUN), Na^+ and K^+ ions. Kidneys were immediately removed and a portion cut and fixed in 10% formaldehyde for histology. The remaining portion was rinsed in ice-cold 1.15% KCl, blotted and weighed. This was then minced with scissors

in 3 volumes of ice-cold 100 mM potassium phosphate buffer, pH 7.4 and homogenized. The homogenates were later centrifuged at 12,500 g for 15 minutes at 4°C and the supernatants, termed the post-mitochondrial fractions (PMF), were aliquoted and used for the enzyme assays.

RESULTS

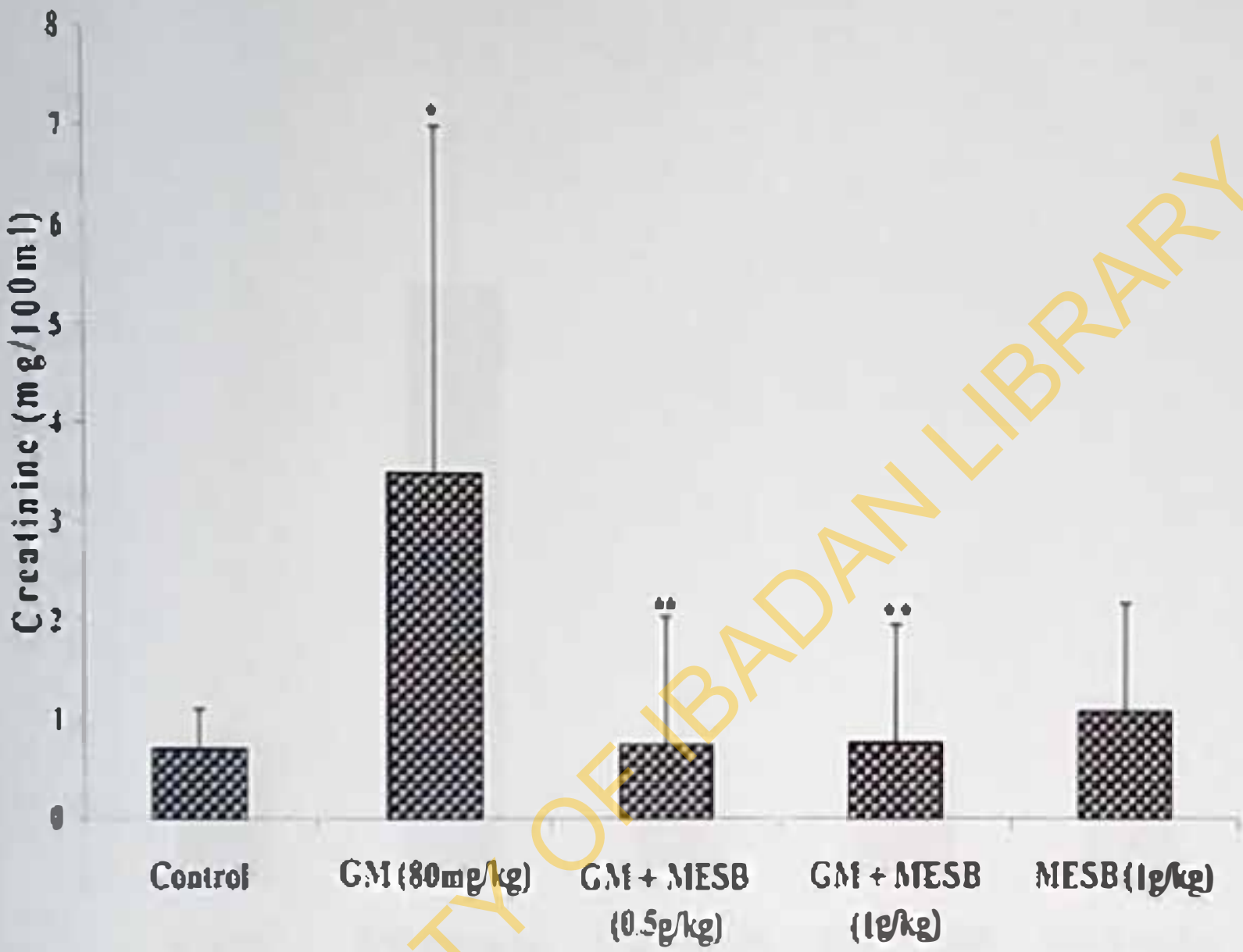


Figure 1A: Effect of methanolic extract of soybean (MESB) on plasma creatinine of normal and gentamicin (GM)-treated rats

*$p<0.001$ when compared with control; **$p<0.001$ when compared with GM group.

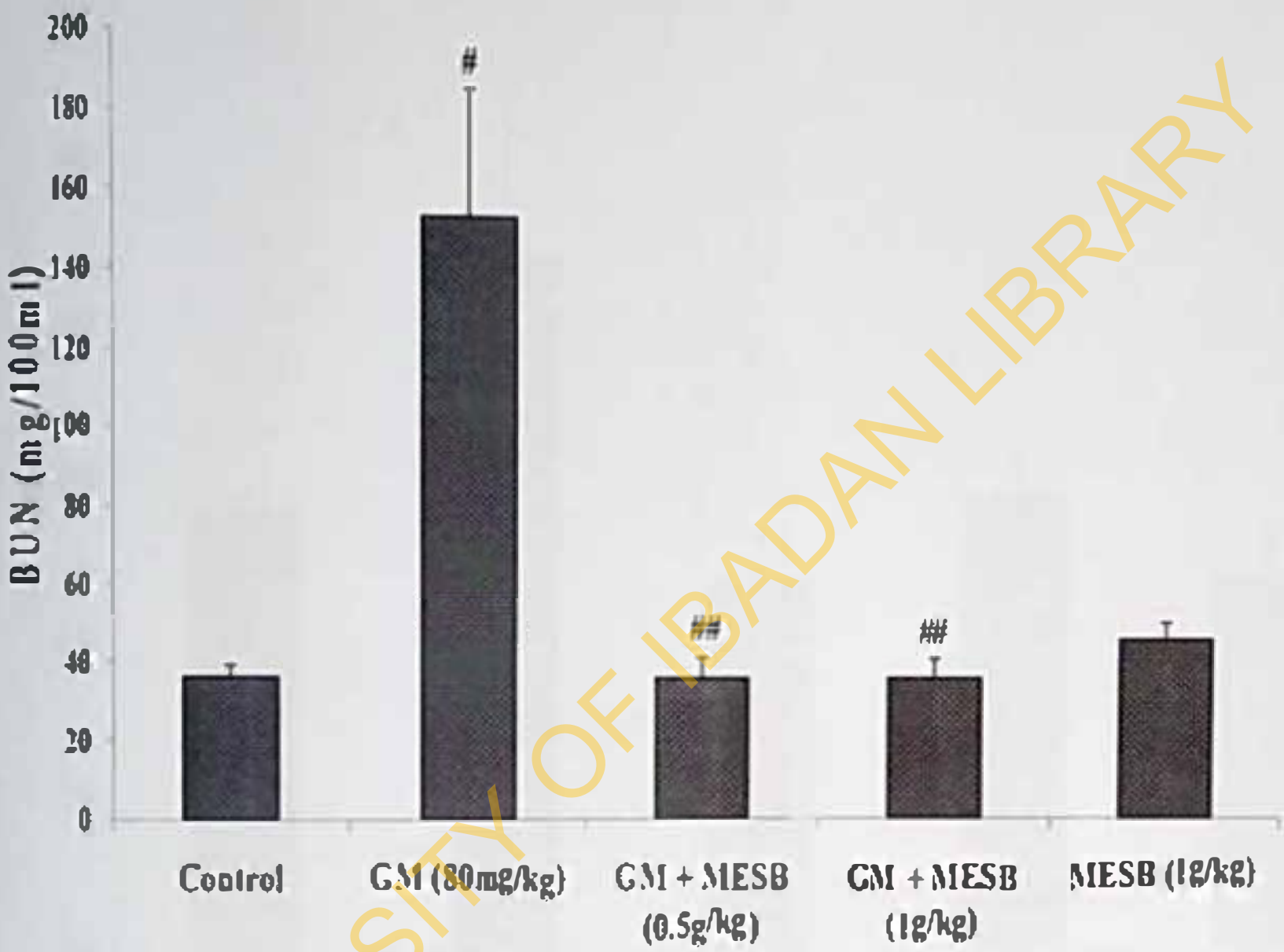


Figure 1B: Effect of methanolic extract of soybean (MESB) on blood urea nitrogen (BUN) of normal and gentamicin (GM)-treated rats.

'p< 0.01 when compared with control; ''p<0.01 when compared with GM group.

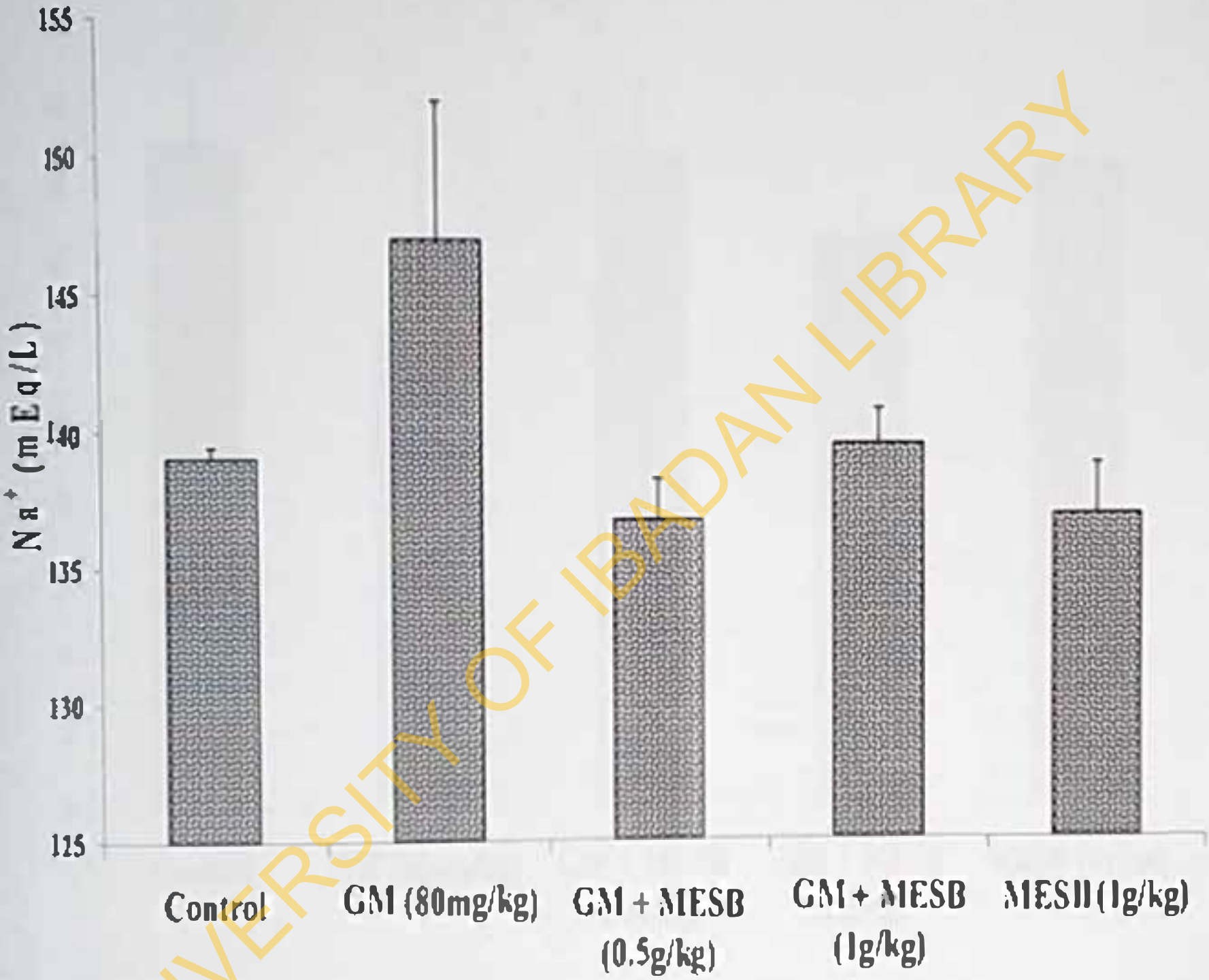


Figure 1C: Effect of methanolic extract of soybean (MESB) on plasma sodium (Na^+) ion level of normal and gentamicin (GM)-treated rats.

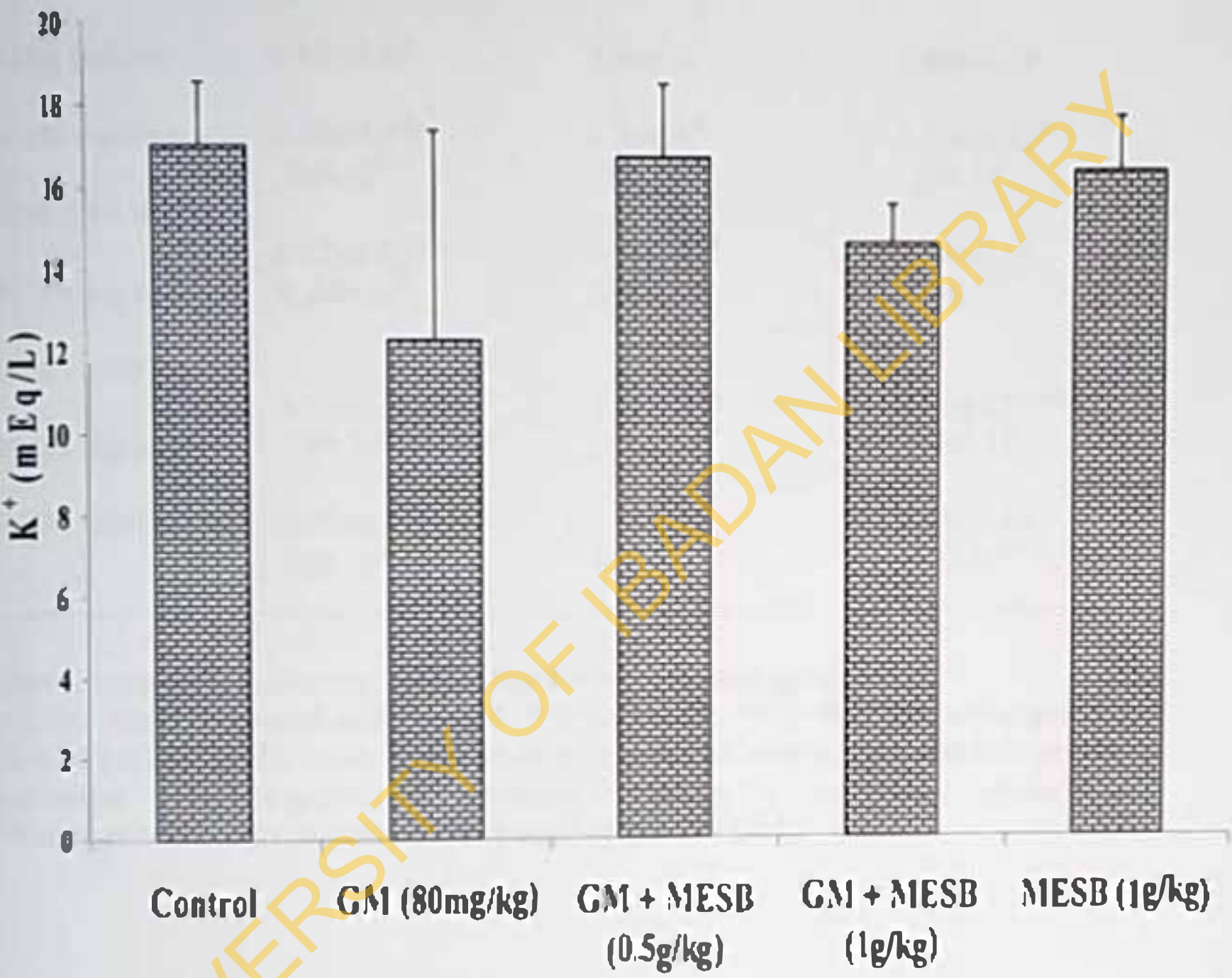


Figure 1D: Effect of methanolic extract of soybean (MESB) on plasma potassium (K^+) ion level of normal and gentamicin (GM)-treated rats.

Table 1A: Effect of methanolic extract of soybean (MESB) on normal and gentamicin (GM) - induced changes in reduced glutathione (GSH) level and glutathione-S-transferase (GST) activity.

Treatment Group	Blood GSH (µg/mg protein)	Renal GSH (µg/g kidney wt)	GST activity (nmol/g kidney wt)
Control (saline)	0.89±0.08	7.9±1.4	2.80±0.18
GM (80 mg/kg)	0.28±0.07* (68.5)[a]	1.3±0.4* (83.5)[a]	1.23±0.22* (56.1)[a]
MESB (500 mg/kg) + GM (80 mg/kg)	0.97±0.07** (-246.4)[b]	3.7±0.9** (-184.6)[b]	1.78±0.15 (-44.7)[b]
MESB (1000 mg/kg) + GM (80 mg/kg)	0.03±0.02*** (89.3)[b]	3.4±0.9** (-161.5)[b]	2.33±0.37** (-89.4)[b]
MESB (1000 mg/kg)	0.67±0.17 (24.7)[a]	1.5±0.4* (81.0)[a]	3.43±0.45 (-22.5)[a]

Values are expressed as mean ± SEM for five rats in each group.
*p<0.001 when compared with control; **p<0.001 and ***p<0.02 when compared with GM group; *p<0.01 and **p<0.05 when compared with control and GM group respectively. Values in parenthesis represent % change; (-) = increase; (+) = decrease
[a]% change relative to control; [b]% change relative to GM.

Table 1B: Effect of methanolic extract of soybean on normal and gentamicin (GM) - induced changes in superoxide dismutase (SOD) activity in rat.

Treatment group	Blood SOD[x]	Renal SOD[x]	Renal CAT[y]
Control (saline)	13.4±3.3	29.2±7.8	8.7±1.8
GM (80 mg/kg)	6.7±4.1* (50.0)[a]	25.0±4.8* (14.4)[a]	6.0±0.5 (31.0)[a]
MESB (500 mg/kg) + GM (80 mg/kg)	8.4±4.8 (-25.4)[b]	27.8±5.6 (-11.2)[b]	6.7±1.8 (-11.7)[b]
MESB (1000 mg/kg) + GM (80 mg/kg)	13.3±8.2# (-98.5)[b]	33.3±16.7# (-33.2)[b]	11.7±3.2# (-95.0)[b]
MESB (1000 mg/kg)	13.4±3.3 (0)[a]	23.3±4.1 (20.2)[a]	10.0±1.4 (-14.9)[a]

Values are expressed as mean ± SEM for five rats in each group.
[x]Activity expressed as units of enzyme required to inhibit auto-oxidation of adrenaline to adrenochrome. 1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the autooxidation of adrenaline to adrenochrome.
[y]Activity expressed as μmol H_2O_2 consumed/min/mg protein.
*Significantly different from control ($p<0.05$); #Significantly different from GM-treated rats ($p<0.05$). Values in parenthesis represent % change; (-) = increase; (+) = decrease
[a] % change relative to control; [b] % change relative to GM.

Table IC: Effect of methanolic extract of soybean on gentamicin (GM)-induced changes in glucose-6-phosphatase (G6Pase) and 5'-Nucleotidase (5'-NTD) activity in rat kidney.

Treatment group	G6Pase (mmol/g protein/min)	5'-NTD (mmol/g protein/min)
Control (saline)	113.23±5.4	150.94±20.6
GM (80 mg/kg)	109.68±4.2 (3.1)[a]	146.63±6.3 (2.9)[a]
MESB (500 mg/kg) + GM (80 mg/kg)	112.64±3.1 (-2.7)[b]	141.74±8.5 (-3.3)[b]
MESB (1000 mg/kg) + GM (80 mg/kg)	91.98±5.7** (16.1)[b]	132.18±7.1 (9.9)[b]
MESB (1000 mg/kg)	85.88±2.6* (24.2)[a]	131.42±6.1 (12.9)[a]

Values are expressed as mean ± SEM for five rats in each group.
*$p<0.01$ and **$p<0.05$ when compared with control and GM-only group respectively.
Values in parenthesis represent % change; (-) = increase; (+) = decrease
[a]% change relative to control; [b]% change relative to GM.

Table 1D: Effect of methanolic extract of soybean (MESB) on gentamicin (GM) - induced changes in γ-glutamyltransferase (γ-GT) and aspartate aminotransferase (AST) activity.

Treatment group	γ-GT activity (units/ml)	AST activity (units/ml)
Control (saline)	5.39±0.9	1.45±0.07
GM (80 mg/kg)	2.16±0.4* (59.9)	1.26±0.04 (13.1)
MESB (500 mg/kg) + GM (80 mg/kg)	2.89±0.3 (-33.8)	1.41±0.05 (-11.9)
MESB (1000 mg/kg) + GM (80 mg/kg)	3.99±0.8** (-84.7)	1.43±0.05 (-13.5)
MESB (1000 mg/kg)	5.36±0.2 (0.6)	1.43±0.04 (1.4)

Values are expressed as mean ± SEM for five rats in each group.
*p<0.02 and **p<0.10 when compared with control and GM-only group respectively.
Values in parenthesis represent % change; (-) = increase; (+) = decrease
(a) % change relative to control; (b) % change relative to GM.

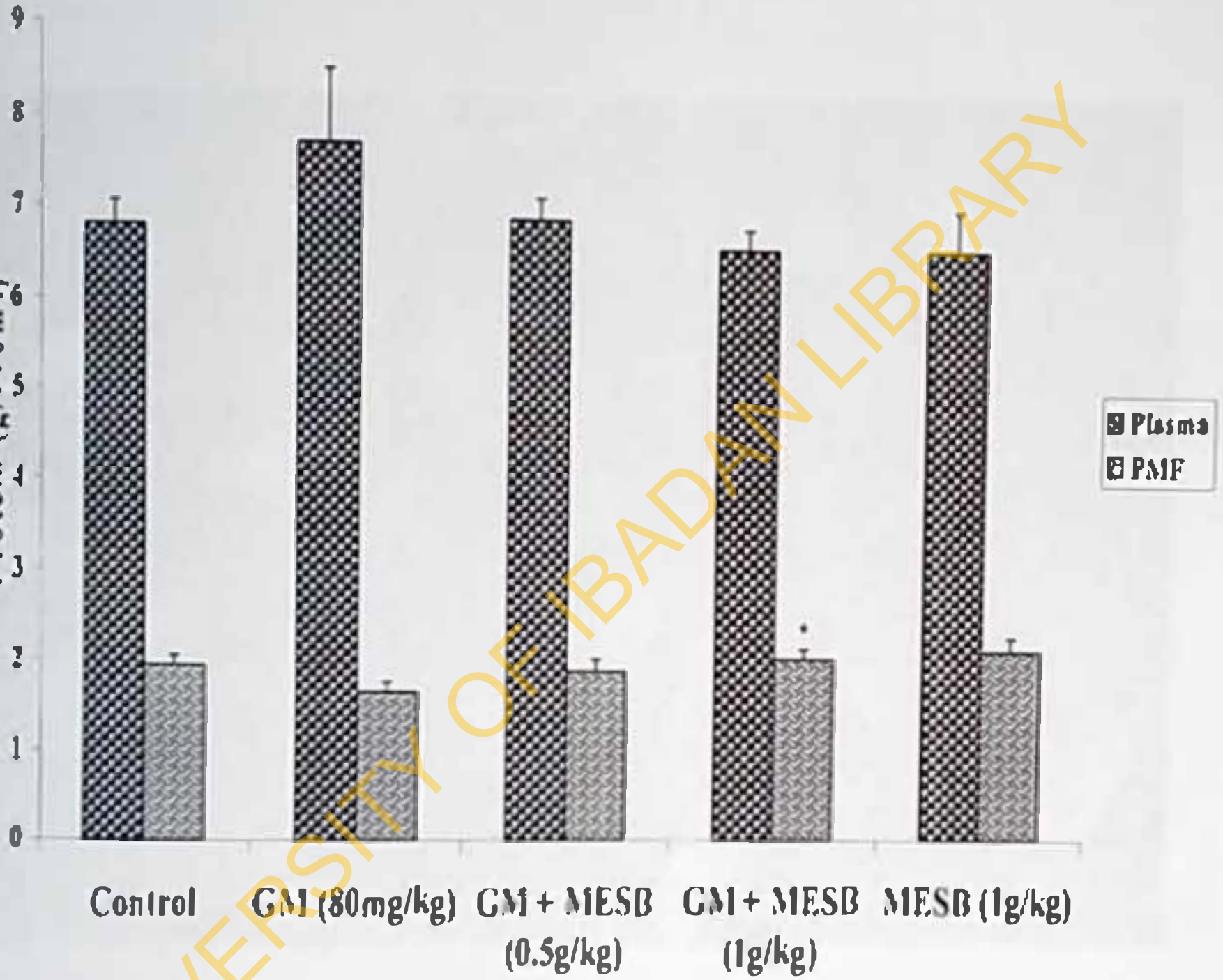


Figure 1E: Effect of methanolic extract of soybean (MESB) on plasma and post-mitochondrial fraction (PMF) total protein concentrations of normal and gentamicin (GM)-treated rats.

* $p<0.05$ when compared with GM group.

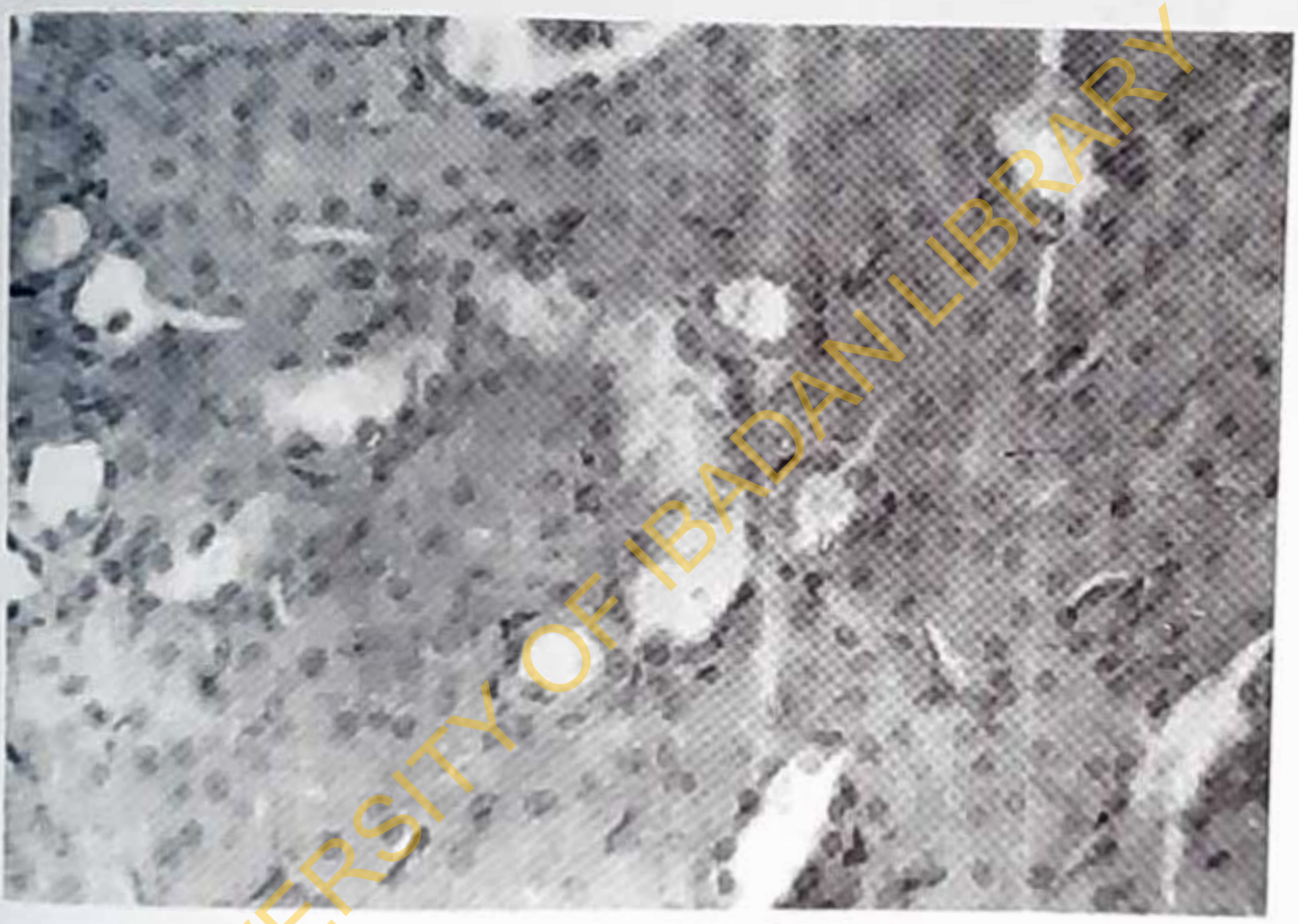


Figure 1F: Section of kidney from rat treated with normal saline (control)

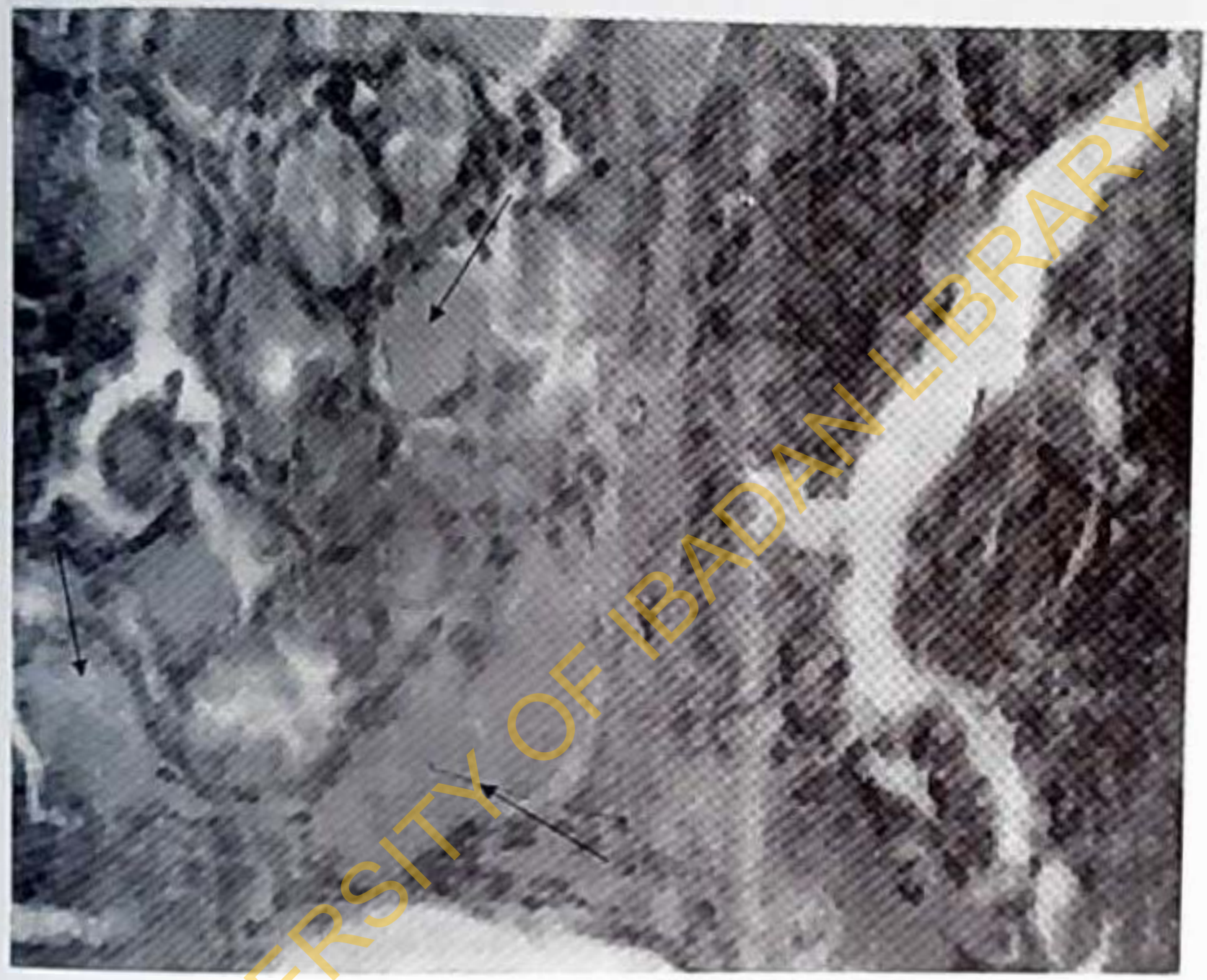


Figure 1G: Section of kidney from rat treated with gentamicin (80 mg/kg. s.c. daily for 12 days). Section shows severe/massive tubular necrosis with deposition of colloid casts within the tubular lumina. —Tubules showing necrosis

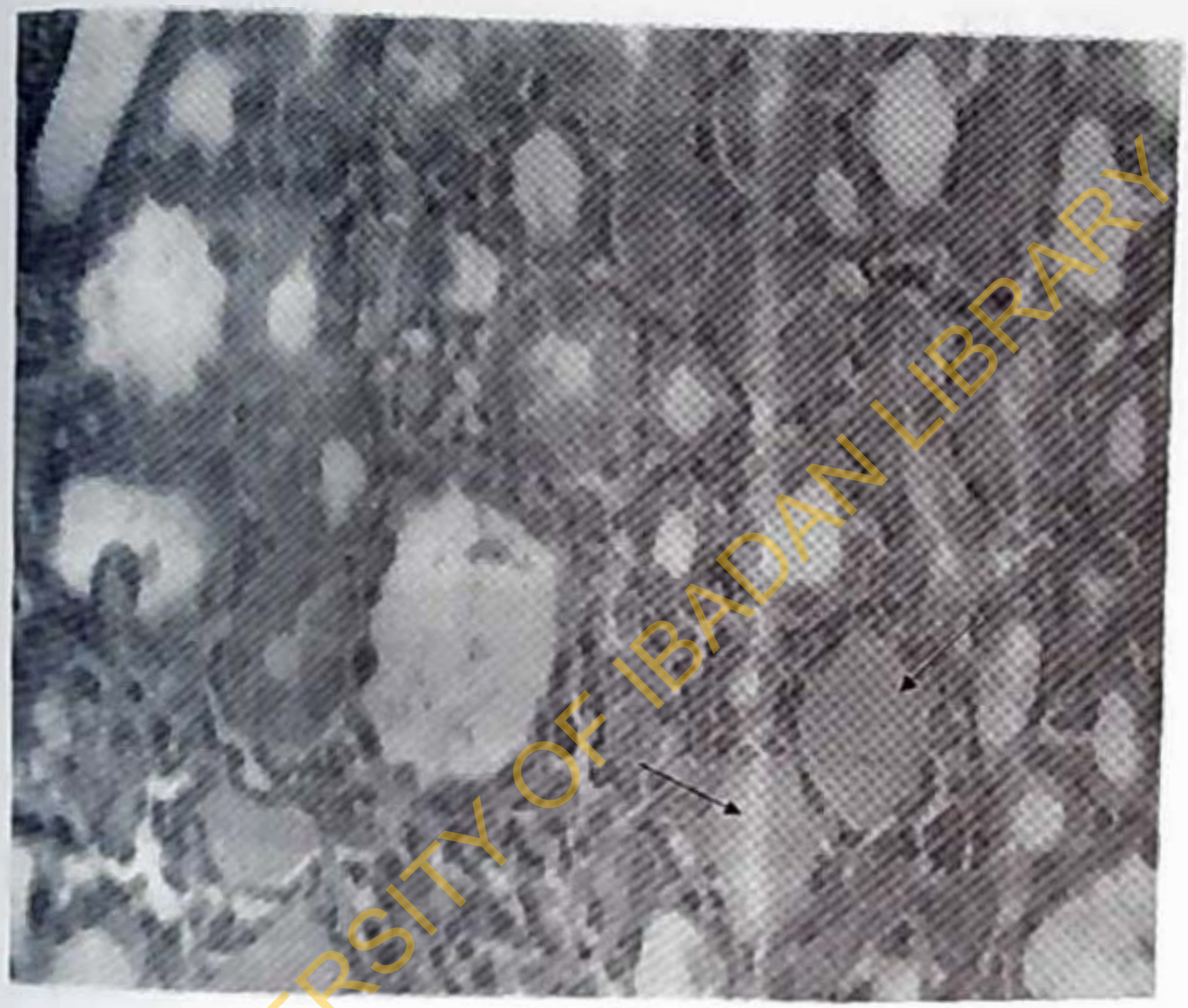


Figure 1H: Section of kidney from rat treated with methanolic extract of soybean (500 mg/kg, p.o.) plus gentamicin (80 mg/kg, s.c.) daily for 12 days. Section shows mild tubular necrosis with some of the tubules containing colloid casts.

⟶ Tubules showing necrosis

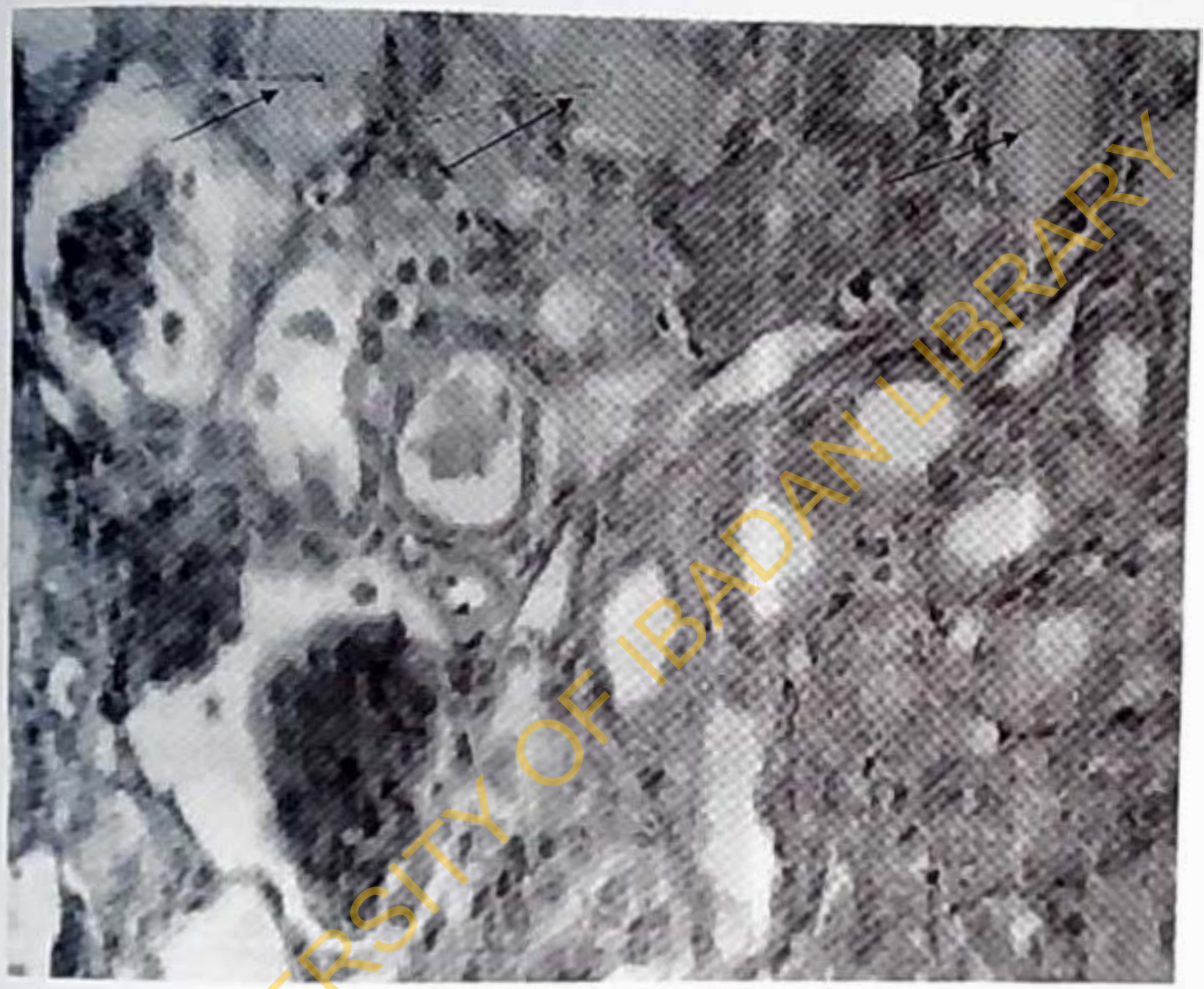


Figure 11: Section of kidney from rat treated with methanolic extract of soy bean (1000 mg/kg, p.o.) plus gentamicin (80 mg/kg, s.c.) daily for 12 days. Section shows moderate tubular necrosis with some of the tubules containing colloid.

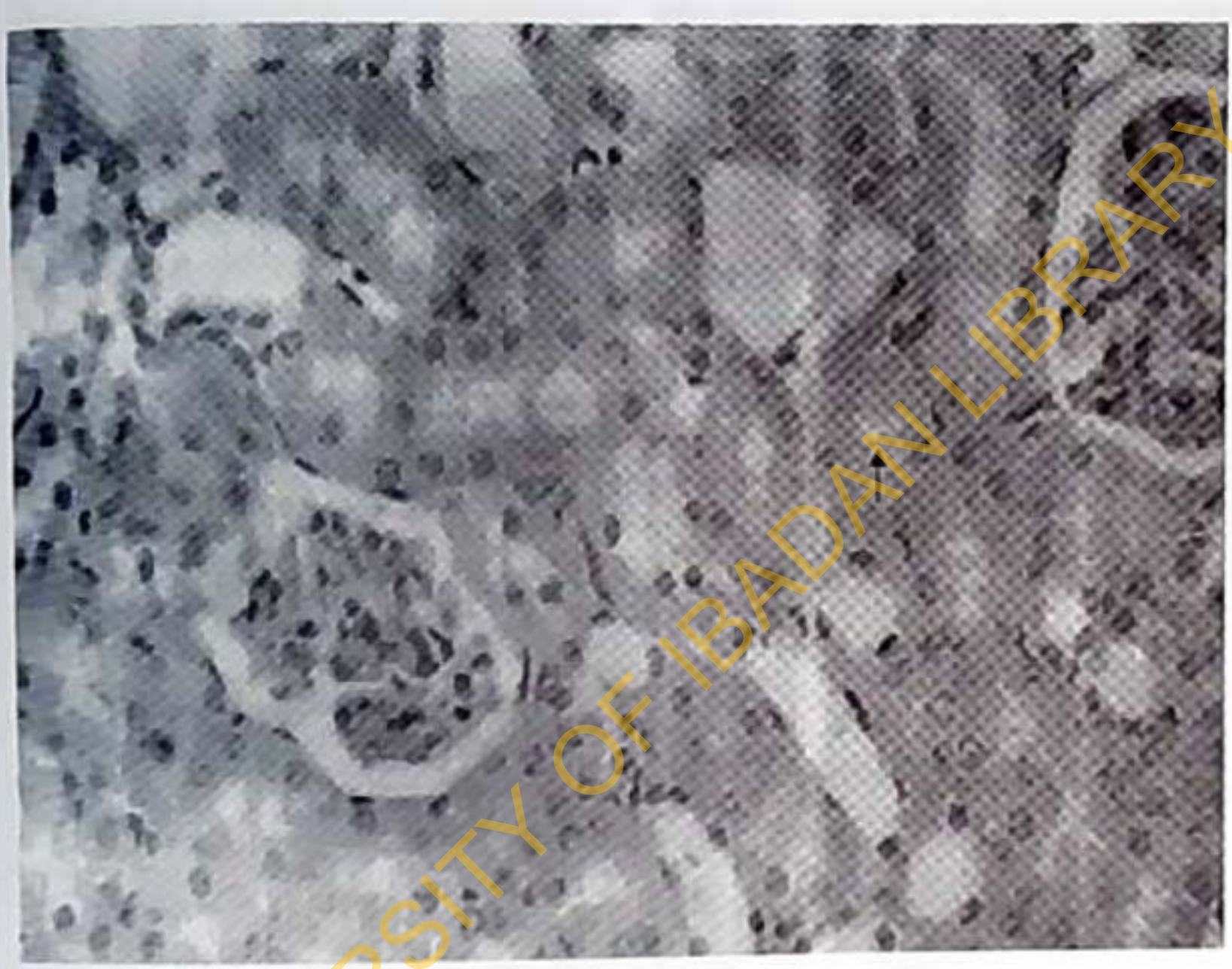
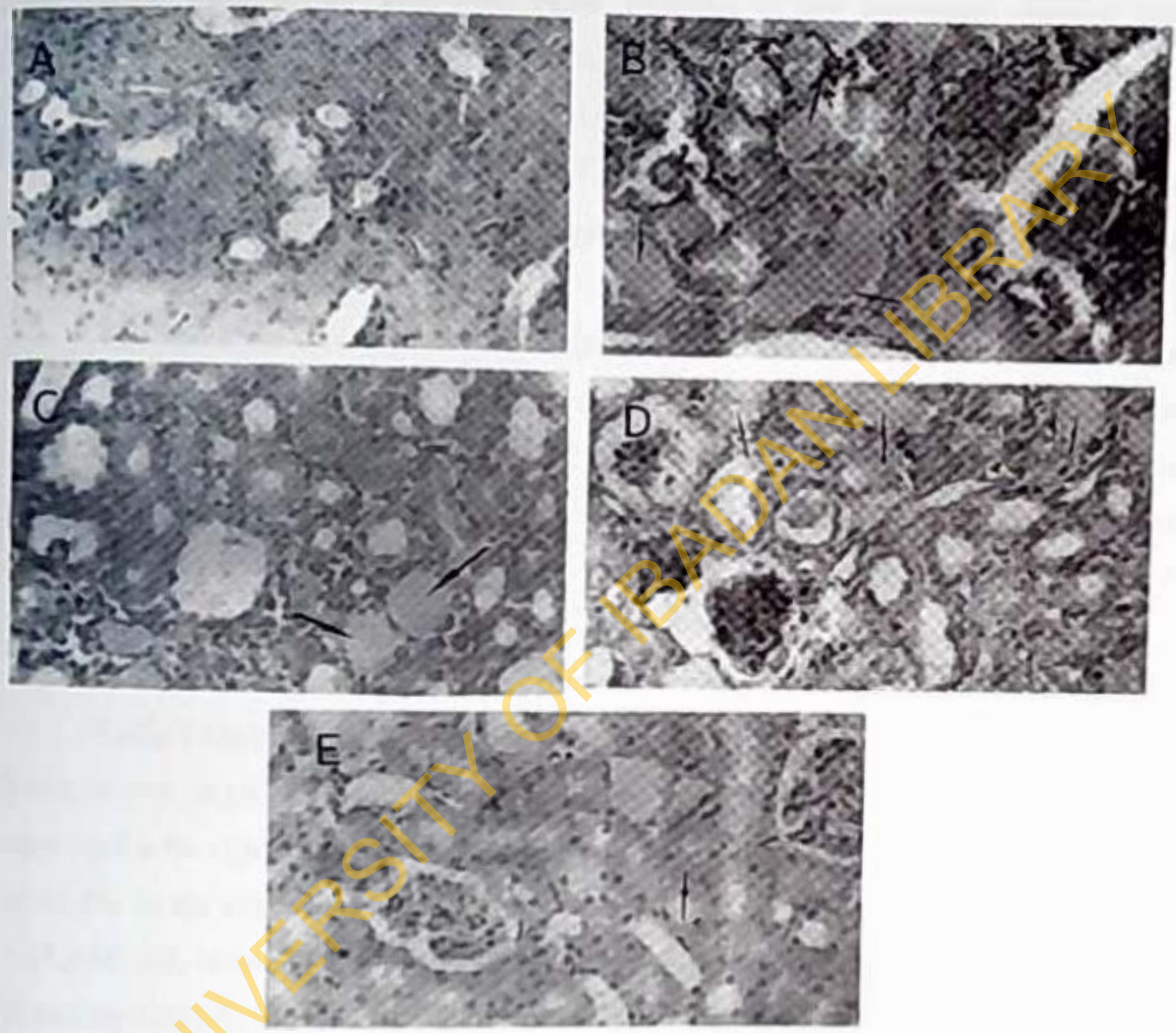


Figure 11: Section of kidney from rat treated with 1000 mg/kg oral dose of the methanolic extract of soybean daily for 12 days. Section shows some mild tubular necrosis.



Summary of photomicrograph of rat kidney section (X400).
A. Control (normal saline) rat kidney section. B. Gentamicin-treated (80mg/kg) group showing severe tubular necrosis. C. Gentamicin (80mg/kg) plus soybean (500mg/kg) treated group with tubules showing mild to moderate necrosis. D. Gentamicin (80mg/kg) plus soybean (1000mg/kg) treated group with moderate tubular necrosis. E. Soybean (1000mg/kg) treated group with mild tubular necrosis.

——— Indicate some of the tubules showing necrosis.

The effect of gentamicin treatment on renal function test is illustrated in Figures 1A to 1D. Gentamicin produced a significant increase in plasma creatinine ($p<0.001$) and BUN ($p<0.01$) concentrations and a moderate increase in plasma Na^+ ion with mild depletion in K^+ ion concentration when compared with the control rats. Pretreatment with MESB significantly improved renal function in the gentamicin-treated rats. Gentamicin-induced elevations in BUN decreased by 76.5%, 76.6% and creatinine by 78.6%, 77.7% following treatment with 500 and 1000 mg/kg MESB respectively ($p<0.05$). The larger dose (1000 mg/kg) of the extract, however, did not seem to offer better protection against gentamicin toxicity when compared with that of the lower (500 mg/kg) dose.

The result on total protein is shown in Figure 1E. Treatment with gentamicin was associated with a decrease in renal but not plasma total protein concentration in which an increase was observed. These changes were however not significant when compared with the control. MESB prevented the decrease in renal total protein and also restored the plasma total protein towards the control values in the gentamicin - treated rats. MESB alone did not significantly affect both renal and plasma total protein concentrations, although a slight increase was observed in the former.

Table I shows the effect of gentamicin administration on blood and renal GSH levels as well as on GST activity in the kidney. GM - induced acute renal failure was associated with significant decrease in GSH level by 68.5% in the blood ($p<0.001$) and by 83.5% in the kidney ($p<0.01$) when compared with control. Pretreatment with 500 mg/kg MESB, however, significantly prevented the gentamicin - induced depletion by increasing GSH levels by 246.4% ($p<0.001$) and 184.6% ($p<0.05$) in blood and kidney respectively. MESB at a dose of 1000 mg/kg alone produced a slight decrease (by 24.7%) in blood GSH, but significantly reduced renal GSH by 81.0% ($p<0.01$) when compared with control. There was further depletion in GSH level in the gentamicin plus MESB (1000 mg/kg) group both in the blood and in the kidney.

Gentamicin treatment significantly decreased renal activity of GST by 56.1% ($p<0.01$) in the rats. MESB when administered at 500 and 1000 mg/kg/day doses significantly increased renal GST activity by 44.7% and 89.4% respectively in the gentamicin - treated rats (Table 1A). MESB (1000 mg/kg) alone increased renal activity of GST by 22.5% relative to control.

The changes observed in whole blood SOD as well as renal SOD and CAT activities are recorded in Table 1B. The extract dose dependently prevented gentamicin

- induced decrease in SOD (both in the blood and in the kidney) and renal CAT activities of rats. MESB significantly attenuated the gentamicin - decreased blood SOD and renal CAT activities. SOD activity in blood increased by 25.4% and 98.5% while renal SOD activity increased by 11.2% and 33.2% following treatment with 500 and 1000 mg/kg/day doses of the extract in the gentamicin - treated rats. Similarly, MESB also at these doses increased renal CAT activity by 11.7% and 95% respectively in the gentamicin - treated rats. The increases in the activities of these enzymes induced by MESB in the gentamicin - treated rats were only significant at 1000 mg/kg dose. MESB when administered alone did not produce any significant change in the activity of these enzymes.

The activities of G6Pase, 5'-NTD, γ-GT and AST in the gentamicin - treated rats and the influence of MESB are shown in Table 1C and Table 1D. Gentamicin treatment caused mild decreases in the renal activities of G6Pase and 5'-NTD. The renal activity of γ-GT however decreased significantly by 59.9% whereas only mild and insignificant decrease (by 13.1%) was observed in AST activity when compared with the control. MESB at a dose of 500 mg/kg increased G6Pase activity non-significantly in the gentamicin - treated rats but caused further decrease in the enzyme activity at a dose of 1000 mg/kg ($p<0.05$). The extract, however, produced an insignificant but dose-dependent decrease in the activity of 5'-NTD in these rats. Pretreatment with MESB protected against GM-induced decreases in renal γ-GT and AST activities and these effects were dose dependent. The protective effect of the extract against the gentamicin - induced decrease in γ-GT and AST was significant at 1000 mg/kg dose when compared with the gentamicin - only group ($p<0.01$). The administration of MESB alone at 1000 mg/kg decreased renal activities of G6Pase and 5'-NTD by 24.2% and 12.9% respectively relative to control whereas it did not produce any change in the activities of γ-GT and AST.

Photomicrographs showing renal morphology in all treatment groups are shown in Figures 1F to 1J. Kidney sections from rats in control group were essentially normal. Gentamicin, however, induced massive tubular necrosis with deposition of proteinacious or colloid casts within the tubular lumina in the rats (Figure 1G). Pretreatment with 500- and 1000- mg/kg doses of MESB reduced gentamicin - induced renal tubular damage with renal section in these groups of rats showing mild to moderate and moderate tubular necrosis respectively (Figures 1H and 1I). The 500 mg/kg dose of the extract appears to show better histological protection against the

gentamicin - induced renal damage. Rats treated with 1000 mg/kg MESB alone showed similar renal histology with those of the control although mild necrosis was observed in very few tubules (Figure 1J). The glomeruli nre spared and the interstitium free from infiltration by chronic inflammatory cells in all the treatment groups (Figures 1F to 1J).

CONCLUSION

The methanolic extract of soybean (MESB) at a relatively low dose has a specific stabilizing effect on the cell membrane and/or other vital cellular macromolecules and protected against gentamicin - induced renal dysfunction and tubular necrosis. The protective or ameliorative effect of the extract appears to be related to the antioxidant polyphenolic content present in soybean.

4.2 EXPERIMENT 2: THE EFFECT OF METHANOLIC EXTRACTS OF SOYBEAN ON CISPLATIN – INDUCED NEPHROTOXICITY.

INTRODUCTION

Several therapeutic agents used in clinical practice have been reported to produce functional impairment and toxic injuries to the kidney and thus contribute a great deal to hospital acquired ARF and the development of nephropathy.

Cisplatin or *cis*-diamminedichloroplatinum (CDDP) and other platinum derivatives are among the most effective chemotheropeutic agents widely used in the treatment of a variety of malignancies, including head and neck, ovarian and testicular cancers. However, the full clinical utility of these drugs (especially cisplatin) is limited by nephrotoxicity, the most common adverse effect, in many cancer patients (Schrier, 2002). While several antineoplastic agents frequently exhibit nephrotoxicity, the platinum derivatives ore among the most frequent compounds leading to renal injury (Schrier, 2002). Approximately 28 to 36% of patients receiving an initial dose (50 to 100-mg/m^2) of cisplatin develop ARF (Lebwohl and Canetta, 1998; Ries and Klastersky, 1986). Since even vigorous hydration has not been effective in eliminating toxicity and the use of diuretics may complicate the electrolyte disturbance induced by cisplatin, discontinuation of cisplatin remains the only option in cases of progressive renal failure. There is a pressing need therefore to protect the kidney while administering effective chemotherapeutic agents, such as cisplatin.

The mechanism of cisplatin nephrotoxicity remains to be fully elucidated. In addition to direct tubular toxicity in the form of apoptosis and necrosis (Arany and Safirstein, 2003), vascular factors (Luke *et al*., 1992; Winston and Safirstein, 1985) and inflammation (Ramesh and Reeves, 2002 and 2003) that have been implicated in the pathogenesis of cisplatin-mediated nephrotoxicity, several other studies have also demonstrated that oxidative stress is involved in the development of this drug's renal tubule injury (Yilmaz *et al*., 2004; Kadikoylu *et al*., 2004; Shino *et al*, 2003). The involvement of oxidative stress is further supported by the fact that free radical scavengers and antioxidants prevented cisplatin-induced nephrotoxicity (Weijl *et al*., 2004; Dickey *et al*., 2005; Gulec *et al*., 2006).

There is increasing evidence that dietary phytoestrogens present primarily in soybeans as isoflavones have a beneficial role in chronic renal disease (Ronich *et al*., 2001). Nutritional intervention studies have shown that consumption of soy-based

protein reduces proteinuria and attenuates renal functional or structural damage in animals and humans with various forms of chronic renal disease (Ranich *et al.*, 2001).

The aim of this experiment therefore was to further evaluate the mechanisms of cisplatin nephrotoxicity, determine the protective effects of soybean using the methanolic extract (MESB) and the probable mechanisms of protection against its renal toxicity. This is aimed at providing agents that will mitigate adverse or toxic effects in patients at risk.

PROCEDURE

Six groups of six rats per group weighing between 204 and 265g were studied in this experiment; Group I (control) received 0.9% NaCl; Group II was treated with CDDP (2 mg/kg/day) intraperitoneally for 5 days; Rats in groups III, IV and V were treated with 250-, 500- and 1000- mg/kg/day oral doses of MESB respectively, one-hour before CDDP injection; Group VI was treated with 1000 mg/kg/day MESB only. All the treatments were given for five days. Rats were sacrificed by cervical dislocation 24-hours after the last injection of CDDP. Blood samples were collected via cardiac puncture and serum separated by centrifugation at room temperature. Kidney samples were immediately removed, homogenized and centrifuged at 4°C to separate the postmitochondrial fraction (PMF) as in experiment I. Renal function was assessed in serum. Biomarkers of oxidative stress, inflammation, plasma membrane and tubular damage were evaluated in the PMF and also in the serum. A portion of the kidney was removed before homogenizing and processed for histology.

RESULTS

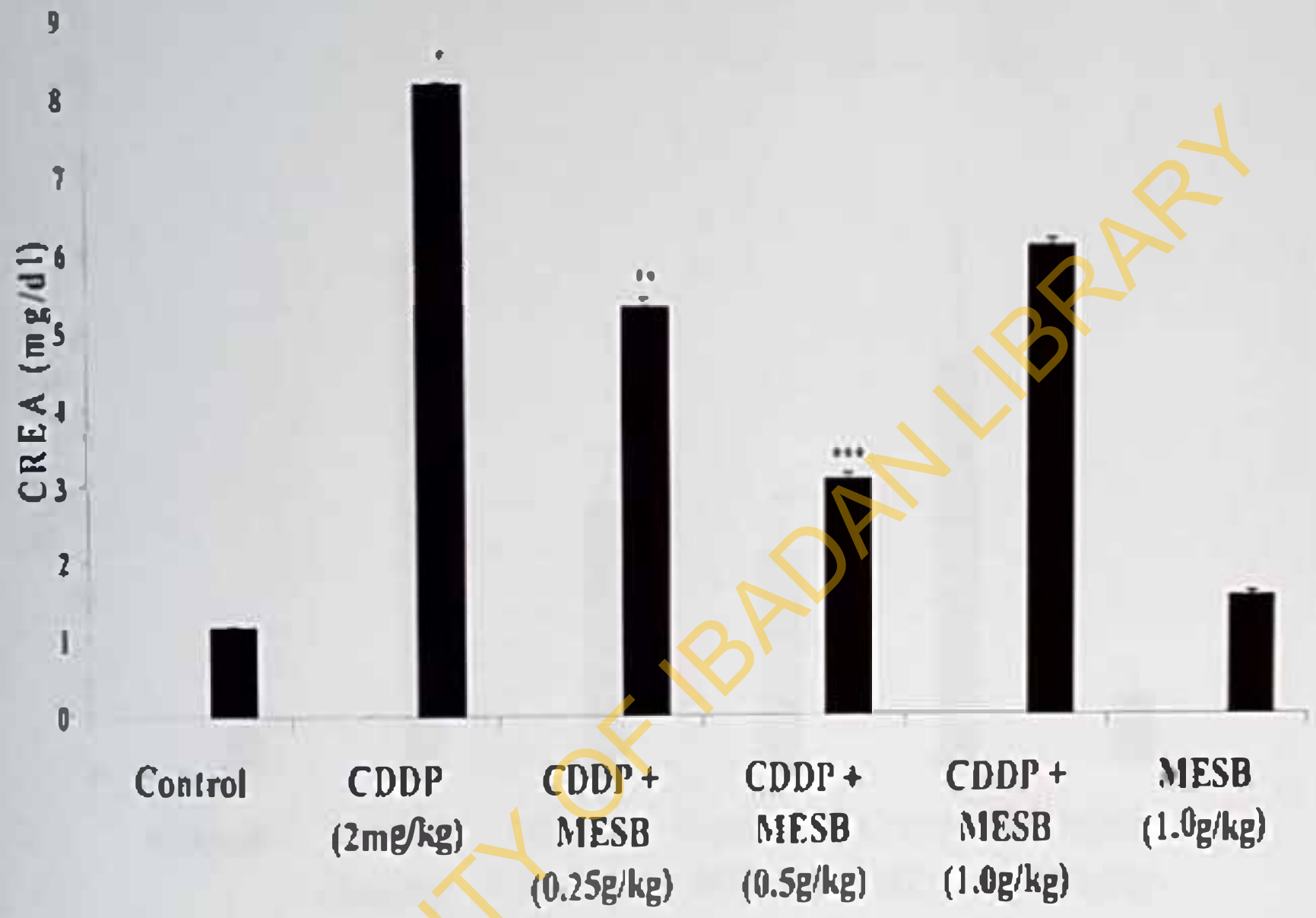


Figure 2A: Effect of the methanolic extract of soybean (MESB) on serum creatinine (CREA) concentration of normal and cisplatin (CDDP) – treated rats.

*p<0.001 when compared with control
**p<0.05 and ***p<0.001 when compared with CDDP group.

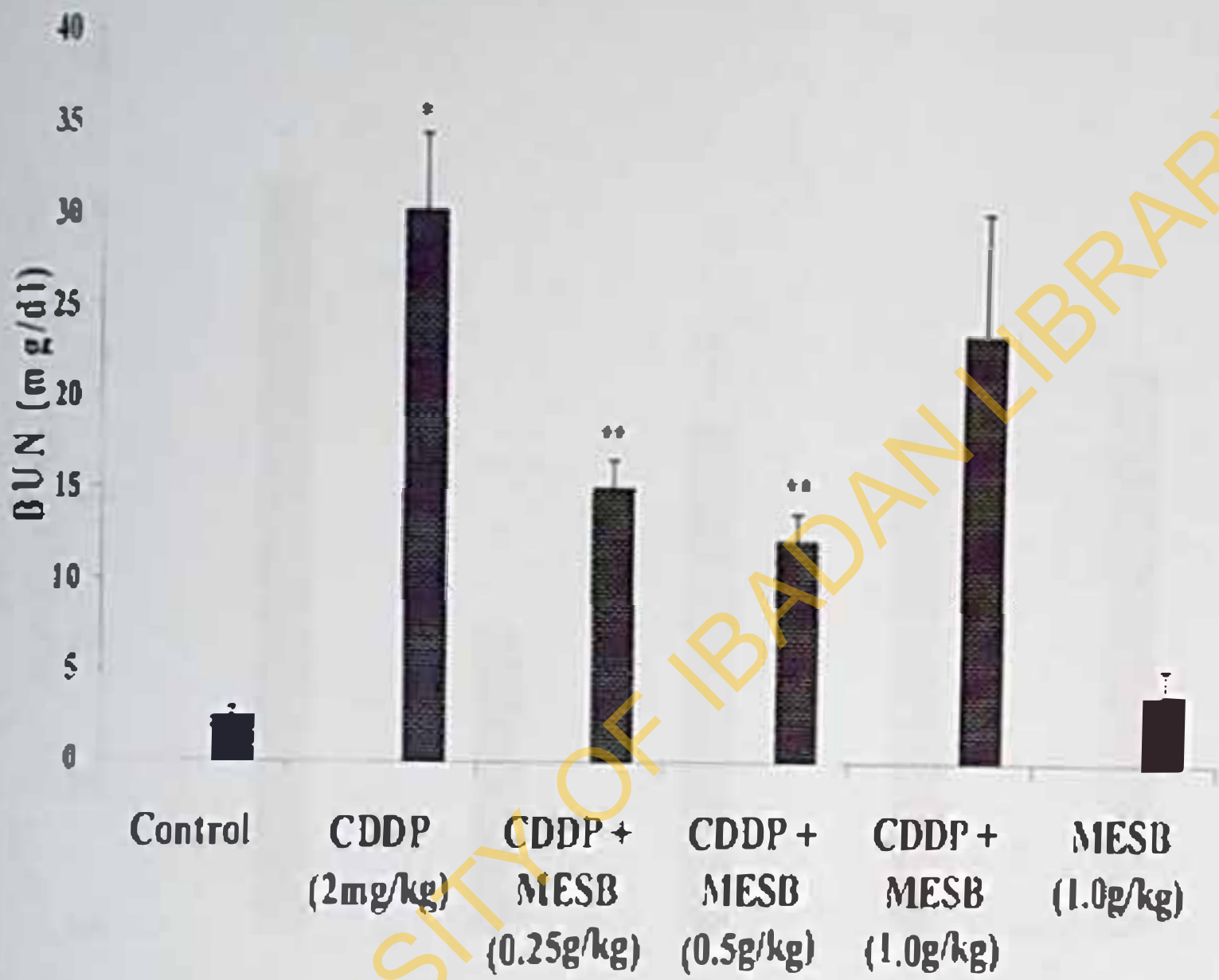


Figure 2B: Effect of the methanolic extract of soybean (MESB) on blood urea nitrogen (BUN) of normal and cisplatin (CDDP) – treated rats.

*$p<0.001$ when compared with control.
**$p<0.001$ when compared with CDDP group.

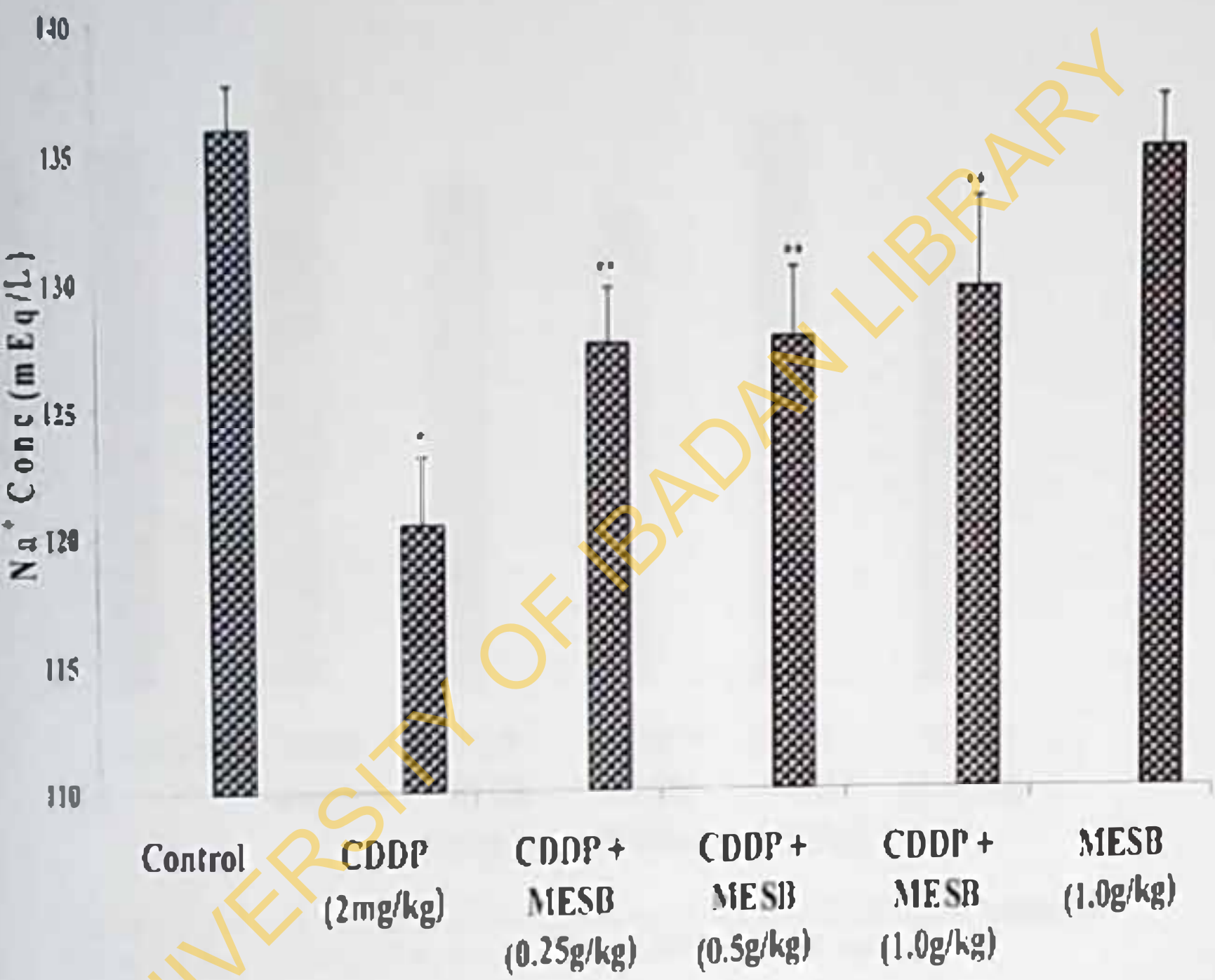


Figure 2C: Effect of the methanolic extract of soybean (MESB) on serum Na^+ concentration of normal and cisplatin (CDDP) – treated rats.

*p<0.001 when compared with control.
**p<0.05 when compared with CDDP group.

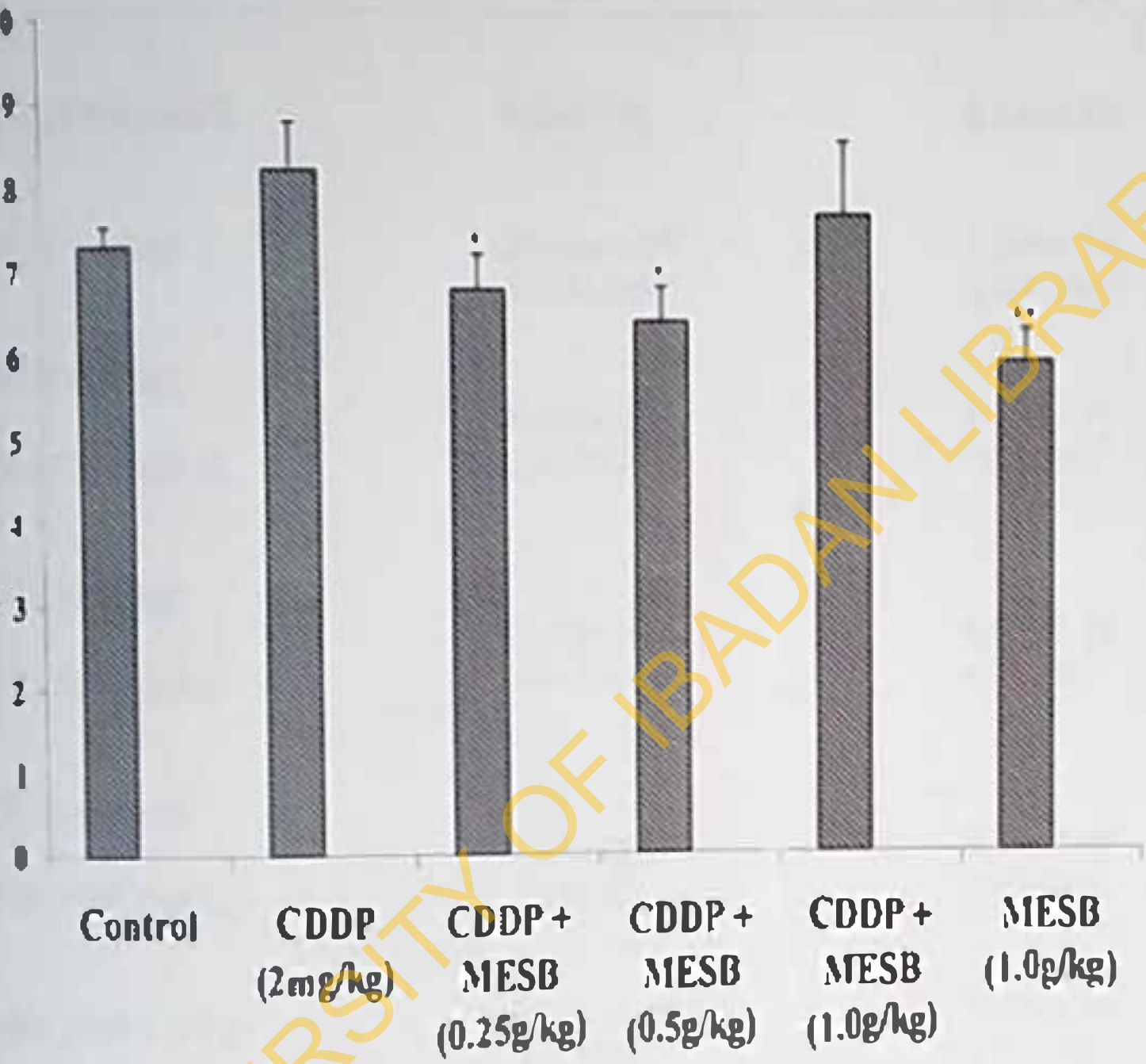


Figure 2D: Effect of the methanolic extract of soybean (MESB) on serum K^+ concentration of normal and cisplatin (CDDP) – treated rats.

*$p<0.001$ and **$p<0.05$ when compared with control.
**$p<0.05$ when compared with CDDP group.

Table 2A: Effect of the methanolic extract of soybean (MESB) on normal and cisplatin (CDDP) – induced increase in myeloperoxidase (MPO) and xanthine oxidase (XO) activities in rat kidney.

Treatment	MPO (units/mg protein)	XO (μmol/min/mg protein)
Control (0.9% NaCl)	9.04±0.96	2.54±0.64
CDDP (2 mg/kg)	34.06±5.85* (-276.8%)[a]	3.24±0.12 (-27.6%)[a]
CDDP (2 mg/kg) + MESB (250 mg/kg)	24.92±5.89 (26.8%)[b]	3.17±1.77 (2.20%)[b]
CDDP (2 mg/kg) + MESB (500 mg/kg)	20.22±1.16 (40.6%)[b]	2.66±0.78 (17.9%)[b]
CDDP (2 mg/kg) + MESB (1000 mg/kg)	31.53±12.50 (7.4%)[b]	4.23±3.07 (30.6%)[b]
MESB (1000 mg/kg)	12.08±1.58 (-33.6%)	2.09±0.48 (17.7%)

Values expressed as mean ± standard error of mean (SEM);
*p<0.01 when compared with control.
Values in parenthesis represent % change; (-) = increase; (+) = decrease
[a] % change relative to control; [b] % change relative to CDDP.

Table 2B: Effect of the methanolic extract of soybean (MESB) on normal and cisplatin (CDDP) – induced increase in combined xanthine oxidase (XO) plus xanthine dehydrogenase activity (XDH) in rat kidney.

Treatment	XO+XDH (µmol/min/mg protein)	XDH (µmol/min/mg protein)
Control (0.9% NaCl)	4.32±0.78	2.60±0.60
CDDP (2 mg/kg)	5.67±0.93 (-31.3%)[a]	4.24±0.57 (-63.0%)[a]
CDDP (2 mg/kg) + MESB (250 mg/kg)	4.42±0.67 (22.0%)[b]	4.24±0.98 (0)
CDDP (2 mg/kg) + MESB (500 mg/kg)	4.20±0.71 (25.9%)[b]	3.97±1.47 (6.4%)[b]
CDDP (2 mg/kg) + MESB (1000 mg/kg)	4.36±0.47 (23.1%)[b]	5.85±2.70 (38.0%)[b]
MESB (1000 mg/kg)	5.27±1.12 (-22.0%)[a]	3.37±0.90 (-29.6%)[a]

Values expressed as mean ± standard error of mean (SEM).
Values in parenthesis represent % change; (-) = increase; (+) = decrease
[a] % change relative to control; [b] % change relative to CDDP.

Table 2C: Effect of the methanolic extract of soybean (MESB) on normal and cisplatin (CDDP) – induced increase in urinary N-acetyl-β-D-glucosaminidase (NAG) activity and serum nitrate/nitrite (NO^-_3/NO^-_2) concentration.

Treatment	NAG (mmol/hr/l)	NO^-_3/NO^-_2 (µmol/l)
Control (0.9% NaCl)	100.9±10.96	4.96±1.51
CDDP (2 mg/kg)	181.3±12.60* (-79.7%)[a]	13.68±1.60* (-175.8%)[a]
CDDP (2 mg/kg) + MESB (250 mg/kg)	113.0±11.9** (37.7%)[b]	8.83±1.28** (35.5)[b]
CDDP (2 mg/kg) + MESB (500 mg/kg)	92.06±7.76*** (49.2%)[b]	8.70±0.52** (36.4%)[b]
CDDP (2 mg/kg) + MESB (1000 mg/kg)	154.74±20.26 (14.6%)[b]	9.05±0.87 (33.8%)[b]
MESB (1000 mg/kg)	129.42±15.20 (-28.3%)	4.52±0.54 (8.9%)

Values expressed as mean ± standard error of mean (SEM).
*p<0.001 when compared with control; **p<0.01 and ***p<0.001 when compared with CDDP group.
Values in parenthesis represent % change; (-) = increase; (+) = decrease
[a] % change relative to control; [b] % change relative to CDDP.

Table 2D: Effect of the methanolic extract of soybean (MESB) on renal catalase (CAT) and superoxide dismutase activities (SOD) in normal and cisplatin (CDDP) – treated rats.

Treatment	CAT (μmol H_2O_2 consumed/min/g tissue)	SOD (Units/g tissue)
Control (0.9% NaCl)	0.726±0.032	1.62±0.13
CDDP (2 mg/kg)	0.500±0.036* (31.1%)[a]	0.385±0.008* (76.2%)[a]
CDDP (2 mg/kg) + MESB (250 mg/kg)	0.558±0.047 (-11.6%)[b]	0.785±0.26 (-103.9)[b]
CDDP (2 mg/kg) + MESB (500 mg/kg)	0.639±0.060** (-27.8%)[b]	1.23±0.21** (-219.5%)[b]
CDDP (2 mg/kg) + MESB (1000 mg/kg)	0.458±0.035 (8.4%)[b]	0.495±0.12 (-28.6%)[b]
MESB (1000 mg/kg)	0.742±0.047 (-22.0%)[a]	0.675±0.26*** (58.3%)[a]

Values expressed as mean ± standard error of mean (SEM).
*p<0.01 and ***p<0.05 when compared with control; **p<0.05 when compared with CDDP group.
Values in parenthesis represent % change; (-) = increase; (+) = decrease
[a] % change relative to control; [b] % change relative to CDDP.

Table 2E: Effect of the methanolic extract of soybean (MESB) on renal glutathione-S-uansferase (GST) activity and reduced glutathione (GSH) level in normal and cisplatin (CDDP) – treated rats.

Treatment	GST (*n*mol/min/g tissue)	GSH (µg/g tissue)
Control (0.9% NaCl)	0.247±0.05	31.52±2.86
CDDP (2 mg/kg)	0.067±0.02* (72.9%)[a]	6.12±1.43** (80.6%)[a]
CDDP (2 mg/kg) + MESB (250 mg/kg)	0.136±0.02 (-103.0%)[b]	15.03±1.03*** (-145.6%)[b]
CDDP (2 mg/kg) + MESB (500 mg/kg)	0.141±0.02 (-110.5%)[b]	21.46±2.53# (-250.7%)[b]
CDDP (2 mg/kg) + MESB (1000 mg/kg)	0.103±0.01 (-53.7%)[b]	8.20±1.45 (-34.0%)[b]
MESB (1000 mg/kg)	0.329±0.08 (-33.2%)[a]	21.63±1.40* (31.4%)[a]

Values expressed as mean ± standard error of mean (SEM).
*p<0.01 and **p<0.001 when compared with control.
***p<0.01 and #p<0.001 when compared with CDDP group.
Values in parenthesis represent % change; (-) = increase; (+) = decrease
[a] % change relative to control; [b] % change relative to CDDP.

Table 2F: Effect of the methanolic extract of soybean (MESB) on renal lipid peroxidation and total serum hydroperoxide (SHPO) in normal and cisplatin (CDDP) – treated rats.

Treatment	LPO (μunits MDA/mg protein)	SHPO (μmol/l)
Control (0.9% NaCl)	4.28±0.75	49.03±3.60
CDDP (2 mg/kg)	17.69±1.58* (-313.0%)[a]	66.72±1.0** (-36.1%)[a]
CDDP (2 mg/kg) + MESB (250 mg/kg)	9.49±0.50*** (46.4%)[b]	65.39±4.03 (2.0%)[b]
CDDP (2 mg/kg) + MESB (500 mg/kg)	8.03±0.76*** (54.6%)[b]	63.39±3.62 (5.0%)[b]
CDDP (2 mg/kg) + MESB (1000 mg/kg)	10.33±1.40*** (41.6%)[b]	63.44±4.41 (4.9%)[b]
MESB (1000 mg/kg)	11.92±0.82* (-178.5%)[a]	66.67±4.41** (-36.0%)[a]

Values expressed as mean ± standard error of mean (SEM).
*p<0.001 and **p<0.01 when compared with control; ***p<0.001 when compared with CDDP group.
Values in parenthesis represent % change; (-) = increase; (+) = decrease
[a] % change relative to control; [b] % change relative to CDDP.

Table 2G: Effect of the methanolic extract of soybean (MESB) on renal glucose-6-phosphatase (G6Pase) and 5'-nucleotidase (5'-NTD) activities in normal and cisplatin (CDDP) – treated rats.

Treatment	G6Pase (mmol/g tissue/min)	5'-NTD (mmol/g tissue/min)
Control (0.9% NaCl)	48.34±0.59	118.9±5.54
CDDP (2 mg/kg)	34.20±1.12* (29.3%)[a]	86.8±7.1* (27.0%)[a]
CDDP (2 mg/kg) + MESB (250 mg/kg)	40.0±2.20 (-17.0%)[b]	92.5±6.58 (-6.7%)[b]
CDDP (2 mg/kg) + MESB (500 mg/kg)	37.7±3.94 (-10.2%)[b]	86.6±9.98 (0.23%)[b]
CDDP (2 mg/kg) + MESB (1000 mg/kg)	32.1±3.77 (6.1%)[b]	83.7±13.0 (3.6%)[b]
MESB (1000 mg/kg)	45.3±0.72 (6.3%)[a]	116.4±3.1 (2.1%)[a]

Values expressed as mean ± standard error of mean (SEM).
*$p<0.05$ when compared with control.
Values in parenthesis represent % change; (-) = increase; (+) = decrease
[a] % change relative to control; [b] % change relative to CDDP.

Table 2H: Effect of the methanolic extract of soybean (MESB) on serum and renal post-mitochondrial fraction (PMF) total protein in normal and cisplatin (CDDP) – treated rats.

Treatment	Serum total protein (g/100ml)	PMF total protein (g/100ml)
Control (0.9% NaCl)	7.83±0.26	3.64±0.13
CDDP (2 mg/kg)	6.39±0.33*	2.21±0.21*
CDDP (2 mg/kg) + MESB (250 mg/kg)	7.41±0.62	2.35±0.19
CDDP (2 mg/kg) + MESB (500 mg/kg)	7.20±0.51	2.27±0.42
CDDP (2 mg/kg) + MESB (1000 mg/kg)	6.89±0.34	2.05±0.70
MESB (1000 mg/kg)	7.29±0.24	2.52±0.23*

Values expressed as mean ± standard error of mean (SEM)
*p<0.05 when compared with control.

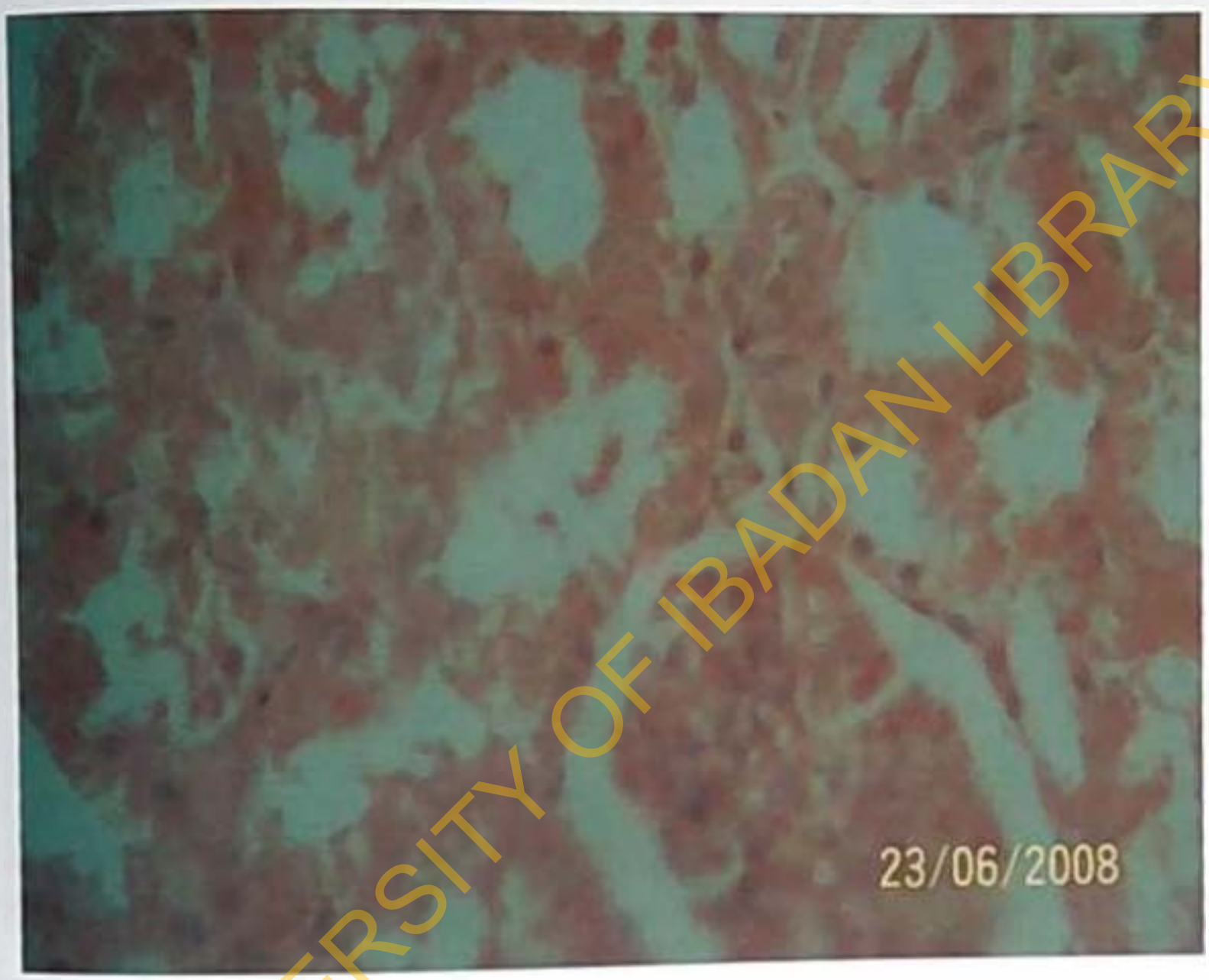


Figure 2E: Section of kidney from rat treated with normal saline (control) Renal morphology appears normal without any visible lesion

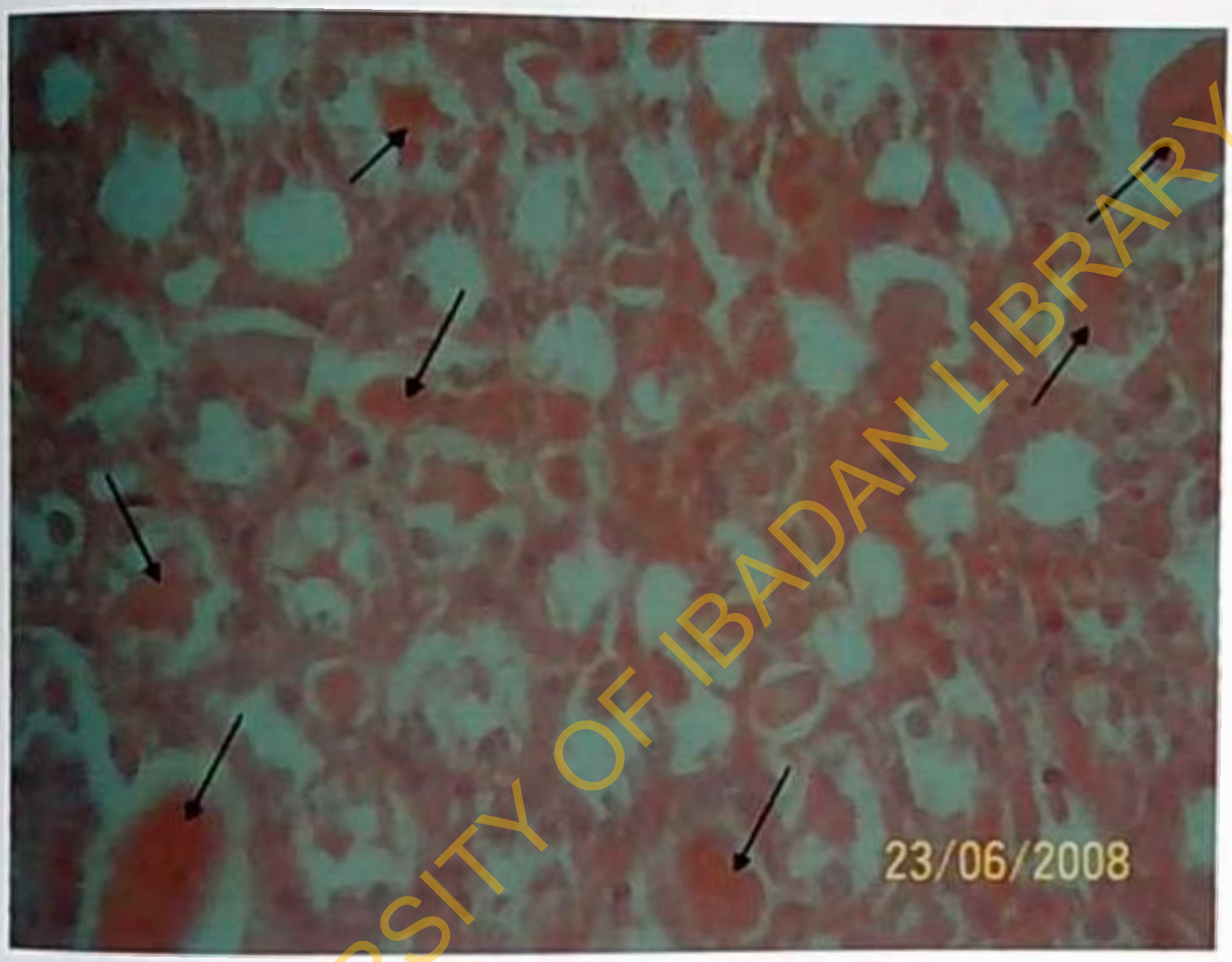


Figure 2F: Section of kidney from rat treated with cisplatin (2 mg/kg. i.p.) daily for 5 days. Section shows severe and generalized tubular epithelial cell necrosis associated with diffuse tubular lumina (hyalinized casts).

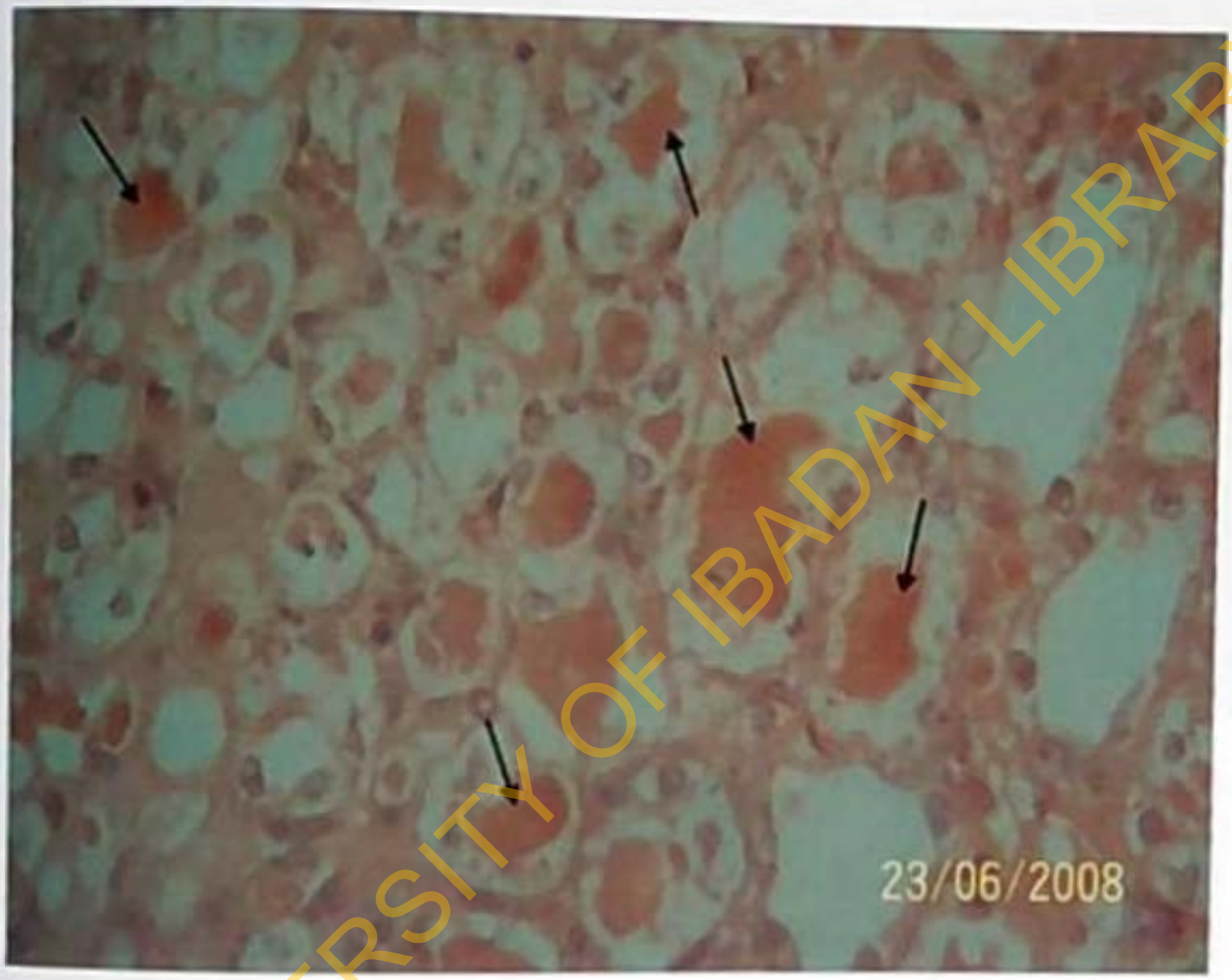


Figure 2G: Section of kidney from rat treated with methanolic soybean extract (250 mg/kg, *p.o.*) plus cisplatin (2 mg/kg, i.p.) daily for 5 days. Section shows generalized degeneration and necrosis of tubular epithelial cells associated with tubular dilatation. Some epithelial cells are detached from the basement membrane into the lumen with cells having a spongy appearance. Most tubules in the medullary zone contain protein casts.

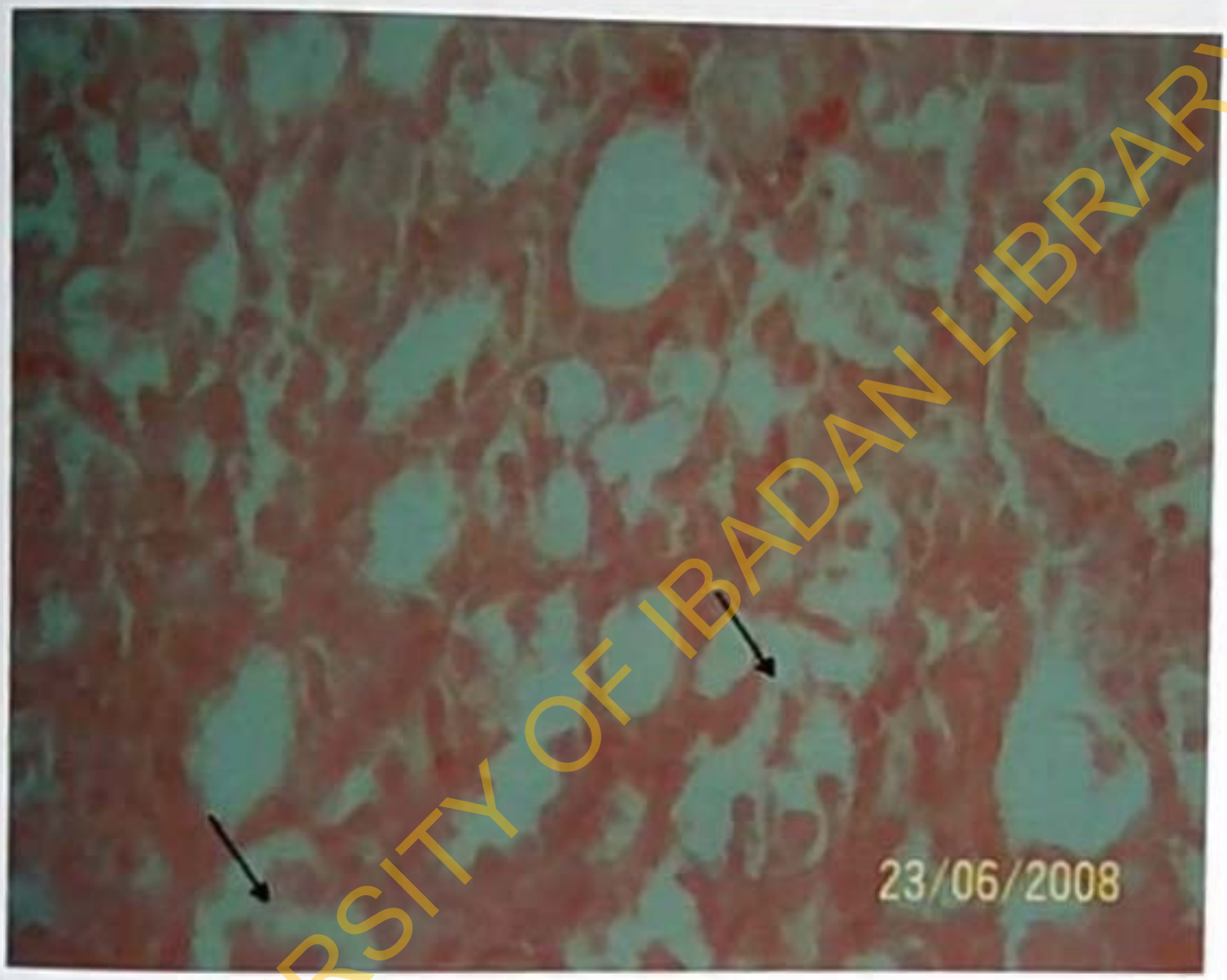


Figure 2H: Section of kidney from rat treated with methanolic soybean extract (500 mg/kg. p.o.) plus cisplatin (2 mg/kg. i.p.) daily for 5 days. Section shows diffuse moderate degeneration and necrosis of tubular epithelial cells associated with tubular dilation. Pinkish protein casts in tubular lumina.

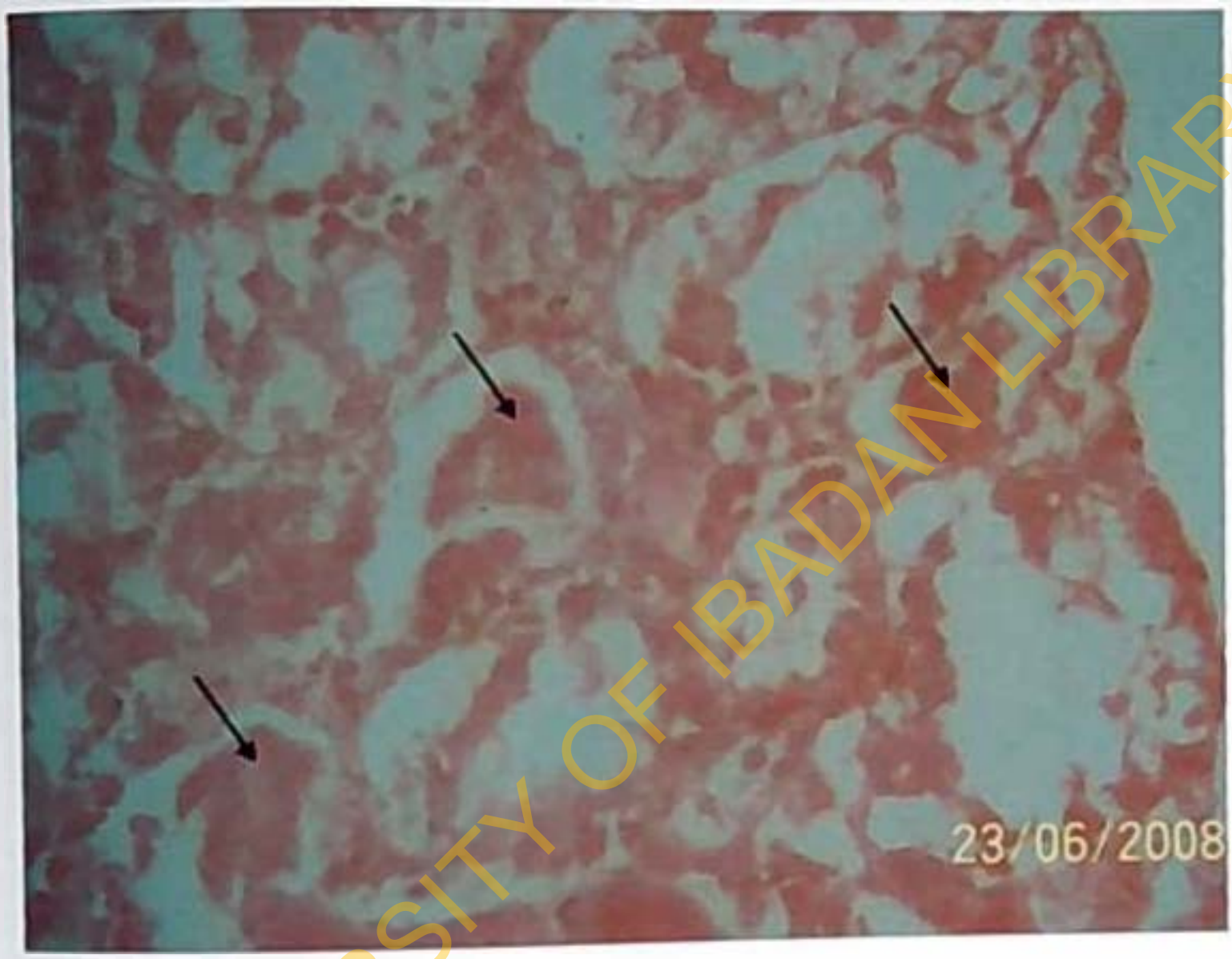
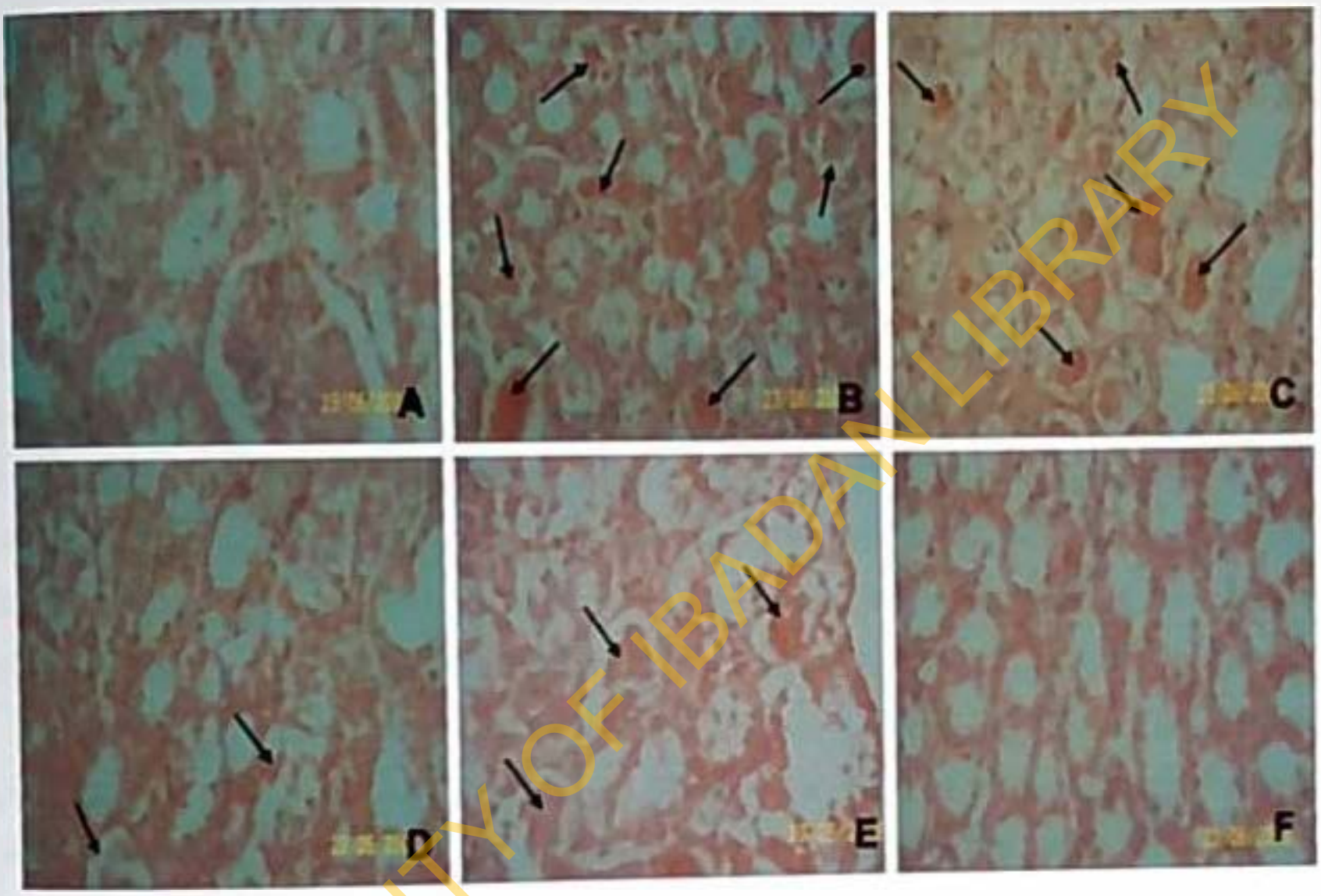


Figure 21: Section of kidney from rat treated with methanolic soybean extract (1000 mg/kg, *p.o.*) plus cisplatin (2 mg/kg. i.p.) daily for 5 days. Section shows generalized and severe degeneration and coagulative necrosis of tubular epithelial cells with dilated hypocellular tubules. Proteinaceous material in tubular lumina. Fibrous thickening of vascular walls. There are a few mononuclear cells in the medullary zone

Figure 2J: Section of kidney from rat treated with methanolic soybean extract (1000 mg/kg, p.o.) daily for 5 days. Section shows very few tubules with mild necrosis observed.



Summary of Photomicrograph of rat kidney sections (X400).
Histological sections of kidney from rat treated with A. Normal saline (control); B. Cisplatin (7mg/kg); C. Cisplatin + MESB (0.25g/kg); D. Cisplatin + MESB (0.5g/kg); E. Cisplatin + MESB (1.0g/kg); F. MESB (1.0g/kg) → indicate some tubules showing necrosis

Results for renal function tests are depicted in Figures 2A to 2D. Cisplatin (CDDP), when administered at a dose of 2 mg/kg/day for 5 days, significantly elevated serum creatinine (SCr) concentration and blood urea nitrogen (BUN) by 61.0% and 1091.8% respectively when compared with control (saline-treated) rats ($p<0.001$). Serum Na^+ ion decreased significantly ($p<0.001$) with a slight increase in K^+ ion concentration following CDDP treatment in the rats. This CDDP-induced acute renal failure was attenuated by MESB. Pretreatment with 250-, 500-, and 1000- mg/kg/day doses of the extract one-hour before CDDP injection (i.p.) reduced SCr by 34.7%, 62.1% and 24.6% and BUN by 49.8%, 59.0% and 21.1% respectively when compared with the CDDP group (Figures 2A and 2B). The reduction of these parameters produced by MESB were statistically significant at 250- and 500- mg/kg doses ($p<0.001$) and at 1000 mg/kg dose (which did not offer better protection than the lower doses) was not significant when compared with the CDDP group. The electrolyte imbalance induced by CDDP was also attenuated by MESB (figures 2C and 2D). MESB (1000 mg/kg) when administered alone produced mild and insignificant increase ($p>0.05$) in these parameters with when compared with control.

CDDP-induced acute renal failure was associated with significant ($p<0.01$) increase in myeloperoxidase (MPO) activity (Table 2A). MPO activity increased in the CDDP-treated rats by 276.8% when compared with control ($p<0.01$). Xanthine oxidase (XO) activity also increased by 27.6% in the CDDP-treated rats, though not statistically significant when compared with control ($p>0.05$). Similarly, there were mild increases in the activities of xanthine dehydrogenase (XDH) as well as combined XO plus XDH (Table 2B). Again, MESB at 250- and 500- mg/kg doses prevented the CDDP-induced increase in the activities of these enzymes, with the 1000 mg/kg dose not providing better protective effect. MESB (1000 mg/kg) when administered alone did not produce any significant changes in these parameters.

Renal dysfunction induced by CDDP was characterized by increases in urinary excretion of N-acetyl-β-D-glucosaminidase (NAG) and serum nitrate/nitrite (NO^-_3/NO^-_2) concentrations (index of nitric oxide production) (Table 2C). Urinary NAG activity and serum NO^-_3/NO^-_2 concentration increased by 79.7% and 175.8% respectively in the CDDP-treated rats when compared with control ($p<0.001$). Urinary NAG activity decreased by 37.7%, 49.2% and 14.6% and serumNO^-_3/NO^-_2 by 35.5%, 36.4% and 33.8% following treatment with 250-, 500- and 1000- mg/kg doses of MESB in the CDDP-treated rats respectively. Decreases in values were only

significantly different from control at 250- and 500- mg/kg doses of MESB ($p<0.01$). There was no significant change in serum NO_3^-/NO_2^- level and a slight increase (though not significant) in NAG activity was observed in rats treated with MESB (1000 mg/kg) only (Table 2C).

CDDP renal toxicity was associated with induction of oxidative stress in the rats. Table 2D shows the effect of MESB administration on catalase (CAT) and superoxide dismutase (SOD) activities in CDDP-treated rats. CAT and SOD activities significantly ($p<0.01$) decreased by 31.1% and 76.2% respectively following treatment with CDDP. MESB prevented this effect by increasing CAT activity by 11.6%, 27.8% and 8.4% and SOD activity by 103.9%, 219.5% and 28.6% at 250-, 500- and 1000- mg/kg doses respectively in the CDDP-treated rats. The increase produced by MESB in the activities of these enzymes was significant at 500 mg/kg dose in the CDDP-treated rats ($p<0.01$).

Treatment with CDDP significantly decreased glutathione-S-transferase (GST) activity ($p<0.01$) and also produced severe depletion of reduced glutathione (GSH) level ($p<0.001$) as shown in Table 2E. MESB mitigated these effects by increasing GST activity by 103.0%, 110.4% and 53.7% and GSH level by 145.6%, 250.7% and 34.0% at 250-, 500- and 1000- mg/kg doses respectively. GST activity increased by 33.2% and GSH level decreased significantly by 31.4% following only MESB (1000mg/kg) administration.

CDDP-induced decrease in antioxidant defense system resulted in significant increase in renal lipid peroxidation (LPO) and total serum hydroperoxide (SHPO) as indicated in table 6. LPO increased by 313.0% ($p<0.001$) and SHPO by 36.1% ($p<0.01$) in the CDDP-treated rats when compared with control. MESB significantly reduced renal LPO by 46.4% and 54.6% at 250- and 500-mg/kg doses respectively ($p<0.001$) and non-significantly by 41.6% at 1000 mg/kg dose when compared with CDDP-treated rats. MESB however did not significantly ($p>0.05$) affect SHPO level as only slight reductions were recorded at all dose levels. MESB (1000 mg/kg), on the other hand, significantly increased LPO ($p<0.001$) and SHPO ($p<0.01$) when administered alone (Table 2F).

The effect of MESB on CDDP-induced decreases in renal glucose-6-phosphatase (G6Pase) and 5'-nucleotidase (5'-NTD) are shown in Table 2G. CDDP significantly decreased G6Pase and 5'-NTD activities by 29.3% and 27.0% respectively when compared with control ($p<0.05$). MESB was most effective in reducing the effect

of CDDP on these enzymes at 250 mg/kg dose, though not significant. MESB increased G6Pase activity by 17.0% and 5'-NTD by 6.7% at this dose (250 mg/kg). MESB (1000 mg/kg) alone also decreased the activities of these enzymes, though not significantly.

CDDP significantly decreased total protein concentrations both in serum and kidney of rats ($p<0.05$) as shown in Table 2H. MESB did not produced significant changes in this parameter but provided moderate increases in values in the CDDP-treated rats.

Histopathological examination of sections from rat kidney treated with cisplatin show severe and generalized tubular epithelial cell necrosis associated with diffuse tubular lumina (hyalinized casts). Kidney sections from rats pretreated with 250-, 500- and 1000- mg/kg doses of MESB before cisplatin administration showed generalized degeneration and necrosis of tubular epithelial cells; diffuse moderate degeneration and necrosis of tubular epithelial cells; and generalized and severe degeneration and coagulative necrosis of tubular epithelial cells respectively with dilated hypocellular tubules and proteinaceous material in tubular lumina in all treatment groups. The 500 mg/kg dose of MESB provided the best histological protection against the renal tubular damage induced by cisplatin.

CONCLUSION

The methanolic extract of soybean (MESB) offered protection against cisplatin - induced renal toxicity by enhancing antioxidant defense, preventing inflammatory processes arising from enhanced polymorphonuclear leukocyte infiltration and accumulation as well as reducing tubular damage and enhancing membrane function in the rats.

43 EXPERIMENT 3: ANTIOXIDANT AND FREE RADICAL SCAVENVING ACTIVITIES OF THE METHANOLIC EXTRACTS OF SOYBEAN *IN VITRO*

INTRODUCTION

There is a growing body of evidence suggesting that free radicals play an important role in the development of tissue damage and pathological events in living organisms (Aruoma, 1998; Lefer and Granger, 2000; Smith *et al.*, 2000; Bhatia *et al.*, 2003; Olinski *et al.*, 2003; Peuchant *et al.*, 2004). Knowledge regarding the chemical nature and mechanisms of action of antioxidants, and their important role in disease prevention and treatment is rapidly evolving (Neergheen *et al.*, 2006).

Flavonoids, a group of naturally occurring polyphenolic compounds ubiquitously found in fruits and vegetables (Aherne and O'Brien, 2002; Hollman and Arts, 2000), have shown potential health benefits arising from the antioxidative effects of these phytochemicals. These properties are attributable to the phenolic hydroxyl groups attached to the flavonoids structure (Jovanovic *et al.*, 1994; Kandaswami and Middleton, 1994). Scavenging of free radicals seems to play a considerable part in the antioxidant activity of flavonoid compounds. In recent years, flavonoids as potent free radical scavengers have attracted a tremendous interest as possible therapeutics against free radical mediated diseases (Middleton *et al.*, 2000). Consumption of soybean-containing diets has been associated with the lower incidence of certain human cancers (Messina and Barnes, 1991; Barnes *et al.*, 1990; Setchell *et al.*, 1984). Genistein, the most abundant isoflavone in soy has been identified as a potent inhibitor of protein tyrosine kinases *in vitro* (Akiyama *et al.*, 1987). In this study, the antioxidant and free radical scavenging activities of MESB was evaluated *in vitro*.

PROCEDURE

The antioxidant activity of MESB was investigated in the trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), reducing power (RP), Fe^{3+}/ascorbate- and 2,2,-azobis(2-amidinopropane) hydrochloride (AAPH)- induced lipid peroxidation models. The radical scavenging activity (RSA) of MESB against chemically generated hydroxyl ($OH^{\cdot}$) radical in the deoxyribose assay, superoxide ($O^{\cdot}_2$), nitric oxide ($NO^{\cdot}$), 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$), and 2,2'-

azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\cdot}$) radicals, *in vitro*.

RESULTS

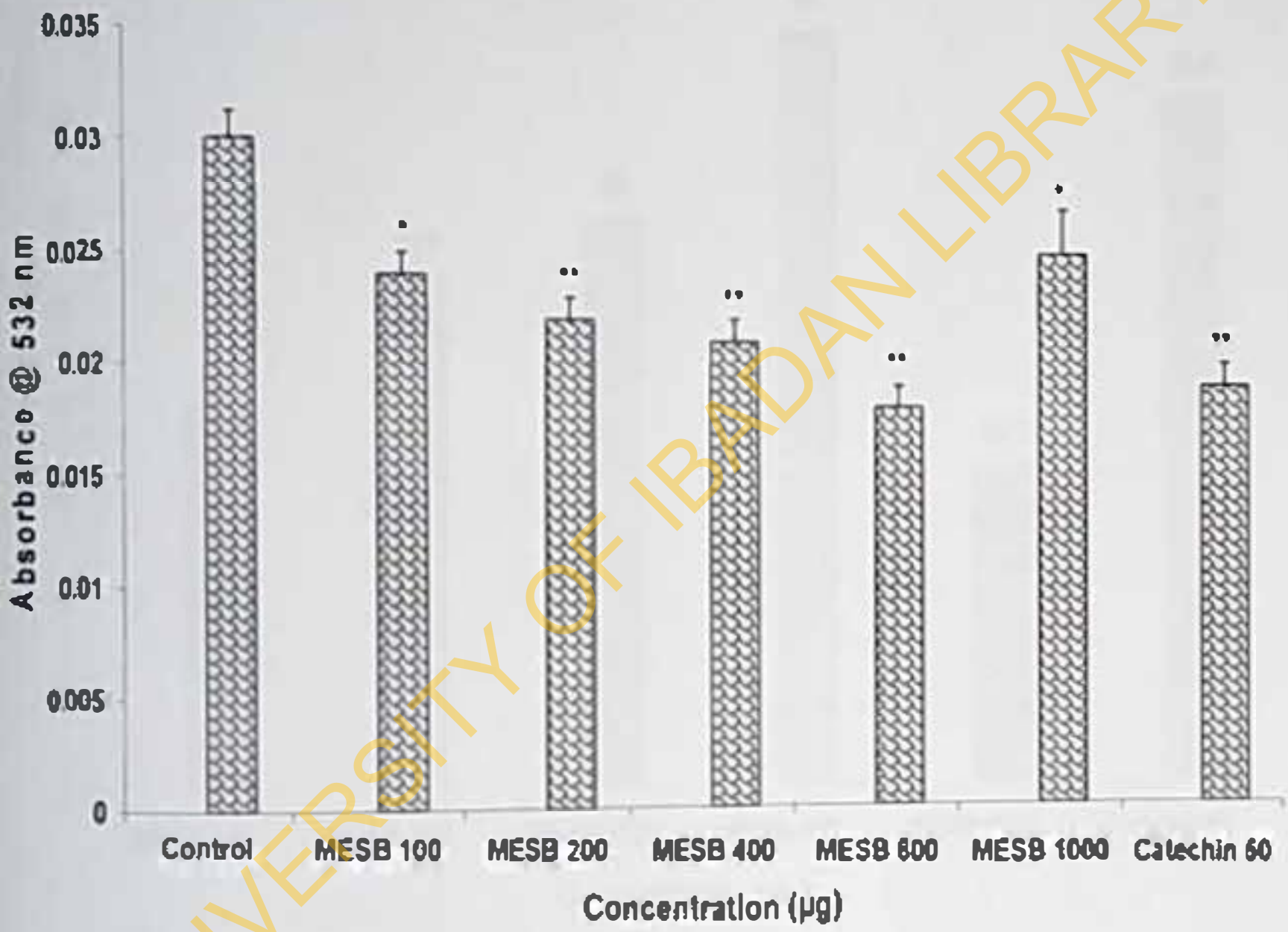


Figure 3A: Effect of methanolic extract of soybean (MESB) on AAPH-induced lipid peroxidation.

*$p<0.01$ and **$p<0.001$ when compared with control.

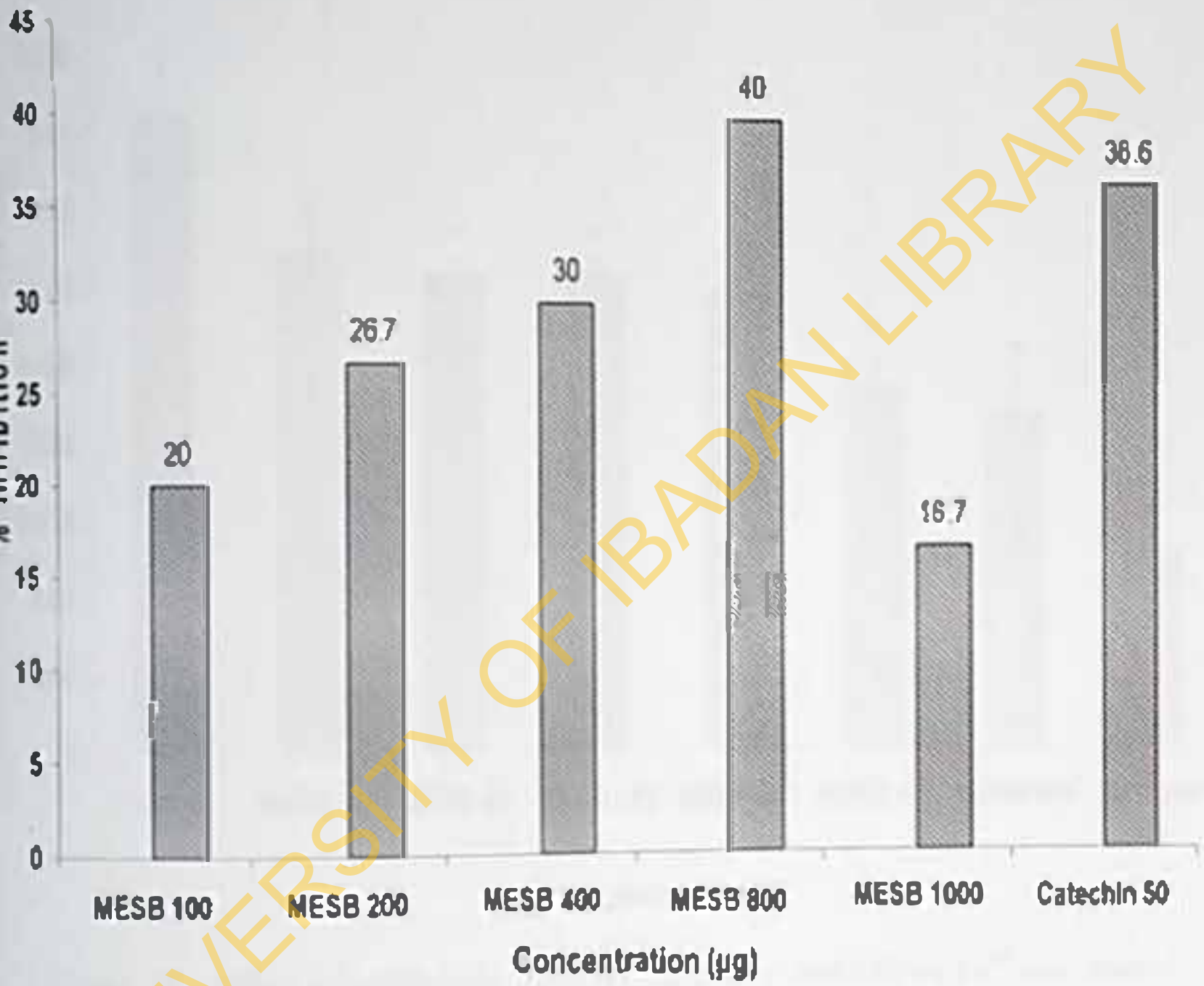


Figure 3B: % Inhibition of AAPH-induced lipid peroxidation by methanolic extract of soybean (MESB) and catechin.

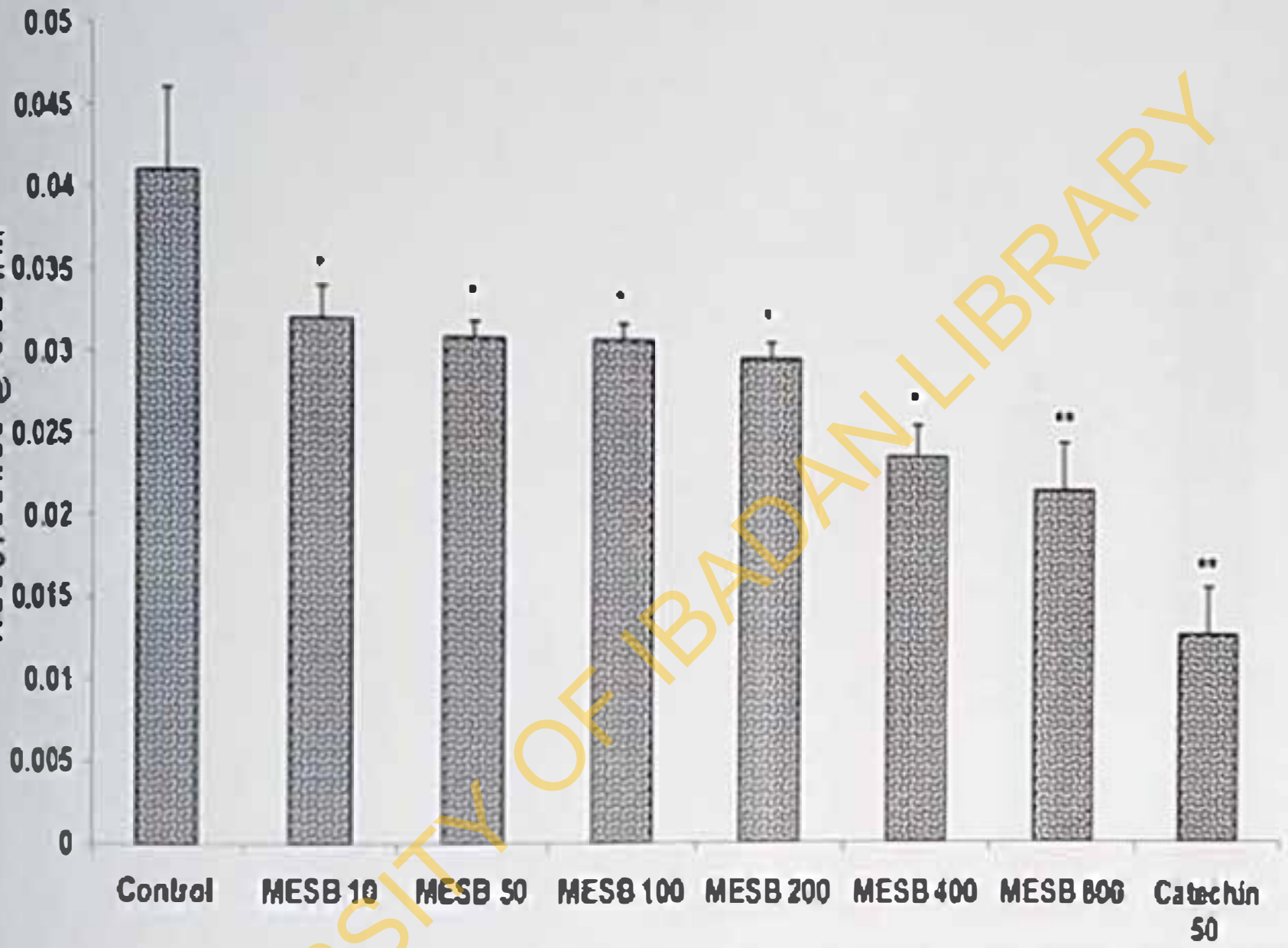


Figure 3C: Effect of methanolic extract of soybean (MESB) on Fe^{2+}/ascorbate-induced lipid peroxidation.

*p<0.05 and **p<0.001 when compared with control.

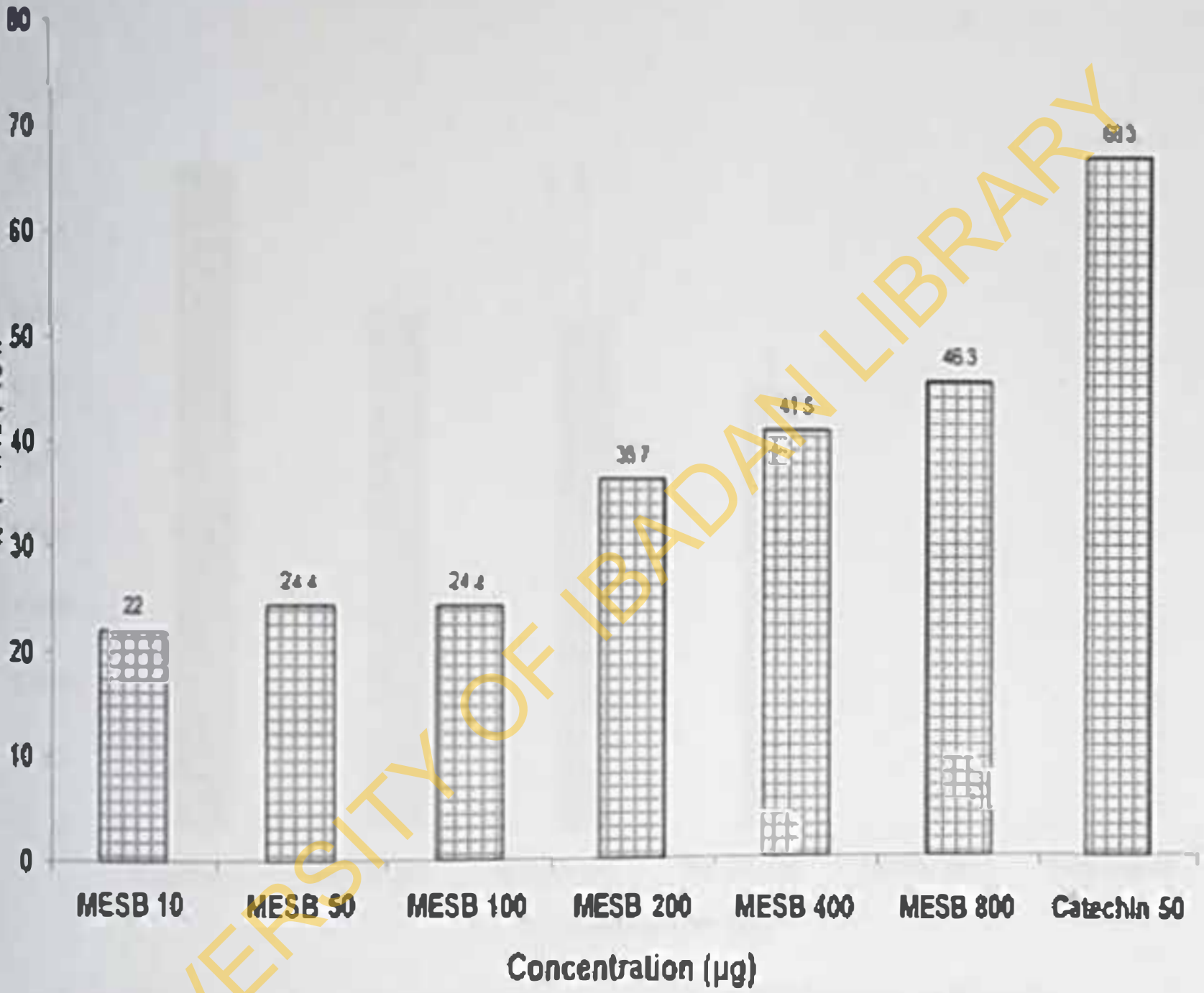


Figure 3D: Effect of methanolic extract of soybean (MESB) on Fe^{2+}/ascorbate-induced lipid peroxidation.

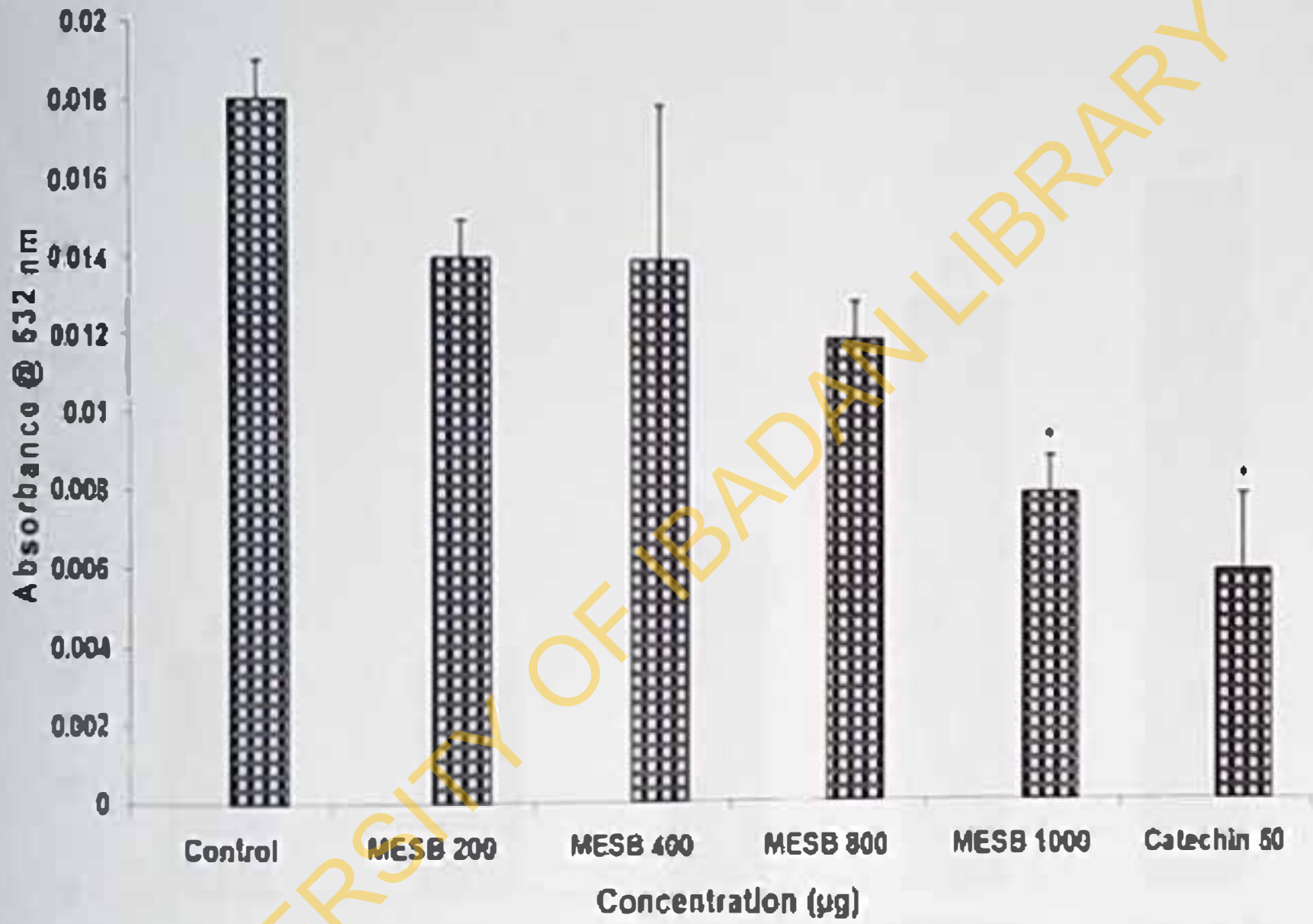


Figure 3E: Inhibition of deoxyribose oxidation by methanolic extract of soybean (MESB)

*p<0.05 when compared with control.

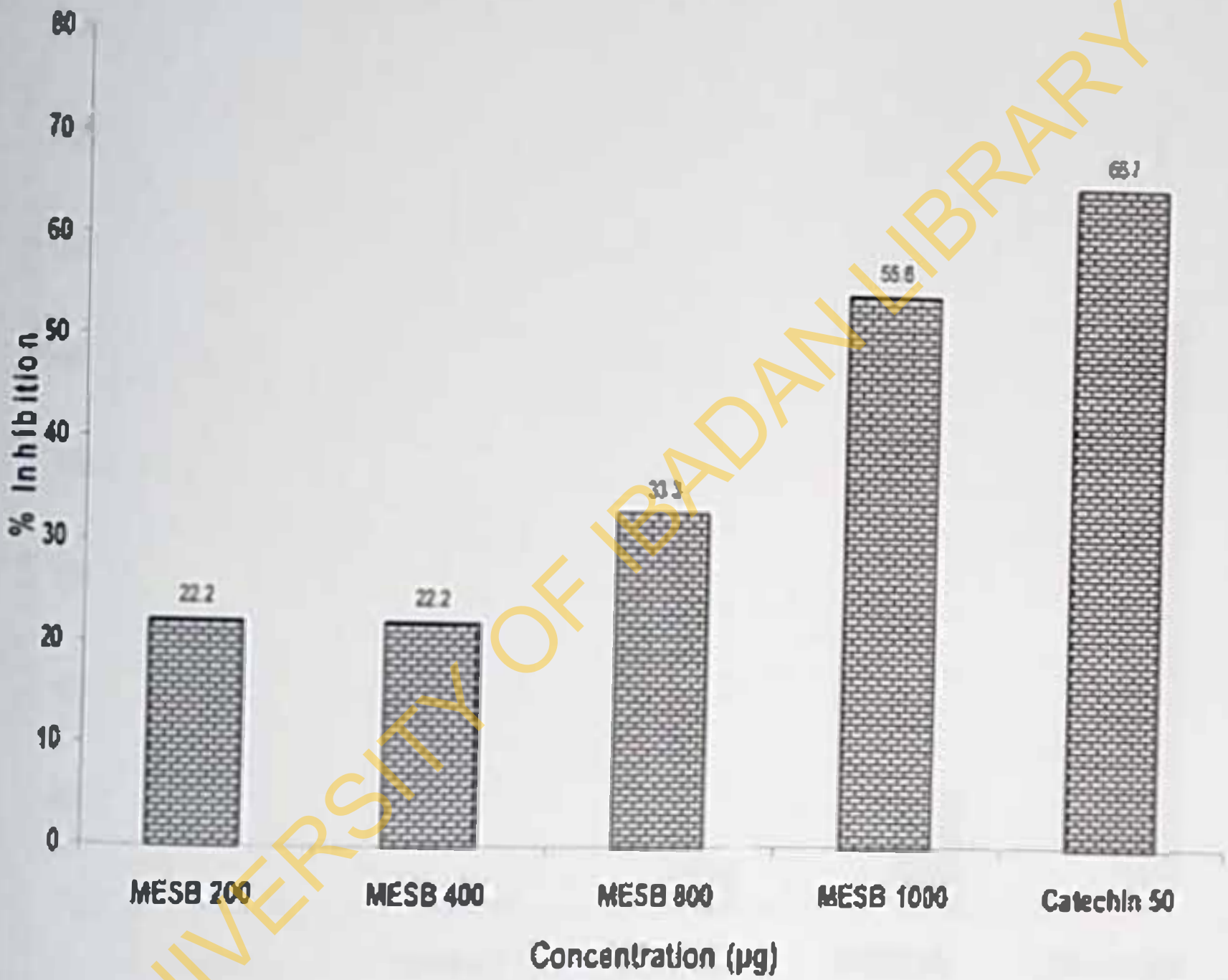


Figure 3F: Inhibition of deoxyribose oxidation by methanolic extract of soybean (MESB)

*p<0.05 when compared with control.

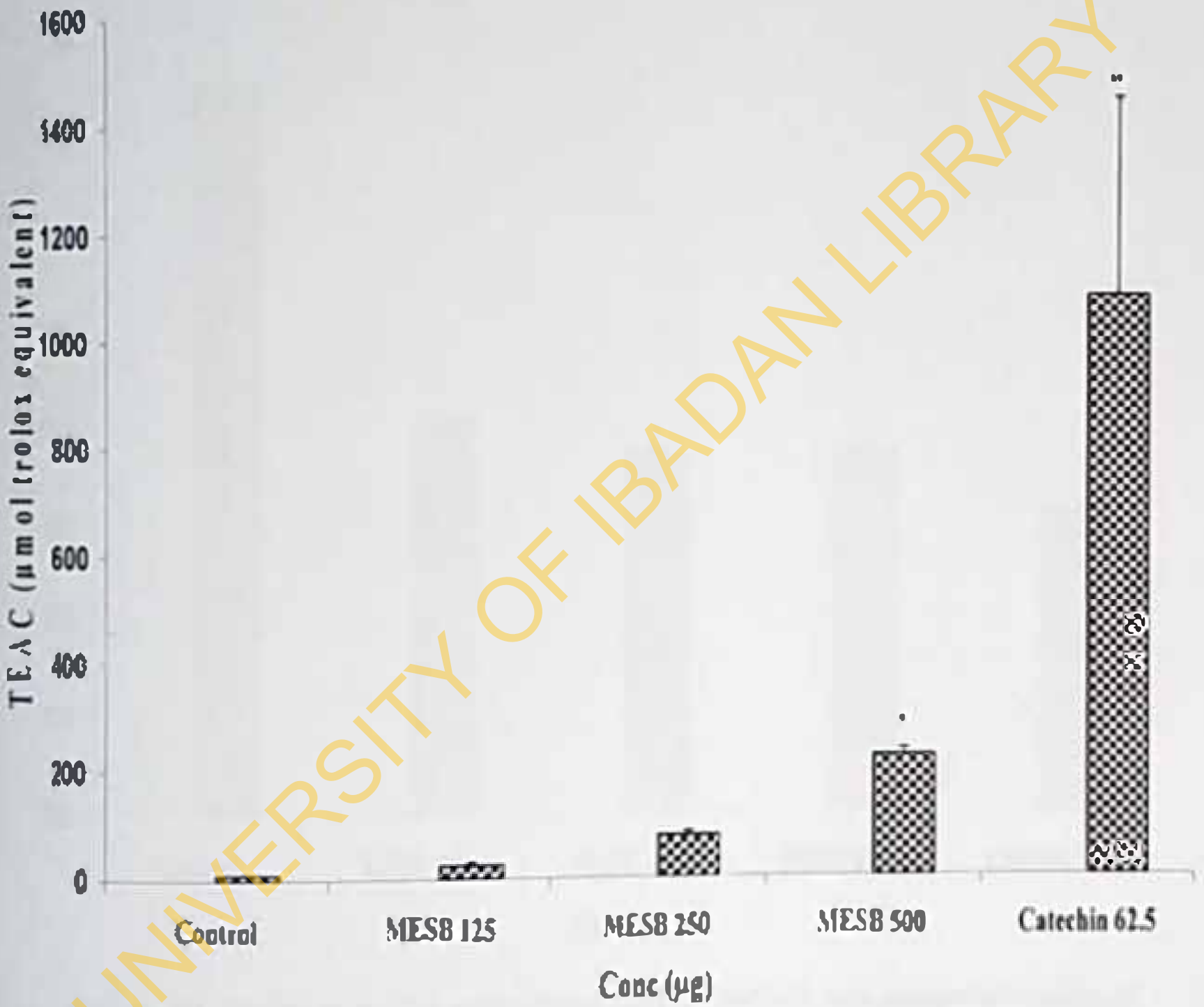


Figure 3G: Trolox antioxidant capacity (TEAC) of methanolic extract of soybean (MESB)

*p<0.05 and **p<0.001 when compared with control.

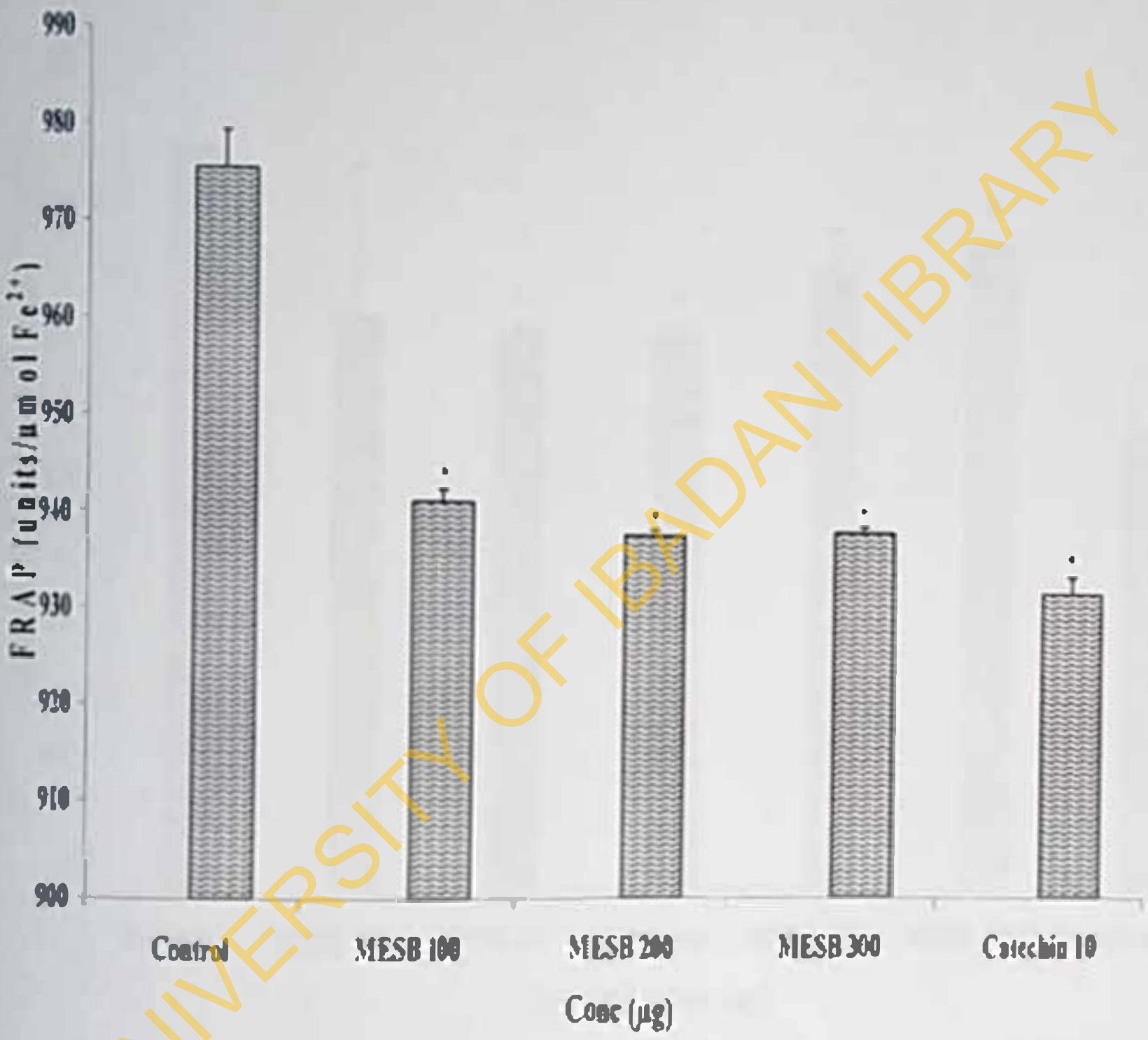


Figure 3H: Ferric reducing antioxidant power (FRAP) of methanolic extract of soybean (MESB)

*p<0.001 when compored with control.

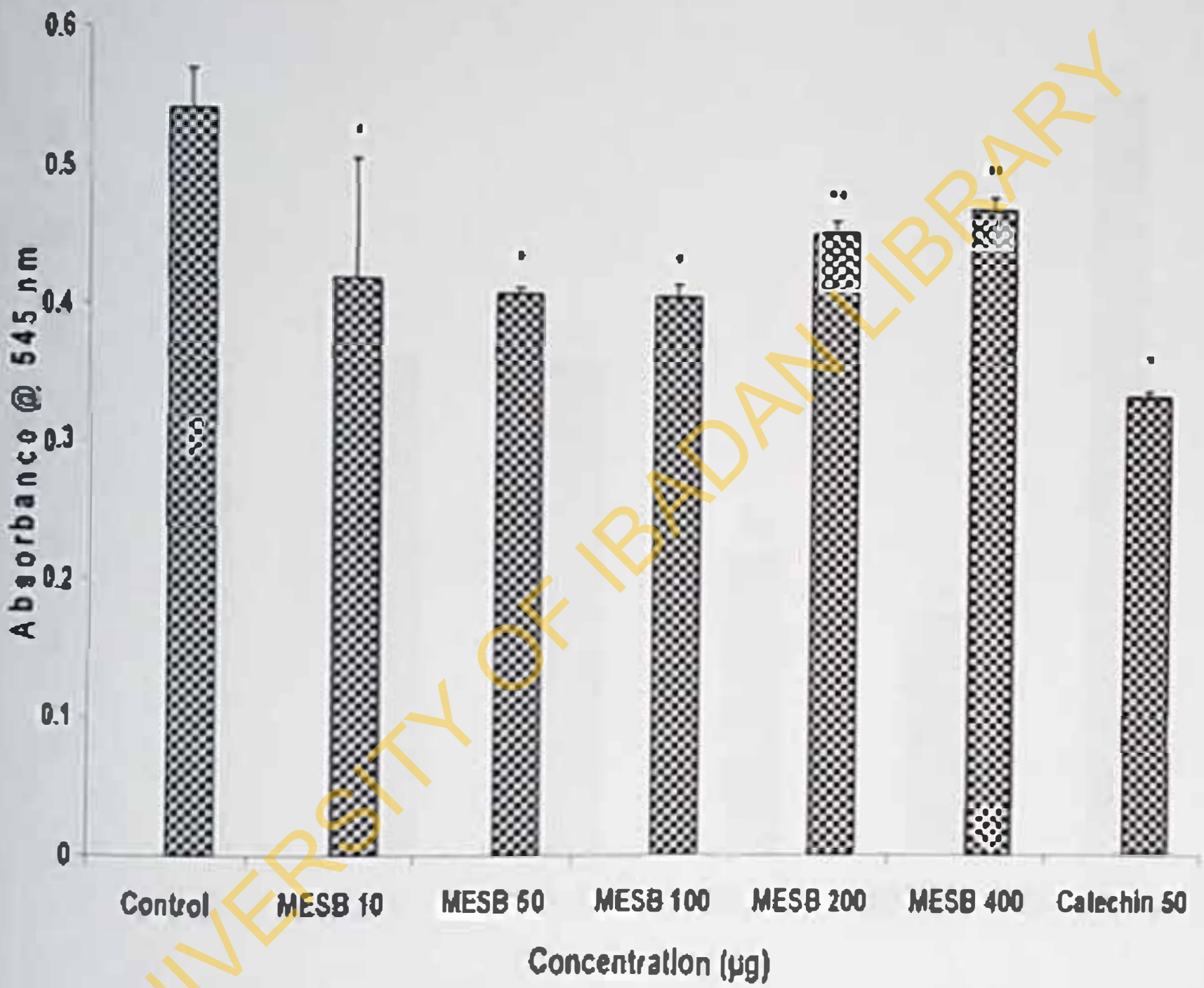


Figure 3I: Nitric oxide ($NO^{\cdot}$) radical scavenging activity (RSA) of methanolic extract of soybean (MESB)

*p<0.001 and **p<0.05 when compared with control

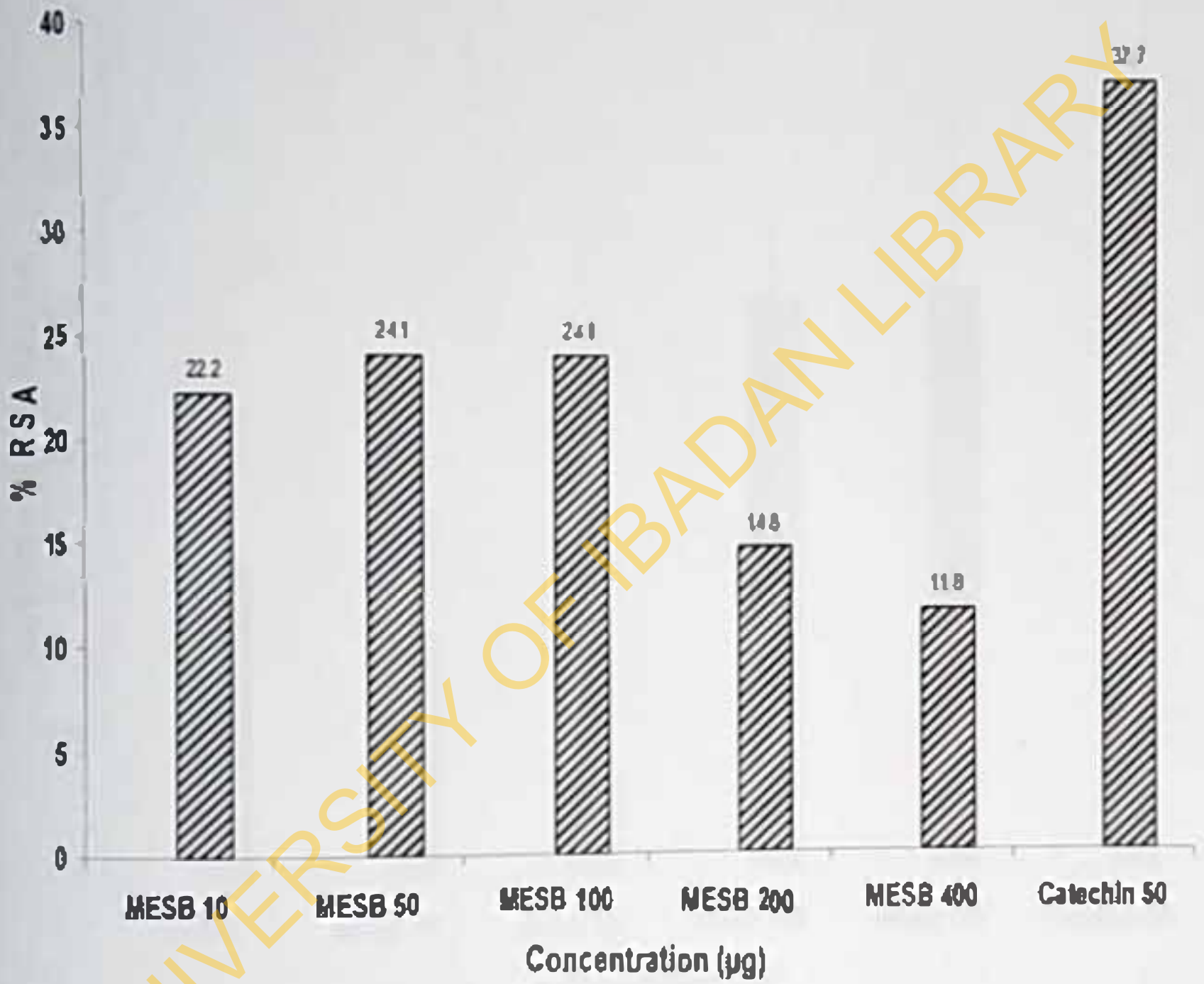


Figure 3J: Nitric oxide ($NO^{\cdot}$) radical scavenging activity (RSA) of methanolic extract of soybean (MESB).

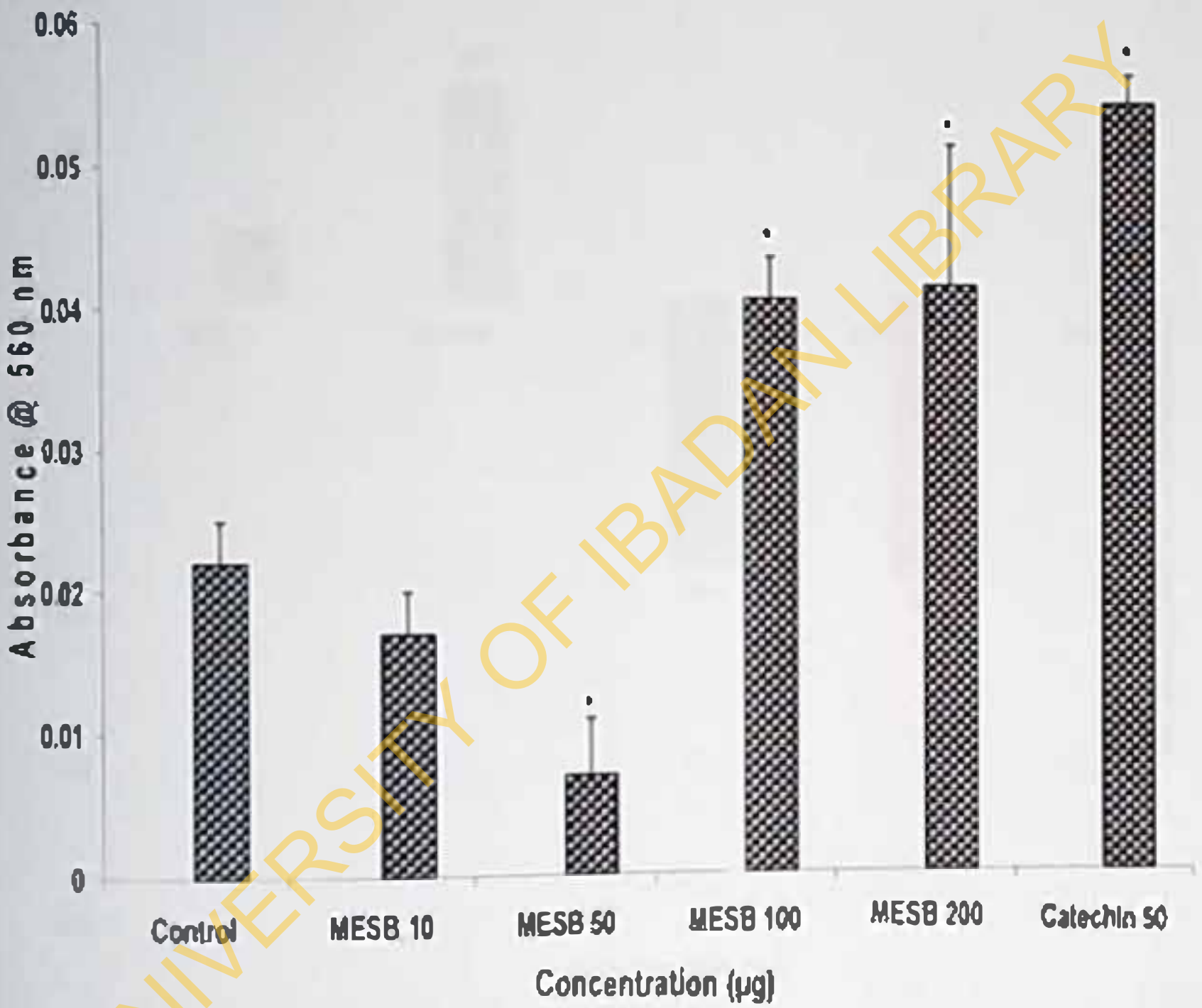


Figure 3K: Superoxide radical (O_2^-) scavenging activity of methanolic extract of soybean (MESB)

*$p<0.05$ when compared with control.

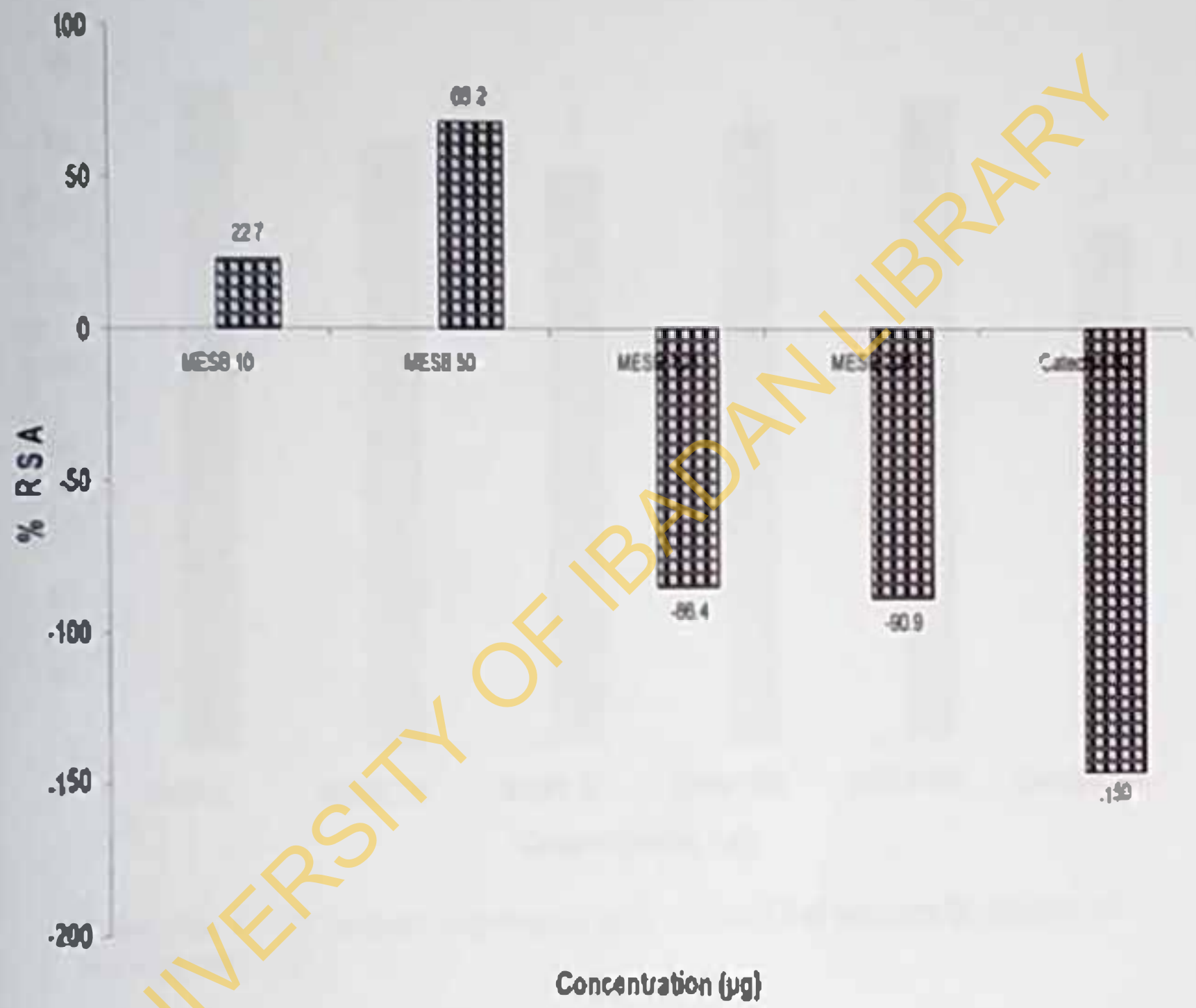


Figure 3L: Superoxide radical (O_2^-) scavenging activity (RSA) of methanolic extract of soybean (MESB).

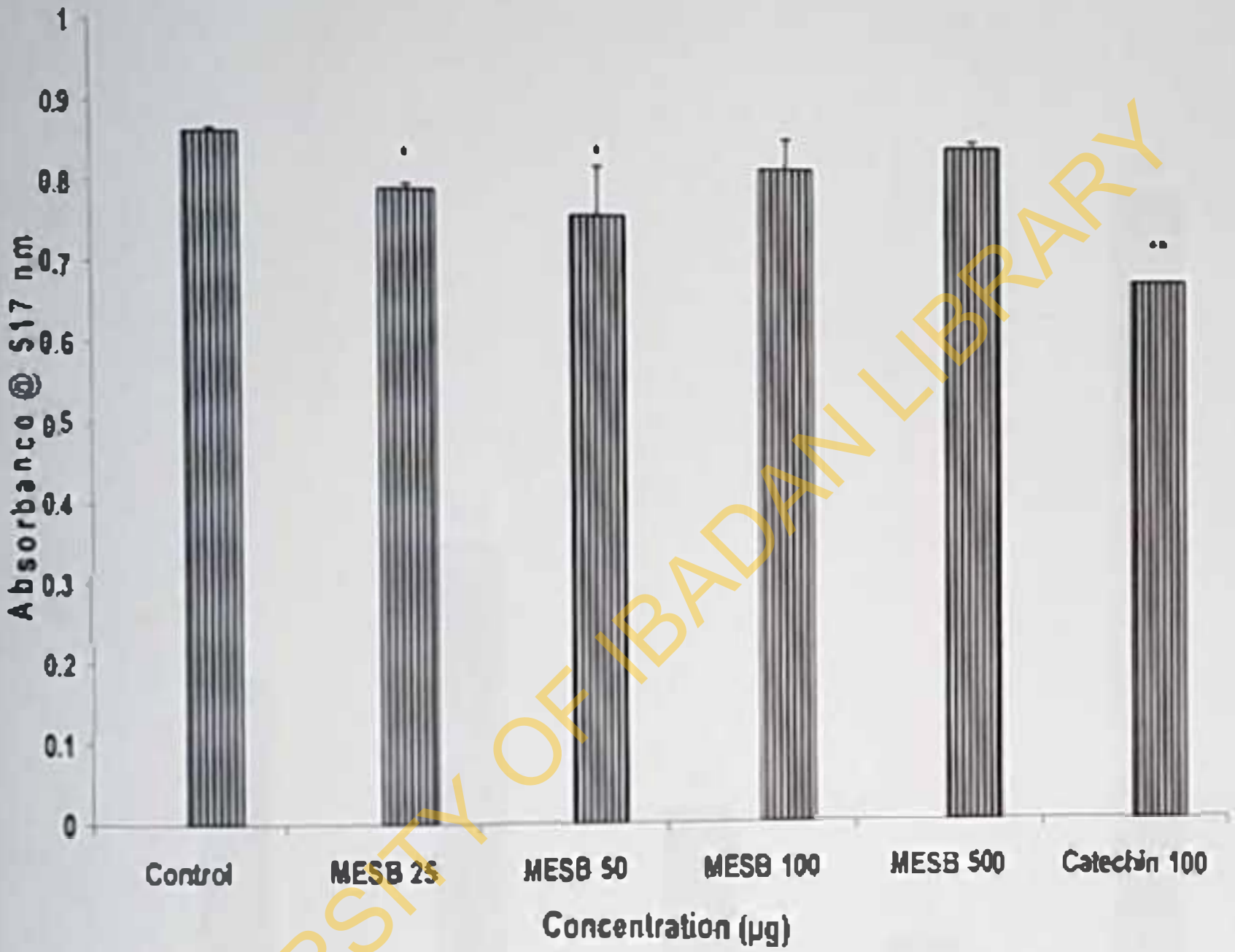


Table 3M: DPPH radical scavenging activity (RSA) of methanolic extract of soybean (MESB).

*p<0.05 and **p<0.001 when compared with control.

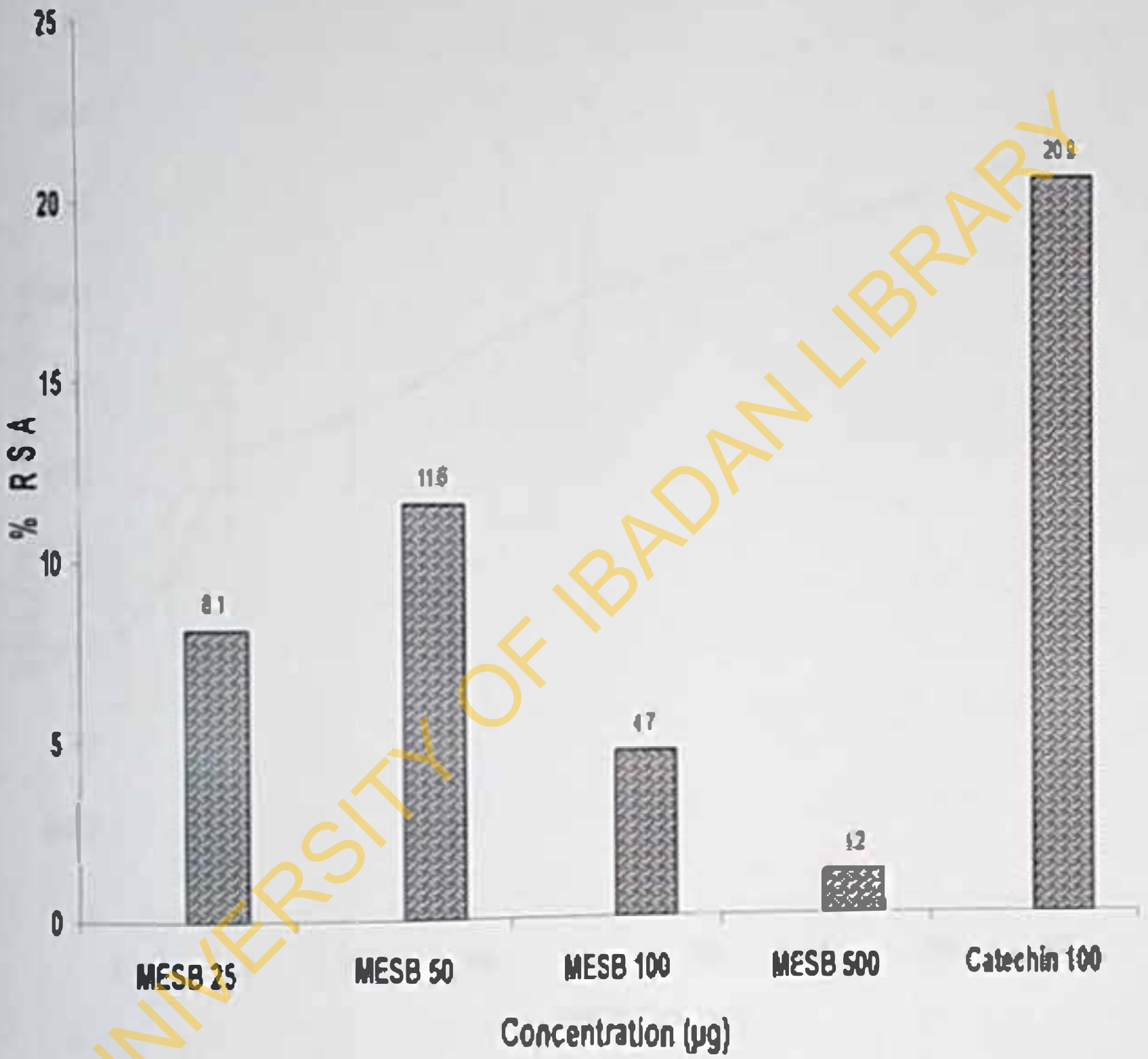


Figure 3N: DPPH radical scavenging activity (RSA) of methanolic extract of soybean (MESB).

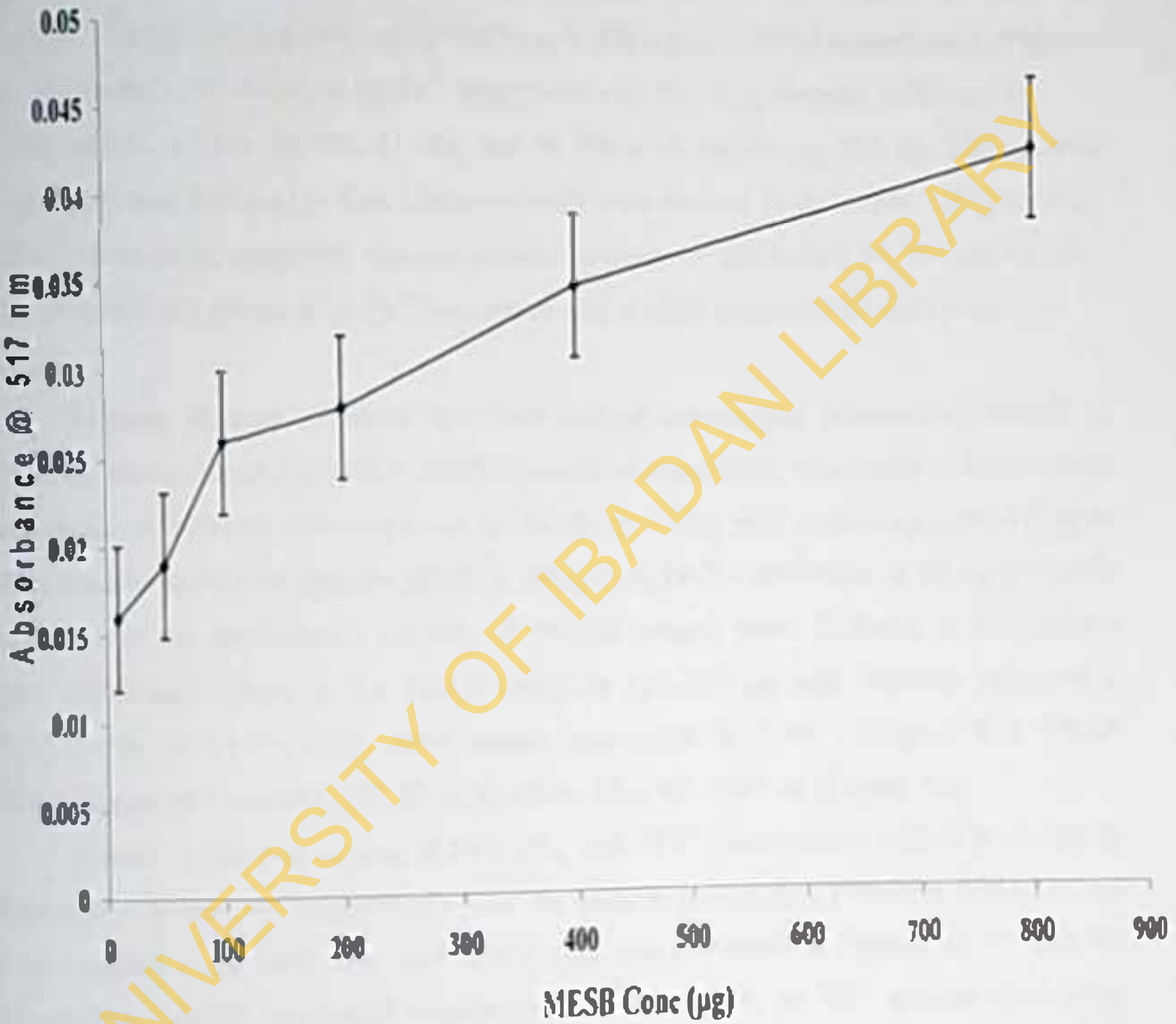


Figure 30: Reducing power of methanolic extract of soybean (MESB)

The antioxidant and free radical scavenging activities of MESB was evaluated. The effect of MESB on AAPH- and Fe^{2+}/ascorbate- induced lipid peroxidation (LPO) are shown in Figures 3A to 3D. MESB significantly inhibited LPO induced by AAPH as indicated by the decrease in absorbance when compared with control (i.e. reaction mixture without MESB) (Figure 3A). LPO was inhibited by 20.0%, 26.7%, 30.0% and 40.0% at 100 µg, 200 µg, 400 µg ($p<0.01$) and 800 µg ($p<0.001$) respectively (Figure 3B). Similarly, LPO induced by Fe^{2+}/ascorbate was also significantly inhibited by 22.0%, 24.4%, 24.4%, 36.7%, 41.5%, and 46.3% at 10 µg, 50 µg, 100 µg, 200 µg, 400 µg ($p<0.05$) and 800 µg ($p<0.001$) respectively with respect to the control (Figure 3D). The reference drug, catechin, showed greater potency by producing 36.7% and 68.3% inhibition of LPO induced by Fe^{2+}/ascorbate and AAPH respectively with respect to control.

Figures 3E and 3F show hydroxyl radical scavenging potential of MESB by inhibiting deoxyribose oxidation. MESB produced significant inhibition of deoxyribose degradation at 1000 µg concentration by 55.6% ($p<0.05$) with respect to control (Figure 3F). Catechin also show greater potency, producing 66.7% inhibition at 50 µg ($p<0.05$). Furthermore, the antioxidant activity of MESB ranged from 24.8±5.6 to 228.0±13.6 µmol trolox equivalent in the TEAC assay at 125-500 µg and catechin exhibited a TEAC value of 1110.0±380 µmol trolox equivalent ($p<0.001$) (Figure 3G). FRAP values decreased from 941.2±1.28 to 938.5±0.57 at 100-300 µg (Figure 3H).

Result of the scavenging of $NO^{\cdot}$, $O^{\cdot}_2$, and $DPPH^{\cdot}$ radicals by MESB is shown in Figures 3I, 3K and 3M respectively with the radical scavenging activities (RSA) of the extract against these ($NO^{\cdot}$, $O^{\cdot}_2$, and $DPPH^{\cdot}$) radicals presented in Figures 3J, 3L and 3N respectively. MESB exhibited maximum RSA of 24.1% on $NO^{\cdot}$ radical ($p<0.001$), 68.2% on $O^{\cdot}_2$ radical ($p<0.05$) and 11.6% on $DPPH^{\cdot}$ radical ($p<0.05$) at 50 µg ($p<0.05$). RSA activity of MESB decreased at concentrations greater than that producing the maximum scavenging effect and tended towards a prooxidant action with $O^{\cdot}_2$. MESB also demonstrated marked reducing power, increasing absorbance values from 0.016±0.007 to 0.043±0.009 at 10-800 µg (Figure 3O). The reducing power of MESB almost compares with that of catechin which increased the absorbance to 0.045±0.016 at 50 µg.

CONCLUSION

The methanolic extracts of soybean (MESB) exhibited significant free radical scavenging and antioxidant activities *in vitro*. The extract also showed prooxidant potential at high concentrations.

4.4 EXPERIMENT 4: PHYTOCHEMICAL ANALYSIS OF THE METHANOLIC EXTRACTS OF SOYBEAN

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is currently the most important source of edible oil and high-quality plant protein for feeding both human and animals worldwide (Zeller 1999, Friedman and Brandon 2001). Originated from mid latitude regions, this species is expanding in tropical areas as a result of the development of new genotypes tolerant to the environmental adversities of these localities (Vasconcelos *et al.*, 1997, Campelo *et al.*, 1999).

It has been reported that the environmental conditions such as latitude, altitude, temperature and precipitation can affect the seed composition as, for example, the protein and oil contents (Zeller 1999; Van Barneveld, 1999). Indeed, Qin *et al.*, (1998) showed that the contents of antinutritional factors were different between raw soybeans produced in two distinct geographical regions. Vollmann *et al.*, (2000) described genetic variations and significant differences in the protein content of soybeans cultivated in distinct climates. Based on these facts, it is reasonable to question whether the soybean cultivars adapted to grow in locality or regions present differences in their seed composition and nutritional profile when compared to cultivars grown in distinct regions around the world. Furthermore, information on the chemical composition and antinutritional and/or toxic protein contents of soybean cultivars exposed to typical tropical environment is scarce. Thus, the present experiment was undertaken to identify some of the known compounds and/or possibly isolate new compounds.

PROCEDURE

Adsorption column chromatography (CC) was performed with Kieselgel 60 (ASTM 70-230 mesh). Gel permeation was achieved using Sephadex LH-20. Reversed phase liquid chromatography (LC) was accomplished with RP-18 (size B, 40-63 mm, E. merck). Thin Layer Chromatography (TLC) analysis was done using analytical silica gel 60 $GF_{254+366}$ pre-coated aluminium backed plates (Merck, 0.25 mm thick). The resulting spots on TLC plates were visualized under UV light (254 nm) and detected by the use of vanillin/H_2SO_4, H_2SO_4, ferric chloride and DPPH spray reagents.

RESULTS

Table 4.1: Fractionation of SBEF on open column Chromatography and detection of antioxidant and phenolic compounds

Fractions	Codes	Weight	Reaction with DPPH	Reaction with $FeCl_3$
1	SBEF1	1.5 g	no activity	no activity
2-3	SBEF2	800 mg	no activity	no activity
4-5	SBEF3	255 mg	no activity	no activity
6-8	SBEF4	176 mg	moderate activity	no activity
9-10	SBEF5	450 mg	moderate activity	moderate activity
11-12	SBEF6	300 mg	moderate activity	moderate activity
13-16	SBEF7	750 mg	moderate activity	moderate activity

Table 4.2: Fractionation of SBEF5 on Sephadex LH-20 and detection of antioxidant and phenolic compounds

Fractions	Codes	Weight	Reaction with DPPH	Reaction with $FeCl_3$
1-2	SBEF5a	65 mg	no activity (pure) 1	no activity (pure)
3	SBEF5b	78 mg	no activity	no activity
4	SBEF5c	74 mg	no activity	no activity
5	SBEF5d	97 mg	no activity	no activny
6-11	SBEF5e	62 mg	no activity (pure) 2	moderate activity (pure)

Reaction with $FeCl_3$

EtOAc : MeOH (8.5 : 1.5)

Reaction with DPPH

EtOAc : MeOH (8.5 : 1.5)

Reaction with 10% H_2SO_4 in MeOH

EtOAc : MeOH (8.5 : 1.5)

Table 4.3: Fractionation of SBBF on open column Chromatography and identification of antioxidant and phenolic compounds

Fractions	Codes	Weight.	Reaction with DPPH	Reaction with $FeCl_3$
1-6	SBBFa	2.1 g	no activity	no activity
7-10	SBBFb	520 mg	weak activity	no activity
11-13	SBBFc	350 mg	moderate activity	no activity
14-18	SBBFd	700 mg	moderate activity	no activity
19-25	SBBFe	1.1 g	moderate activity	no activity

Table 4.4: Fractionation of SBBF on Reversed phase liquid chromatography (LC) and detection of antioxidant and phenolic compounds

Fractions	Codes	Weight.	Reaction with DPPH	Reaction with $FeCl_3$
1	SBBFd1	60 mg	No activity	no activity
2-7	SBBFd2	270 mg	moderate activity	no activity
8-22	SBBFd3	33 mg	weak activity	no activity
23-26	SBBFd4	109 mg	No activity	no activity

Table 4.5: Fractionation of SBBFd2 on Sephadex LH-20 and determination of antioxidant activity and phenolic group

Fractions	Codes	Weight.	Reaction with DPPH	Reaction with $FeCl_3$
1-2	SBBFd2a	65 mg	no activity	no activity
3-4	SBBFd2b	78 mg	moderate activity (pure) 3	no activity (pure)
5-6	SBBFd2c	74 mg	no activity	no activity
7	SBBFd2d	97 mg	moderate activity (pure) 4	no activity (pure)
8-12	SBBFd2e	62 mg	no activity	no activity

Dichloromethane : Ethanol (7 : 3)

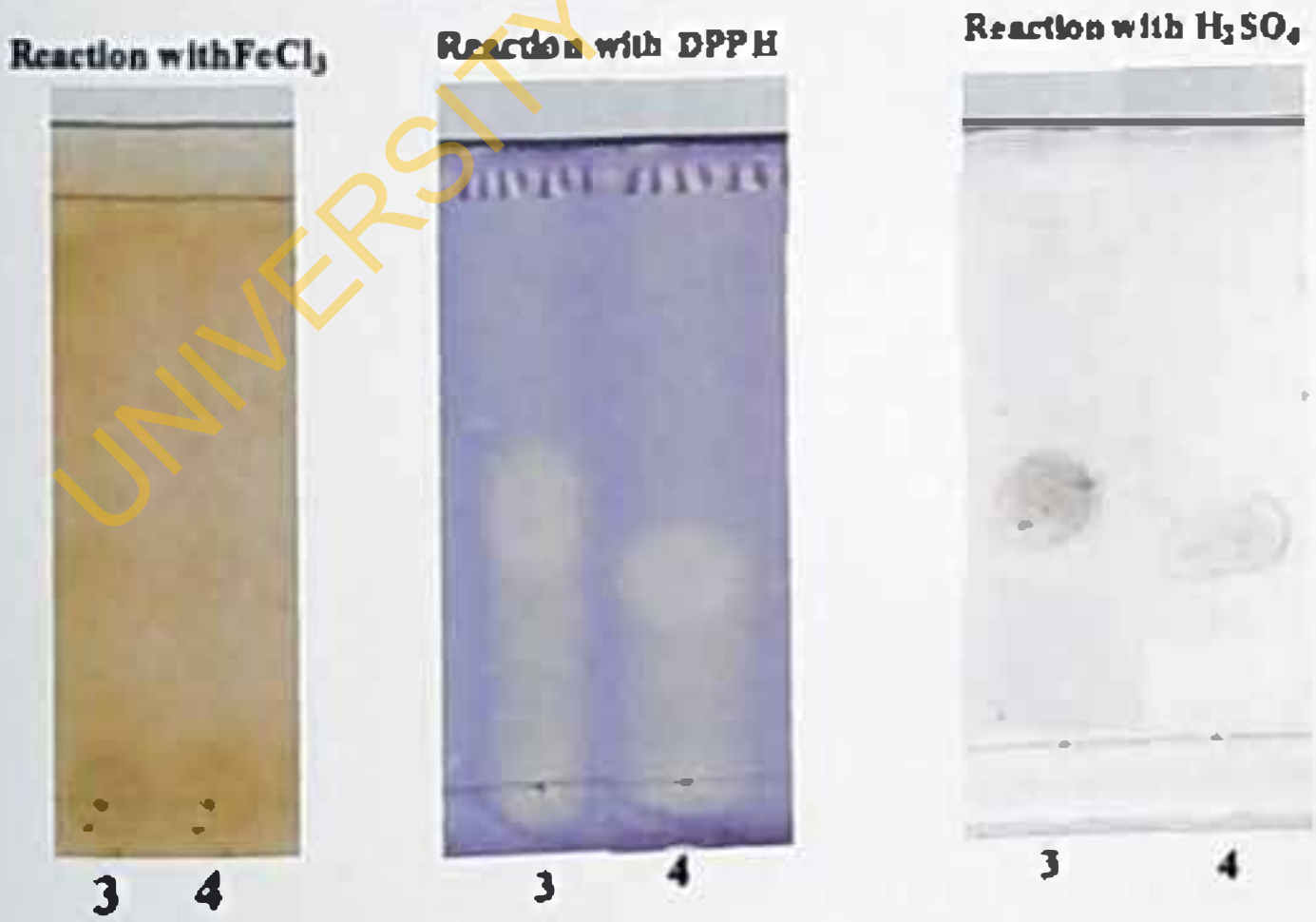


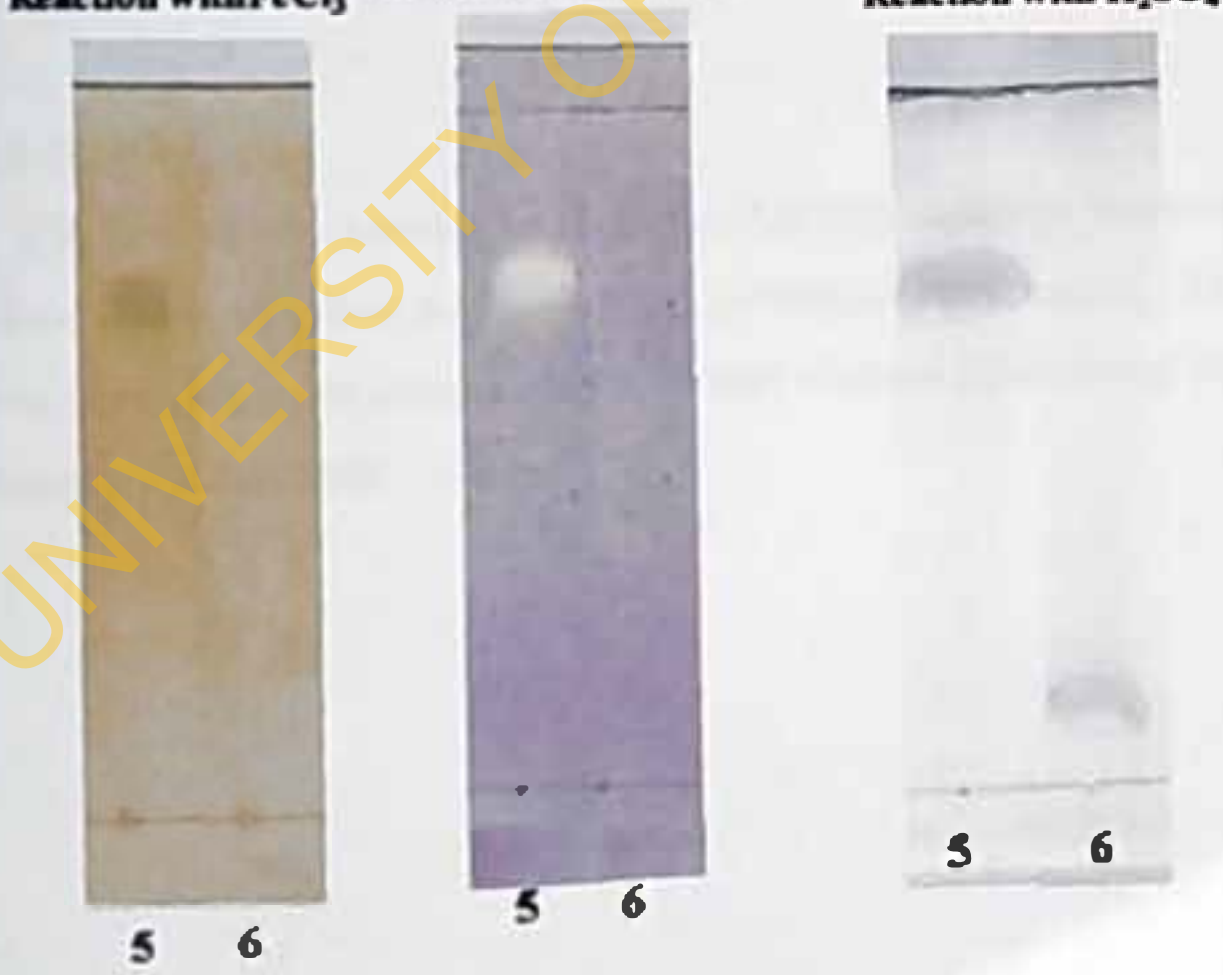
Table 4.6: Fractionation of SBBFb on Reversed Phase Liquid Chromatography and determination of antioxidant activity and phenolic group.

Fractions	Codes	Weight.	Reaction with DPPH	Reaction with $FeCl_3$
1-4	SBBFb1	210 mg	weak activity	no activity
5-10	SBBFb2	75 mg	very weak activity	no activity
11-18	SBBFb3	26 mg	no activity	no activity
19-23	SBBFb4	125 mg	no activity	no activity

Table 4.7: Fractionation of SBBFb1 on Sephadex LH-20 and determination of antioxidant activity and phenolic group

Fractions	Codes	Weight.	Reaction with DPPH	Reaction with FeCl$_3$
1-4	SBBFb2a	65 mg	No activity	no activity
5-6	SBBFb2b	78 mg	strong activity (pure) 5	weak activity (pure)
6-8	SBBFb2c	74 mg	No activity	no activity
9-10	SBBFb2d	97 mg	No activity (pure) 6	no activity (pure)

Dichloromethane : Ethanol (7 : 3)



Compound 1 showed neither antioxidant nor phenolic property.

Compounds 2 showed moderate phenolic property with ferric chloride without antioxidant property. The development of blue-black colouration spot on the orange background in compound 2 is stronger than compound 5 which also demonstrated phenolic property.

Compounds 3 and 4 demonstrated moderate antioxidant properties and the reaction with DPPH was within 2-3 minutes but without phenolic property at all.

Compound 5 showed both antioxidant and phenolic properties. However, the development of yellow spot on the purple background in compound 5 was within 1 minute (almost immediate) after spraying with DPPH while that of compound 3 and 4 showed up gradually about 4 minutes later, indicating that 5 has higher antioxidant activity than 3 and 4. This fact was further supported by the strong intensity of the yellow spot of 5.

Compound 6 was neither phenolic nor antioxidant in its reactions with ferric chloride and DPPH respectively.

However all isolated compounds reacted with the general purpose detecting reagent (sulphuric acid)

CONCLUSION

The methanolic extract of soybean (MESB) contains predominantly dihydroxy and trihydroxy phenolics with varying antioxidative strength. Also present in the extract are non-phenolic compounds with and without antioxidant properties as well as non-phenolic antioxidants.

CHAPTER FIVE

5.1 DISCUSSION

The incidence or prevalence of renal injury arising from the use of therapeutic drugs continues to soar, posing serious challenges to therapeutic outcomes in clinical practice. The mortality rate of patients with acute renal failure (ARF) has remained between 25 to 75% despite the use of various pharmacological agents (Thadhani *et al.*, 1996). Several therapeutic agents used in clinical practice have been reported to produce functional impairment and toxic injuries to the kidney and thus contribute a great deal to hospital acquired ARF and the development of nephropathy. The reason for this is not unconnected with the fact that the kidney is the major organ of excretion and as a result is exposed to large amount of parent and active metabolites of drugs and other xenobiotics. It becomes imperative therefore that deliberate efforts are directed towards protecting the kidney from injuries and damage during therapies with agents that predispose to renal pathologies.

Gentamicin and cisplatin are among the very useful chemotherapeutic agents that contribute to this ever increasing incidence of hospital acquired acute renal failure. Interestingly, the mortality rate from ARF has not changed significantly over the last 40 years. Several clinical and experimental strategies have been employed to reduce the predisposition to or mitigate this renal dysfunction and injury arising from the use of therapeutic agents but very little success has been achieved in this regard. The need therefore for studies aimed at clearly identifying the cellular and molecular mechanisms of nepbrotoxicity of these therapeutic drugs, and search for effective agents or compounds to protect against their toxicity to allow for safer use in patients at risk become imperative.

5.1.1 Gentamicin Nephrotoxicity and Protective effect of MESNA

The goal of reducing or protecting against aminoglycoside nephrotoxicity has attracted much effort and attention over the last two decades (Mingeot-Leclercq and Tulkens, 1999), but very little success has been achieved in this regard.

There is substantial evidence implicating the participation of reactive oxygen species in the pathogenesis of gentamicin nephropathy and administration of free radical scavengers like metalothioncin, selenium and vitamin E has provided some benefit in ameliorating this toxicity (Yang *et al.*, 1991; Ademuyiwa *et al.*, 1990).

This study evaluated the effect of defatted MESB on gentamicin – induced nephrotoxicity. Isoflavones are the major phenolic compounds in soybean and they are known for their biological activities including oestrogenic, antioxidant and antitumour activities (Messina *et al.*, 1994; Knight and Eden, 1996). It was on this basis that MESB was screened for possible chemopreventive action against gentamicin nephrotoxicity.

Results from this study show that rats treated with gentamicin developed marked acute renal dysfunction or damage as evident by the significant elevation in plasma creatinine and blood urea nitrogen (indicators of impaired glomerular function). This was also associated with significant hyperuraemia and mild K^+ ion depletion. Renal histology also revealed massive tubular necrosis with deposition of colloid casts within the tubular lumina. Pre-treatment with 500 mg/kg/day of defatted MESB significantly improved renal function and histology. A higher dose of the extract (1000 mg/kg/day) did not offer greater protection but instead produced mild increases in plasma creatinine and urea concentrations, suggesting some interference with glomerular function.

The relationship between gentamicin - oxidative stress and nephrotoxicity was further established in this present study. Gentamicin nephropathy was associated with decreased activity of SOD and CAT in the kidney, as already observed in several studies. SOD and CAT enzymes are major primary antioxidant defense components that primarily catalyze the dismutation of superoxide radical ($O_2^{\cdot}$) to H_2O_2 and decomposition of H_2O_2 to H_2O respectively (McCord and Fridovich, 1969; Cheng *et al.*, 1981). The decreased SOD and CAT activity induced by gentamicin results in accumulation of $O_2^{\cdot}$ and H_2O_2 which react with metal ions to promote additional radical generation, with the release of the particularly reactive hydroxyl radical (Stadtman, 1990). Hydroxyl radicals react at nearly diffusion-limited rates with any component of the cell, including lipids, DNA and proteins. The net result of this non-specific free radical attack is a loss of cell integrity, enzyme function, and genomic stability (Gille *et al.*, 1994). The involvement of these reactive oxygen species (ROS) in the impairment of glomerular filtration rate (GFR) has been reported (Hughes *et al.*, 1996). Moreover, the elevation in glomerular antioxidant enzymes protects renal function against the injury induced by ROS (Yoshika *et al.*, 1990). The protective effect of MESB therefore could be attributed to its antioxidant properties. The increase in the activity of SOD and CAT produced by the extract in the gentamicin treated rats prevents exaggerated production of ROS and oxidative damage. In addition, the renal amount of nitric oxide,

which plays an important role in the regulation of GFR (Baylis and Qin, 1996), may be decreased as a consequence of its reaction with the excessive amount of $O_2^{\cdot}$ because of the low activity of SOD (Oury *et al.*, 1996). It is therefore reasonable to suggest that MESB may also be preserving renal nitric oxide levels by increasing SOD activity, thus preventing $O_2^{\cdot}$ accumulation and reducing the production of the toxic reaction product, peroxynitrite anion ($ONOO^-$) arising from the reaction between $NO^{\cdot}$ and $O_2^{\cdot}$.

The results from this study further reveal that gentamicin nephrotoxicity is associated with decrease in renal GST activity and excessive GSH utilization resulting in depletion of cellular GSH level. Since GSH is a hydroxyl radical scavenger, result from this study correlates with other findings that hydroxyl radical may play a role in gentamicin nephrotoxicity (Shah and Walker, 1992). Pretreatment with MESB reduced the gentamicin-induced renal depletion of GSH and decrease in GST activity. This protective effect on renal GST was dose dependent and significant at 1000 mg/kg. Treatment with the extract alone (1000 mg/kg) produced a moderate increase in GST activity, thus explaining the preservation of GST activity in the gentamicin - treated rats even when the extract alone at this dose is also capable of reducing renal GSH. The apparent decrease in both renal and blood GSH levels with MESB treatment at 1000 mg/kg dose in the gentamicin - treated rats may be associated with increased utilization of GSH both as a hydroxyl radical scavenger and as a substrate for GST – catalyzed reactions. This process usually facilitates detoxification and excretion but may also be involved in the biosynthesis of certain compounds such as leukotriene C_4 (LTC_4) (Nicholson *et al.*, 1993), and prostaglandins (Ujihara *et al.*, 1988). PGE_2 can be transformed to the dehydration product prostaglandin A_2 (PGA_2), and PGE_1 to PGA_1 (Suzuki *et al.*, 1988), both of which are potentially toxic (Bogaards *et al.*, 1997). PGA_2 – GSH conjugates may be involved in the cytotoxicity of PGA_2 (Parker and Ankel, 1992). Although Assael *et al.*, (1985), had earlier hypothesized that the increase in PGE_2 observed in their work causes vasodilatation in the kidney to maintain normal renal blood flow and GFR during the development of gentamicin nephrotoxicity, the increased PGE_2 production may actually be contributing to the renal damage through its transformation to the cytotoxic form, PGA_2–GSH conjugates. It's possible therefore that increased leukotrienes and prostaglandins biosynthesis and their conjugation products play significant role in gentamicin nephrotoxicity and MESB at high doses may be exerting some influence on this pathway to cause increase GSH depletion as observed in this study.

Furthermore, many GSH conjugates undergo further enzymatic modification by hydrolysis of the glutathione – S – conjugate at the γ-glutamyl bond. This reaction is catalyzed by the enzyme γ-GT. In addition to hydrolysis, γ-GT can catalyze transpeptidation or auto-transpeptidation. γ-GT is an enzyme localized in the cell membrane of many cell types including kidney tubules. The kidney, in fact, has been shown to have the highest activity in several mammals studied, including humans (Hinchman and Ballatori, 1990). The decreased activity of this enzyme due to gentamicin treatment as observed in this study may also play a role in gentamicin nephrotoxicity by impairing further enzymatic modification of cytotoxic GSH conjugates. The activity of AST, which is also located in the proximal tubule, (Guder and Ross, 1984) was decreased. 5'-NTD and G6Pase (suitable marker enzymes for plasma membranes) were similarly decreased in the gentamicin - treated rats. The inactivation of these plasma membrane marker enzymes reveals membrane damage from gentamicin - induced oxidative stress, causing disruption in membrane function and its eventual collapse. All these may contribute to the proximal tubular damage during gentamicin treatment as also observed in several studies. The MESB in this study increased G6Pase activity, though not significantly, at a dose of 500 mg/kg/day but caused a significant decrease in the activity of the enzyme at 1000 mg/kg/day in the gentamicin - treated rats. The activity of 5'-NTD, however was decreased dose – dependently in these rats, though not significant. Treatment with the extract alone (1000 mg/kg/day dose) decreased the activities of these enzymes, the effect being stronger on G6Pase. This seems to further suggest that MESB in large doses may induce mild to moderate impairment of renal function and possibly in part, by impairing tubular membrane function. In addition, the 1000 mg/kg dose of MESB did not offer greater histological protection than the 500 mg/kg dose against gentamicin - induced renal toxicity.

It seems possible therefore, that the defatted MESB at a relatively low dose has a specific stabilizing effect on the cell membrane and/or other vital cellular macromolecules and can thus protect against gentamicin - induced microsomal damage or tubular necrosis. The protective effects of the extract appear to be related to the antioxidant polyphenolic content present in soybean.

5.1.2 Cisplatin Nephrotoxicity and Protective effect of MESB

Cisplatin (*cis*-diammincdichloroplatinum II, CDDP) and other platinum derivatives are among the most effective chemotherapeutic agents widely used in the treatment of a variety of malignancies, including head and neck, ovarian and testicular cancers. However, the full clinical utility of these drugs (especially cisplatin) is limited by nephrotoxicity, the most common adverse effect in many cancer patients (Schrier, 2002). While several antineoplastic agents frequently exhibit nephrotoxicity, the platinum derivatives are among the most frequent compounds leading to renal injury (Schrier, 2002). Approximately 28 to 36% of patients receiving an initial dose (50- to 100- mg/m^2) of cisplatin develop ARF (Lebwohl and Canetta, 1998; Ries and Klastersky, 1986). Since even vigorous hydration has not been effective in eliminating toxicity and the use of diuretics may complicate the electrolyte disturbance induced by cisplatin, discontinuation of cisplatin remains the only option in cases of progressive renal failure. Thus, there is a pressing need to protect the kidney while administering effective chemotherapeutic agents, such as cisplatin.

The mechanism of cisplatin nephrotoxicity remains to be fully elucidated. In addition to direct tubular toxicity in the form of apoptosis and necrosis (Arany and Safirstein, 2003), vascular factors (Luke *et al.*, 1992; Winston and Safirstein, 1985) and inflammation (Ramesh and Reeves, 2002 and 2003) that have been implicated in the pathogenesis of cisplatin - mediated nephrotoxicity, several other studies have also demonstrated that oxidative stress is involved in the development of this drug's renal tubule injury (Baliga *et al.*, 1998; Nath and Norby, 2000; Tsutsumishita *et al.*, 1998; Matsushima *et al.*, 1998; Yilmaz *et al.*, 2004; Davis *et al.*, 2001; Kadikoylu *et al.*, 2004; Shino *et al.*, 2003). The involvement of oxidative stress is further supported by the fact that free radical scavengers and antioxidants prevented cisplatin - induced nephrotoxicity (Sener *et al.*, 2000; Weijl *et al.*, 2004; Satoh *et al.*, 2003; Ham *et al.*, 2001; Davis *et al.*, 2001; Tsuruya *et al.*, 2003; Dickey *et al.*, 2005; Gulec *et al.*, 2006).

The protective benefit of antioxidant food supplement against cisplatin toxicity has been reported (Avci *et al.*, 2008). There is increasing evidence that dietary phytoestrogens present primarily in soybeans as isoflavones have a beneficial role in chronic renal disease (Ranich *et al.*, 2001). Nutritional intervention studies have shown that consumption of soy-based protein reduces proteinuria and attenuates renal functional or structural damage in animals and humans with various forms of chronic renal disease (Ranich *et al.*, 2001). Having demonstrated the protective benefit of the

methanolic extract of soybean (MESB) against renal injury induced by gentamicin (Ekor *et al.*, 2006), it was therefore hypothesized in this study that the antioxidant polyphenolic compounds in soybean with demonstrable antitumour activity (Messina *et al.*, 1994; Knight and Eden, 1996; Wei *et al.*, 1995; Barnes *et al.*, 1990) may provide the same protective benefit in cisplatin - mediated nephrotoxicity. To justify the rationale for this present hypothesis, the optimism that the anticarcinogenic effect of soybean may synergize with that of cisplatin while at the same time protecting the kidney from damage by the latter was expressed. If this happens, the therapeutic efficacy and clinical utility of cisplatin would be greatly enhanced. In addition, the increased risk of nephrotoxicity and renal failure in patients who should benefit from aminoglycoside plus cisplatin combination would also be reduced.

In this study, nephrotoxicity was induced at an intraperitoneal dose of 2 mg of cisplatin per kg body weight for 5 consecutive days (Leonard *et al.*, 1971), which is a well accepted duration of treatment in clinical practice. Assessment of renal function 24-hours after administration of cisplatin revealed the induction of acute renal failure in the rats when compared with saline-treated or control group ($p<0.001$). This was characterized by significant increases in serum creatinine (SCr) and blood urea nitrogen (BUN) levels (markers of impaired glomerular function) with severe tubular necrosis following treatment with cisplatin. This was also associated with marked increase in urinary excretion of N-acetyl-β-D-glucosaminidase (NAG, biomarker of tubular damage) and significant hyponatraemia with mild elevation in serum K^+ ion concentration (electrolyte disturbance similar to clinical observation in some patients). Due to the renal excretion of cisplatin, the kidney accumulates a higher effective concentration of the drug than any other organ (Ikari *et al.*, 2005). This accumulation preferentially affects the terminal proximal tubule and the distal nephron and can cause either apoptosis or necrosis, depending on exposure time and concentration (Ikari *et al.*, 2005). Low, prolonged doses of cisplatin typically induce apoptosis, whereas short exposures to higher doses of cisplatin, as used in this study, cause necrosis (Lieberthal *et al.*, 1996). The diagnosis of ARF is based on increases in BUN, SCr or urinary excretion of enzyme in tubular cells (NAG or low molecular protein, beta-2-microglobulin [β_2MG]) (Zhou *et al.*, 2006). The increase in urinary NAG in ARF is induced by the leakage of tubular enzyme or by defective tubular reabsorption and this could be associated with necrosis of the proximal convoluted tubules, the primary site of drug accumulation, in the cisplatin - treated rats (Thadhani *et al.*, 1996; Star, 1998,

Kuhlmann *et al*., 1997). Ingestion of defatted MESB one-hour before cisplatin injection each day throughout the period of administration significantly attenuated the ARF and protected against morphological damage induced by cisplatin in the rats in this study. MESB significantly ($p<0.05$) prevented the increases in SCr, BUN and urinary excretion of NAG induced by cisplatin treatment when administered at 250- and 500-mg/kg doses. The electrolyte disturbance produced was also ameliorated in these rats. The highest dose of 1000mg/kg used in this study did not offer better protection against this toxicity when compared with the lower doses. The extract also at this dose (1000 mg/kg) produced slight but insignificant elevation of these parameters. This is similar to previous observations made in this study, thus validating the findings (Ekor *et al.*, 2006).

Results from this study further lend support to reports from several studies that provided evidence that the cellular events in cisplatin - mediated nephrotoxicity are a consequence of reactive oxygen species (ROS) generation, which produces oxidative renal damage (Yilmaz *et al*., 2004; Davis *et al*., 2001; Kadikoylu *et al*., 2004; Shino *et al*., 2003). Cisplatin in this study significantly ($p<0.05$) decreased the activities of the renal enzymic antioxidants SOD, CAT and GST when compared with the control rats. This was accompanied by severe GSH depletion and increased lipid peroxidation in the renal tissues of the cisplatin - treated rats. This renal oxidative stress may have resulted from the build-up of ROS such as $O_2^{\cdot}$ and H_2O_2 following the decrease in the activities of the renal enzymic antioxidants. The increased generation of $O_2^{\cdot}$ and H_2O_2 leads to increase production of the more reactive hydroxyl ($OH^{\cdot}$) radicals via Fenton and Haber-Weiss reactions (Stadtman, 1990). $OH^{\cdot}$ radicals react at nearly diffusion-limited rates with any component of the cell including lipids, DNA and proteins. The net result of this non-specific free radical attack is a loss of cell integrity, enzyme function and genomic stability (Gille *et al*., 1994; Halliwell, 1993). All these explain the increased lipid peroxidation as evident by the significant elevation in renal MDA and serum total hydroperoxides as well as the depletion of renal GSH observed in the cisplatin-treated rats in this study. This result agrees with findings from other studies (Somani *et al*., 2000; Saad and Al-Rikabi, 2002; Davis *et al*., 2001; Borrego *et al*., 2004; Gonzalez *et al*., 2004). Results from this study however, show that MESB via its antioxidant property is capable of ameliorating cisplatin - induced oxidative renal damage. The extract reversed the decrease in SOD and CAT activities and GSH level resulting in lowering of the renal MDA and total serum hydroperoxides in cisplatin - treated rats.

The increase in GST activity produced by the extract may also contribute to its protective effect by enhancing cisplatin detoxification which has been reported to occur via adduct formation with GSH (Rudin *et al.*, 2003).

In addition, the increase in nitric oxide (NO) production by cisplatin as indicated by the elevated serum nitrate/nitrite concentration was significantly ($p<0.01$) attenuated by the extract. The involvement of ROS in the impairment of GFR has been reported (Hughes *et al.*, 1996) and renal NO plays an important role in the regulation of GFR (Baylis and Qin, 1996). It was suggested earlier that MESB by increasing SOD activity, might prevent O_2^- accumulation and reduce the production of the toxic reaction product, peroxynitrite, which can arise from reaction with NO^- to protect against gentamicin nephrotoxicity (Ekor *et al.*, 2006). Evidence from the present study seems to suggest in addition that the extract is capable of decreasing excessive NO generation that characterize cisplatin - induced nephrotoxicity and ARF. The effect of the extract in lowering serum nitrate/nitrite concentration (index of NO production) in this study was significant ($p<0.01$) at 250- and 500-mg/kg doses when compared with the rats treated with cisplatin only. The highest dose (1000 mg/kg) of the extract, however, did not provide greater decrease in NO level when compared with the lower doses, and also did not produce any significant change in NO production when administered alone (i.e. without cisplatin injection).

Furthermore, there is increasing evidence that strongly suggests the role of inflammation in the pathophysiology of acute renal injury (Bonventre, 2004; Burne *et al.*, 2001; Friedewald and Rabb. 2004; Okusa, 2002). Results from this study show that cisplatin - induced renal injury can cause polymorphonuclear leukocyte infiltration and accumulation as indicated by the significant increase in myeloperoxidase (MPO) activity in the cisplatin - treated rats. This observation which supports inflammatory mechanism in cisplatin nephrotoxicity corroborates similar findings from recent studies (Gulec *et al.*, 2006; Liu *et al.*, 2006; Li *et al.*, 2005; Arany and Safirstein, 2003; Ramesh and Reeves, 2002). It is believed that the infiltrating phagocytes (neutrophils and macrophages) can lead to the release of cytokines/chemokines (Bonventre, 2004; Friedewald and Rabb, 2004; Kelly *et al.*, 1999; Okusa, 2002; Ramesh and Reeves, 2002, 2003; Lu *et al.*, 2008). Cytokines, particularly tumour necrosis factor-α (TNF-α), have been shown to contribute to cisplatin-induced renal injury and to co-ordinate the activation of a large network of chemokines and cytokines in the kidney following cisplatin injection (Ramesh and Reeves, 2002). The ability of MESB to decrease the

renal activity of MPO in the cisplatin - treated rats suggests its protective effect is also related to its ability to reduce leukocyte infiltration and subsequently down-regulate some components of the inflammatory mechanisms that contribute to this injury. In fact, it will not be unreasonable to speculate that inhibition of either TNF-α production or its activity is involved in the protection afforded by MESB against the renal dysfunction and structural damage observed in this study. This speculation is largely predicated on recent experimental findings by Ramesh and Reeves (2004 and 2002). Previous observation by Jones and Shoskes (2000) in which quercetin prevented cisplatin – induced cellular injury and upregulation of chemokines in the renal cortex *in vitro*, also strengthens this speculation. This is also in line with the suppression of NF-κB activation, cytokine/chemokine expression and neutrophils infiltration reported by Li *et al.* (2005) in the study of the ameliorative effect of fibrates pretreatment in cisplatin – induced renal dysfunction.

Similarly, the role of MPO in vascular pathology has recently been highlighted. MPO is abundant in phagocytes and catalyzes the reaction between H_2O_2 and Cl^- to produce HOCl and other oxidizing species (Winterbourn *et al.*, 2000). It also utilizes $NO\cdot$ to generate reactive nitrogen species, thereby reducing $NO\cdot$ bioactivity and increasing oxidative stress (Eiserich *et al.*, 2002; Gaut *et al.*, 2000). The decrease in renal activity of MPO produced by MESB in this study may enhance NO bioactivity, improve renal haemodynamics and reduce oxidative stress associated with the increased MPO activity in the cisplatin - treated rats. Again, the highest dose (1000 mg/kg) of MESB did not offer better protection against this cisplatin - mediated activation of polymorphonuclear leukocyte infiltration and inflammation in comparison with the lower doses (250- and 500- mg/kg). The extract (1000 mg/kg) when administered alone did not produce significant change in MPO activity when compared with the control.

Results from this study also demonstrate that the protective benefit offered by MESB against nephrotoxicity and acute renal failure induced by cisplatin may also be related to its ability to inhibit renal xanthine oxidase (XO) activity. The activity of this enzyme (XO) increased following cisplatin treatment as was also observed by Gulec *et al.* (2006). XO is an enzyme of purine catabolism that catalyzes the conversion reactions of hypoxanthine to xanthine and xanthine to uric acid with by-product of toxic superoxide radical. In this regard it is a key enzyme between purine and free radical metabolism (Gulec *et al.*, 2006). XO exists primarily as xanthine dehydrogenase

(XDH) in healthy tissue. A proposed pathway for the formation of O_2^- radicals included the transformation of XDH to XO during ishaemic period. Xanthine degradation by XO, not by XDH, also leads to the formation of H_2O_2 and OH^- radicals. The increase in XO and combined XO plus XDH activities following cisplatin treatment may enhance generation of ROS and partly be responsible for increased lipid peroxidation and oxidative damage that characterized this drug's toxicity in this study. MESB through its antioxidant activity and possibly by interfering with purine metabolism may inhibit this pathway of ROS production and thus reduce the effect of the induction of XO activity in cisplatin-mediated nephropathy. The highest dose of MESB, again did not offer greater decrease in the activities of these enzymes.

Cisplatin nephrotoxicity in this study was also associated with plasma membrane damage as indicated by the significant decrease in activities of renal glucose-6-phosphatase (G6Pase) and 5'-nucleotidase (5'-NTD). This may result from direct tubular injury or peroxidative damage caused by exaggerated ROS production and inactivation of these membrane-bound enzymes. MESB only attenuated this plasma membrane damage at 250 mg/kg dose. The extract alone (1000 mg/kg) produced mild decreases in the activities of these enzymes.

It is pertinent to conclude here again for the purpose of emphasis that, although MESB at high dose preserved the activities of antioxidant enzymes and renal function, its ability to decrease the activities of G6Pase and 5'-NTD when administered alone and also to decrease GSH level when administered with cisplatin and with gentamicin from previous experiment (Ekor *et al*., 2006), suggest that the extract in large doses may probably produce prooxidant effects.

5.1.3 Free Radical Scavenging and Antioxidant Activities of MESB

In recent years, flavonoids have attracted a tremendous interest as possible therapeutics against free radical mediated diseases (Middleton *et al*., 2000). Scavenging of free radicals has been suggested to play a considerable part in the antioxidant activity of these compounds. The antioxidant and antipromotional effects of genistein, the most abundant isoflavone in soy have been reported (Wei *et al*., 1995). Consumption of soybean-containing diets has been associated with the lower incidence of certain human cancers (Messina and Barnes, 1991; Barnes *et al*., 1990; Setchell *et al*., 1984). Results from previous experiments in this study have demonstrated the ability of the methanolic extract of soybean (MESB) to protect against gentamicin- and cisplatin- induced

nephrotoxicity and acute renal failure. The protective action by this extract was suggested to be largely dependent on the observed antioxidant activity and in addition, anti-inflammatory property in the case of cisplatin – mediated nephropathy. It was also suggested that these actions in these experimental models of nephrotoxicity were related to the total phenolic content of MESB. To validate these speculations and provide explanations for the effects observed *in vivo*, the antioxidant and free radical scavenging activity of the extract was evaluated *in vitro*.

Phenolic compounds have been reported to function as antioxidants by virtue of their ability to donate hydrogen to stabilize reactive and unstable free radicals (Rice-Evans *et al.*, 1996). Indeed, results from this experiment indicate that phenolic compounds may make a major contribution to the antioxidant capacity of the MESB. The reducing power increased with increasing concentration of the extract (result depicted in figure 31), indicating the presence of electron donors which could react with free radicals to convert them into more stable products and to terminate radical chain reactions. The $FeCl_3/K_3Fe(CN)_6$ system has been shown to offer a sensitive method for the "semi-quantitative" determination of dilute concentrations of polyphenolics, which participate in the redox reaction (Amarowicz *et al*., 2004). In this assay, MESB causes the reduction of Fe^{3+}/ferricyanide complex to the ferrous form, and the Fe^{2+} was monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung *et al*., 2002). The absorbance value produced by MESB at 800 µg (0.043±0.009) compares with that of the reference standard, catechin (0.045±0.016), at 50 µg indicating that the reducing power of MESB was much less than that of catechin. The absorbance values ranged between 0.016±0.007 to 0.043±0.009 at MESB concentration of 10-800 µg. Though less potent than catechin, polyphenolics in MESB appear to function as good electron and hydrogen-atom donors and therefore should be able to terminate radical chain reactions by converting free radicals to more stable products.

It is clear that due to the complex nature of the different phytochemical classes, the antioxidant capacities of plant extracts cannot be evaluated using a single method. This justifies the use of a multiple-method approach in antioxidant activity assessment as recommended in literature (Frankel and Meyer, 2000; Aruoma, 1996, 2003; Aruoma *et al*., 1996). In this study, a number of well-established *in vitro* assays were used to characterize the direct antioxidant actions of MESB. It is well known that antioxidants can seize the free radical chain of oxidation and form stable free radicals which would not initiate or propagate further oxidation. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) has

been used extensively as a free radical to evaluate reducing substances (Schimada *et al.*, 1992; Duh and Yen, 1997). MESB in this study exerted significant scavenging effect on DPPH· Radical at lower concentrations (25- and 50- µg). This is depicted by the significant decrease in absorbance values ($p<0.05$) of reaction mixtures containing MESB at these concentrations when compared with control (without MESB) (result shown in Table 3H). It is known that a freshly prepared DPPH· Solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades or disappears when an antioxidant molecule can quench DPPH· (by providing hydrogen atoms or by electron donation, conceivably via a free radical attack on the DPPH· Molecule) and convert them to a colourless or bleached product (i.e. 1,1-diphenyl-2-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance (Yamaguchi *et al.*, 1998). This present result corroborates the detection of phenolic compounds with antioxidant property on spraying spotted fractions from MESB with DPPH solution on TLC, which gave distinct yellow colour. The scavenging of DPPH· Radical by MESB, however, decreased with increasing concentration. The scavenging activity against DPPH· Radical was also lower than those of the standard, catechin.

Furthermore, the antioxidant activity of MESB was also demonstrated in the TEAC system and catechin also showed greater potency than MESB in this system. The TEAC system involved the potential scavenging of an artificially generated $ABTS^{\cdot+}$ radical (Campos and Lissi, 1997). MESB increased the TEAC values by 148%, 682%, 2280% at 250 µg, 250 µg and 500 µg respectively. The high $ABTS^{\cdot+}$ radical scavenging activity demonstrated by MESB attests to its antioxidant potential and explains the observed *in vivo* antioxidant activity and the protective effects against gentamicin- and cisplatin-induced renal oxidative damage. MESB on the other hand, did not seem to exhibit antioxidant activity in the FRAP assay. In this assay, it is expected that reduction of ferric to ferrous ion at low pH should cause a coloured ferrous-tripyridyltriazine complex to form. The decrease in FRAP values observed with MESB does not seem to correlate with the increased reducing power recorded in the $FeCl_3/K_3Fe(CN)_6$ system. The reason for this is not very clear at the moment. However, the stability of the active components in MESB or the possible interference or interaction of these components with tripyridyltriazine in the reaction system may have accounted for this.

Also, MESB produced 22-56% inhibition of deoxyribose oxidation at concentration range of 200-1000 µg. The reference drug, catechin, produced 67% inhibition at 50 µg, once again, demonstrating its greater potency over MESB as an antioxidant. The deoxyribose method is a simple assay to determine the rate constants of hydroxyl radicals production (Halliwell *et al.*, 1987). The mixture of $FeCl_3$-EDTA, H_2O_2 and ascorbate, when incubated with deoxyribose in phosphate buffer (pH 7.4), generates $OH^{\cdot}$ Radicals which attack the deoxyribose and result in a series of reactions that cause the formation of MDA. The decrease in deoxyribose degradation produced by MESB as measured by the reduced MDA formation suggests a competition with deoxyribose for the availability of $OH^{\cdot}$ Radicals. Thus, the dose-dependent $OH^{\cdot}$ Radical scavenging activity demonstrated by MESB in this study further explains its effectiveness *in vivo* in attenuating gentamicin- and cisplatininduced renal oxidative damage. The scavenging of $OH^{\cdot}$ Radical in the deoxyribose assay was comparable to previous studies conducted on extracts from several medicinal plants (Schinella *et al.*, 2002; Munasinghe *et al.*, 2001; Neergheen *et al.*, 2006). *Monimiastrum acutisepalum*, in the study carried out by Neergheen *et al.* (2006) provided 32% inhibition of deoxyribose degradation and showed weaker scavenging activities toward $ABTS^{\cdot+}$ radical and hypochlorous acid. This was attributed to the fact that the assays used to determine the antioxidant activity were based on different mechanisms with different expected results, thereby indicating that extracts showing poor antioxidant properties in one assay system should not be categorized as poor sources of antioxidants. This has been described as an important issue for studies to assess the antioxidant potentials of bioactive components in both food plants and medicinal plants (Aruoma, 2003).

MESB in this study also exhibited marked scavenging effects on $NO^{\cdot}$ and $O_2^{\cdot-}$ radicals at moderately low concentrations (Table 3G). The scavenging effect on these radicals decreased with increasing concentration of the extract and became pro-oxidant at concentrations ≥ 100 µg for $O_2^{\cdot-}$ radicals. MESB produced 23% and 68% scavenging activity on $O_2^{\cdot-}$ radical and 22% and 24% on $NO^{\cdot}$ radical at 10 µg and 50 µg respectively. The inhibitory effect on NBT reduction in this study is primarily due to $O_2^{\cdot-}$ scavenging. This effect by MESB in addition to its observed inhibitory activity on xanthine oxidase *in vivo*, would make the extract very effective in preventing $O_2^{\cdot-}$ radical accumulation in biological systems. Similarly, the scavenging of $NO^{\cdot}$ in this *in vitro* study also correlates with the decrease in nitrate/nitrite levels (index of $NO^{\cdot}$ production) observed *in vivo* in cisplatin-treated rats. Overproduction of NO and its

metabolites, in particular the deleterious molecule peroxynitrite ($ONOO^-$) have been suggested to contribute to numerous pathological conditions associated primarily with inflammatory disorders (Clancy and Abramson, 1995). The indirect scavenging of $ONOO^-$ by MESB as demonstrated in its ability to scavenge $O_2^{\cdot-}$ and $NO^{\cdot}$ *in vitro*, coupled with its ability to decrease $NO^{\cdot}$ production and MPO activity *in vivo* re-iterates earlier suggestion of possible involvement of an anti-inflammatory mechanism in the protection against cisplatin nephrotoxicity. Similar reports have been made in other studies. Kobuchi *et al.* (1997) revealed that *Ginko biloba* extract (Egb 761) not only directly acts as a NO scavenger, but also inhibits NO production in LPS/IFN-γ-activated macrophages by concomitant inhibition of induction of iNOS mRNA and the enzyme activity of iNOS. Similar effects were reported with epigallocatechin gallate (Chan *et al.*, 1997). In addition, Sheu *et al.* (2001) and Kim *et al.* (1999) also reported on the inhibition of NO production in LPS-activated macrophages by isoflavones, including genistein, similar to Kobuchi *et al.* (1997) or Chan *et al.* (1997). These reports support this present result and the soybean isoflavones present in MESB might be partly responsible for the $NO^{\cdot}$ scavenging activities and by extension, the anti-inflammatory activity of the extract.

Data from AAPH- and Fe^{2+}/ascorbate- induced lipid peroxidation assays further establishes the antioxidant and free radical scavenging properties of MESB. The effectiveness of MESB to inhibit lipid peroxidation in these two models was comparable. MESB produced 20-40% and 24-46% in AAPH and Fe^{2+}/ascorbate systems respectively at concentration range between 100-800 µg. This result demonstrates the ability of MESB to scavenge peroxyl radical generated by AAPH and $OH^{\cdot}$ As well as other radicals generated in the Fe^{2+}/ascorbate system. The direct scavenging effects on ROS observed earlier and the inhibition of AAPH and Fe^{2+}/ascorbate-induced lipid peroxidation provide further evidence for the antioxidant and protective effects demonstrated by the extract *in vivo*.

In conclusion, MESB has demonstrated significant antioxidant and free radical scavenging activities *in vitro*. These results correlate with observations made *in vivo* and thus provide explanations for the remarkable protective effects of MESB in nephrotoxicity and oxidative damage induced by gentamicin and cisplatin. This points to the potential benefits and future use of soybean in oxidative and free radical pathologies.

5.1.4 Phytochemical Analysis of MESB

It has been reported that the environmental conditions such as latitude, altitude, temperature and precipitation can affect the soybean seed composition as, for example, the protein and oil contents (Zeller 1999; Van Barneveld, 1999). Indeed, Qin *et al.* (1998) showed that the contents of anti-nutritional factors were different between raw soybeans produced in two distinct geographical regions. Vollmann *et al.* (2000) described genetic variations and significant differences in the protein content of soybeans cultivated in distinct climates. Based on these facts, it is reasonable to question whether the soybean cultivars adapted to grow in locality or regions present differences in their seed composition and nutritional profile when compared to cultivars grown in distinct regions around the world. Furthermore, information on the chemical composition and anti-nutritional and/or toxic protein contents of soybean cultivars exposed to typical tropical environment is scarce. Thus, the present experiment was undertaken to attempt to identify some of the known compounds and/or possibly isolate new compounds.

Results from this experiment indicated the presence of different classes of phenolic compounds mainly of the dihydroxy group in the ethyl acetate fraction, SBEF. Some phenolics with trihydroxy groups together with other non-phenolic compounds were also identified.

Phenolics exhibiting antioxidant activity using the DPPH spray system were detected in ethyl acetate fractions – SBEF5, SBEF6 and SBEF7 – obtained on open chromatography (Table 4.2). Further fractionation of SBEF5 on Sephadex LH-20 yielded SBEF5a, SBEF5b, SBEF5c, SBEF5d and SBEF5e (Table 4.3). SBEF5a (compound 1) and SBEF5e (compound 2) were obtained as chromatographically pure compounds while others contained mixture of compounds. SBEF5e (compound 2) which reacted with $FeCl_3$ is the only phenolic compound obtained from SBEF5 and belongs to the trihydroxy group. This phenolic compound does not seem to possess antioxidant property in the DPPH spray system. Other fractions obtained from SBEF5 (i.e. SBEF5a, SBEF5b, SBEF5c and SBEF5d) are non-phenolic and did not exhibit antioxidant property in the DPPH spray model.

Furthermore, fractionation of the n-butanol fraction of the soybean extract (SBBF) on open column chromatography yielded SBBFa, non-phenolic non-antioxidant, together with SBBFb, SBBFc, SBBFd and SBBFe which are non-phenolic compounds exhibiting antioxidant properties (Table 4.4). Subsequent fractionation of

SBBF and SBBFd2 on reversed phase liquid chromatography and Sephadex LH-20 respectively yielded the non-phenolic antioxidant compounds, SBBFd2b (compound 3) and SBBFd2d (compound 4), in their pure form (Tables 4.5 and 4.6). Similady, fractionation of SBBFb1 on Sephadex LH-20 yielded SBBFb2b (compound 5) SBBFb2d (compound 6). Compound 6 is a non-phenolic compound which also did not exhibit antioxidant property on spraying with DPPH. Compound 5 on the other hand is a phenolic compound with antioxidant property (Table 4.7). The reaction of compound 5 with DPPH was immediate and colour development more intense when compared with those produced by compounds 3 and 4, indicating that it's a stronger antioxidant.

In summary,

- Compound 1 showed neither antioxidant nor phenolic property.
- Compounds 2 showed moderate phenolic property with ferric chloride without antioxidant property. The development of blue-black colouration spot on the orange background in compound 2 is stronger than compound 5 which also demonstrated phenolic property.
- Compounds 3 and 4 demonstrated moderate antioxidant properties and the reaction with DPPH was within 2-3 minutes but without phenolic property at all.
- Compound 5 showed both antioxidant and phenolic properties. However, the development of yellow spot on the purple background in compound 5 was within 1 minute (almost immediate) after spraying with DPPH while that of compounds 3 and 4 showed up gradually about 4 minutes later, indicating that 5 has higher antioxidant activity than 3 and 4. This fact was further supported by the strong intensity of the yellow spot of 5.
- Compound 6 was neither phenolic nor antioxidant in its reactions with ferric chloride and DPPH respectively.
- All isolated compounds, however, reacted with the general purpose detecting reagent (sulphuric acid).

CONCLUSION

The relationship between gentamicin and cisplatin nephrotoxicity and oxidative stress was further established in this study.

The methanolic extracts of soybean (MESB) attenuated the nephrotoxicity and acute renal dysfunction induced by gentamicin and cisplatin in rat by reinforcing the antioxidant defense system *in vivo*. The inflammatory component associated with cisplatin renal toxicity was ameliorated by the extract. MESB at a relatively low dose probably has a specific stabilizing effect on the cell membrane and/or other vital cellular macromolecules and can thus protect against gentamicin- and cisplatin-induced renal damage and tubular necrosis. MESB exhibited significant free radical scavenging and antioxidant activities, *in vitro*. Phytochemistry revealed the presence of antioxidant phenolics with dihydroxy and trihydroxy groups. Also present in the extract are non-phenolic compounds with and without antioxidant properties as well as non phenolic antioxidants.

MESB therefore, via antioxidant and free radical scavenging activities, in addition to possible anti-inflammatory effect of the phenolic and non-phenolic compounds present in it, offered protection against gentamicin- and cisplatin- mediated nephropathy. Data from this study point to the potential benefit of soybean as possible useful adjunct in patients receiving cisplatin and gentamicin therapy.

1. Data from this study provide information for the first time that renal injury which may progress to acute renal failure following therapy with gentamicin and cisplatin during bacterial and cancer chemotherapy respectively may be ameliorated with a dietary supplement or co-administration of soybean. This reveals additional potential therapeutic or chemoprophylactic benefit of naturally occurring constituents present in soybean.
2. This study has contributed to the body of existing evidence which had implicated the participation of free radicals and disruption of the antioxidant defense system in the pathophysiology of gentamicin- and cisplatin- induced nephrotoxicity and suongly supports the involvement of inflammatory mechanism in the toxicity mediated by the latter.
3. The safety of soybean and its products on consumption had remained controversial. This study provides information on the possible pro-oxidant potential of soybean when consumed at high dose levels and therefore cautions on indiscriminate consumption of soybean or its products.
4. The isoflavones are generally believed to be the predominant phenolic compounds and antioxidants in soybean. This study identified and isolated for the first time two non-phenolic antioxidant compounds (in addition to some phenolic antioxidants already known to be present) in soybean.
5. This study provides useful information for future clinical trials involving evaluation of chemoprophylactic or therapeutic potentials of soybean constituents as well as the safe and effective doses in iatrogenic or other forms of renal injury in patients at risk.

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APPENDIX

CALCULATIONS

IN VIVO ASSAYS

i. Creatinine, Urea and Protein Concentrations

$$\text{Concentration (mg/100ml)} = \frac{\text{O.D. of test}}{\text{O.D. of standard}} \times \text{Concentration of standard}$$

ii. Catalase activity

The mononuclear velocity constant, K, for the decomposition of H_2O_2 by catalase was determined by using the equation for a first-order reaction:

$$K = 1/t \log S_0/S$$

where S_0 is the initial concentration of H_2O_2 and S is the concentration of the peroxide at t min. The values of the K are plotted against time in minutes and the velocity constant of catalase $K_{(0)}$ at 0 min determined by extrapolation.

The catalase contents of the enzyme preparation were expressed in term of Katalase feiabigkeit or 'Kat.f' according to von Euler and Josephson (1927).

$$\text{Kat. f} = \frac{K_{(0)}}{\text{mg protein/ml}}$$

iii. Superoxide Dismutase (SOD) activity

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

where A_0=absorbance after seconds

A_1=absorbance 150 seconds

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

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

iv. Glutathione-S-transferase activity

The extinction coefficient of CDNB = $9.6mm^{-1}\ Cm^{-1}$

$$\text{GSH-S-transferase activity} = \frac{\frac{OD/min}{9.6}}{0.03ml\ mg\ protein} \times 1$$

= µmole/min/mg protein.

v. Malondialdehyde (MDA) level

$$\text{MDA (units/mg/protein)} = \frac{\text{Absorbance x volume of mixture}}{E_{532nm} \text{ x volume of sample x mg protein}}$$

vi. Urinary N-acetyl-β-D-glucosaminidase activity

$$\text{Enzyme activity} = \frac{S \times (OD_{SA} - OD_{RB})}{OD_{ST} - OD_{RB}}$$

where S = activity of NAG Calibrant (1013µmol/h/l)

OD_{SA} = sample absorbance

OD_{ST} = Calibrant absorbance

OD_{RB} = reagent blank absorbance

vii. Myeloperoxidase (MPO) activity

$$\text{MPO (units/mg protein)} = \frac{13.5(\Delta OD/min)}{\text{mg protein or mg dry tissue weight.}}$$

viii. Xanthine Dehydrogenase and Xanthine Oxidase

Enzyme activities were expressed in nanomoles/min and mg protein (nmol min^{-1} mg $prot^{-1}$) by the following formula:

$$\text{Enzyme activity (nmol } min^{-1} \text{ mg } prot^{-1}) = \frac{\text{Absorption}_{340}/min \times (\text{cuvette vol in L/sample vol in mL})}{(1.1 \times 10^4\ M^{-1}\ ABS_{340})^{-1} \times \text{mg protein}^{-1}}$$

where: 1nmole urate/min = units and the millimolar extinction coefficient is $1.1 \times 10^4 M^{-1}$.

ix. γ-Glutamyltransferase (γ-GT) activity

γ-GT activity was calculated using the following formula;

$$\frac{\text{A/min X total assay volume (ml)}}{\text{E x light path (cm) x sample volume (ml)}} \quad X \quad 100 \quad = \text{U/L } \gamma\text{-GT}$$

A/min = change in Absorbance per minute

1000 = factor for converting ml to litre

E = Molar absorptivity of p-nitoraniline

$9.9 cm^2$ / µmol at 405 nm

$$\text{factor} \quad \frac{2.2 \text{ ml x } 1000}{9.9 \text{ X km X } 0.2 \text{ ml}} = 1111$$

U/L = 1111 X A 404 nm

x. Calculation of Glucose-6-Phosphatase activity

$$\text{Activity} = \text{product liberated} = \frac{\text{ODT}}{\text{ODS}} \text{ x } \frac{\text{Conc of std}}{1}$$

= xnM

0.15ml microsomal fraction liberated 'x' conc of product
1.0ml microsomal fraction liberated 'n' mg/ml protein
= n x 6.6nM

Hence 1.0ml microsomal fraction liberates $\frac{6.6\text{nM}}{\text{mg/ml protein}}$

Time for reaction is 30 minutes

Concentration of standard = 0.01M
= 10.000µM

$$\text{Activity} = \frac{\text{ODT}}{\text{ODS}} \text{ x } \frac{\text{conc of std}}{1} \text{ x } \frac{6.6}{1} \text{ x } \frac{1}{\text{mg/ml protein}} \text{ x } \frac{1}{20} \text{ x } \frac{10}{1}$$

$$= \frac{\text{ODT}}{\text{ODS}} \text{ x } 10{,}000 \text{ x } \frac{6.6}{1} \text{ x } \frac{1}{\text{mg/ml protein}} \text{ x } \frac{1}{20} \text{ x } 10$$

$$= \frac{ODT}{ODS} \times 660000 \times \frac{1}{\text{mg/ml protein}} \times \frac{1}{20}$$

xi. Calculation of 5'- Nucleotidase activity

$$\text{Activity} = \text{product liberated} = \frac{ODT}{ODS} \times \frac{\text{Conc of std}}{1}$$

$$= xnM$$

0.15ml microsomal fraction liberated 'x' conc of product
1.0ml microsomal fraction liberated 'n' mg/ml protein
$= n \times 6.6nM$

Hence 1.0ml microsomal fraction liberates $\frac{6.6nM}{\text{mg/ml protein}}$

Time for reaction is 30 minutes

1.0ml microsomal fraction liberated $\frac{6.6n}{\text{mg/ml}} \times \frac{1}{30}$

1 unit enzyme = 1μM

1.0ml microsomal fraction liberates $\frac{ODT}{ODS} \times \frac{\text{conc of std}}{1} \times \frac{6.6}{1} \times \frac{1}{\text{mg/ml protein}}$

$\frac{1}{30} \times \frac{10}{1} \times 1\mu M$

$$= \frac{ODT}{ODS} \times \frac{40,000}{1} \times \frac{6.6}{\text{mg/ml protein}} \times \frac{1}{30} \times \frac{10}{1} \times 1\mu M$$

$$= \frac{ODT}{ODS} \times \frac{2640000}{30\text{mg/ml protein}} \left.\begin{matrix} \\ \\ \end{matrix}\right\} \begin{matrix} \text{enzyme} \\ \text{unit} \end{matrix}$$

Concentration of standard = 0.04M
= 40,0000μM

IN VITRO ASSAYS

i. Inhibition of Deoxyribose degradation (Deoxyribose assay)

$$\% \text{ Inhibition} = \frac{A_{unsh} - A_{test}}{A_{unsh} - A_{blank}} \times 100$$

where A_{unsh} = absorbance of control (without extract)

A_{test} = absorbance of sample with extract

A_{blank} = absorbance of blank

ii. 1,1-Dipheny-2-picrylhydrazyl (DPPH) Radical Scavenging assay

$$\% \text{ RSA} = 100 \times (1 - A_E/A_D)$$

where A_E is the absorbance of the solution when extract has been added at a particular level, and A_D is the absorbance of the DPPH solution without extract

iii. Inhibition of Fe^{2+}/ascorbate and 2,2'-Azobis (2-amidinopropane) hydrochloride (AAPH) - induced Lipid peroxidation

$$\% \text{ Inhibition} = \frac{A_{unsb} - A_{test}}{A_{unsb} - A_{blank}} \times 100$$

where A_{unsb} = absorbance of control (without extract)

A_{test} = absorbance of sample with extract

A_{blank} = absorbance of blank

iv. Nitric oxide and Superoxide Radical Scavenging assays

$$\% \text{ RSA} = \left[1 - \frac{\Delta \text{Abs of sample}}{\Delta \text{Abs of control}}\right] \times 100$$

TABLES

Table 3A: Total Phenolic Content of Methanolic Extract of Soybean (MESB)

Extract	Total Phenolic Content (mg/g extract)
MESB	50.5 ± 1.2

Value is Mean ± SEM of five replicate analyses, and expressed as (+) – Catechin equivalent.

Table 3B: Effect of methanolic extract of soybean (MESB) on AAPH-induced lipid peroxidation.

Concentration (µg)	Absorbance	% Inhibition
Control	0.030±0.0012	-
MESB		
100	0.024±0.001•	20.0
200	0.022±0.001••	26.7
400	0.021±0.001••	30.0
800	0.018±0.001••	40.0
1000	0.025±0.002•	16.7
Catechin		
50	0.019±0.001	36.6

Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates.

•p<0.01 and ••p<0.001 when compared with control.

Table 3C: Effect of methanolic extract of soybean (MESB) on Fe^{2+}/ascorbate-induced lipid peroxidation.

Concentration (µg)	Absorbance	% Inhibition
Control	0.041±0.005	-
MESB		
10	0.032+0.002*	22.0
50	0.031+0.001*	24.4
100	0.031±0.001*	24.4
200	0.030±0.001*	36.7
400	0.024±0.002*	41.5
800	0.022±0.003**	46.3
Catechin		
50	0.013±0.003**	68.3

Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates.

*$p<0.05$ and **$p<0.001$ when compared with control.

Table 3D: Inhibition of deoxyribose oxidation by methanolic extract of soybean (MESB)

Concentration (µg)	Absorbance	% Inhibition
Control	0.018±0.001	-
MESB		
200	0.014±0.001	22.2
400	0.014±0.004	22.2
800	0.012±0.001	33.3
1000	0.008±0.001*	55.6
Catechin		
50	0.006±0.002*	66.7

Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates.

*p<0.05 when compared with control.

Table 3E: Nitric oxide ($NO^{\cdot}$) radical scavenging activity (RSA) of methanolic extract of soybean (MESB)

Concentration (µg)	Absorbance	% RSA
Control	0.54±0.029	-
MESB		
10	0.42±0.087*	22.2
50	0.41±0.005*	24.1
100	0.41±0.01*	24.1
200	0.46±0.01**	14.8
400	0.48±0.009**	11.8
Catechin		
50	0.34±0.004*	37.7

Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates.

*p<0.001 and **p<0.05 when compared with control.

Table 3F: Superoxide radical (O_2^-) scavenging activity of methanolic extract of soybean (MESB)

Concentration (µg)	Absorbance	% RSA
Control	0.022±0.003	-
MESB		
10	0.017±0.003	22.7
50	0.007±0.004*	68.2
100	0.041±0.003*	-86.4
200	0.042±0.010*	-90.9
Catechin		
50	0.055±0.002*	-150.0

Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates.

*p<0.05 when compared with control.

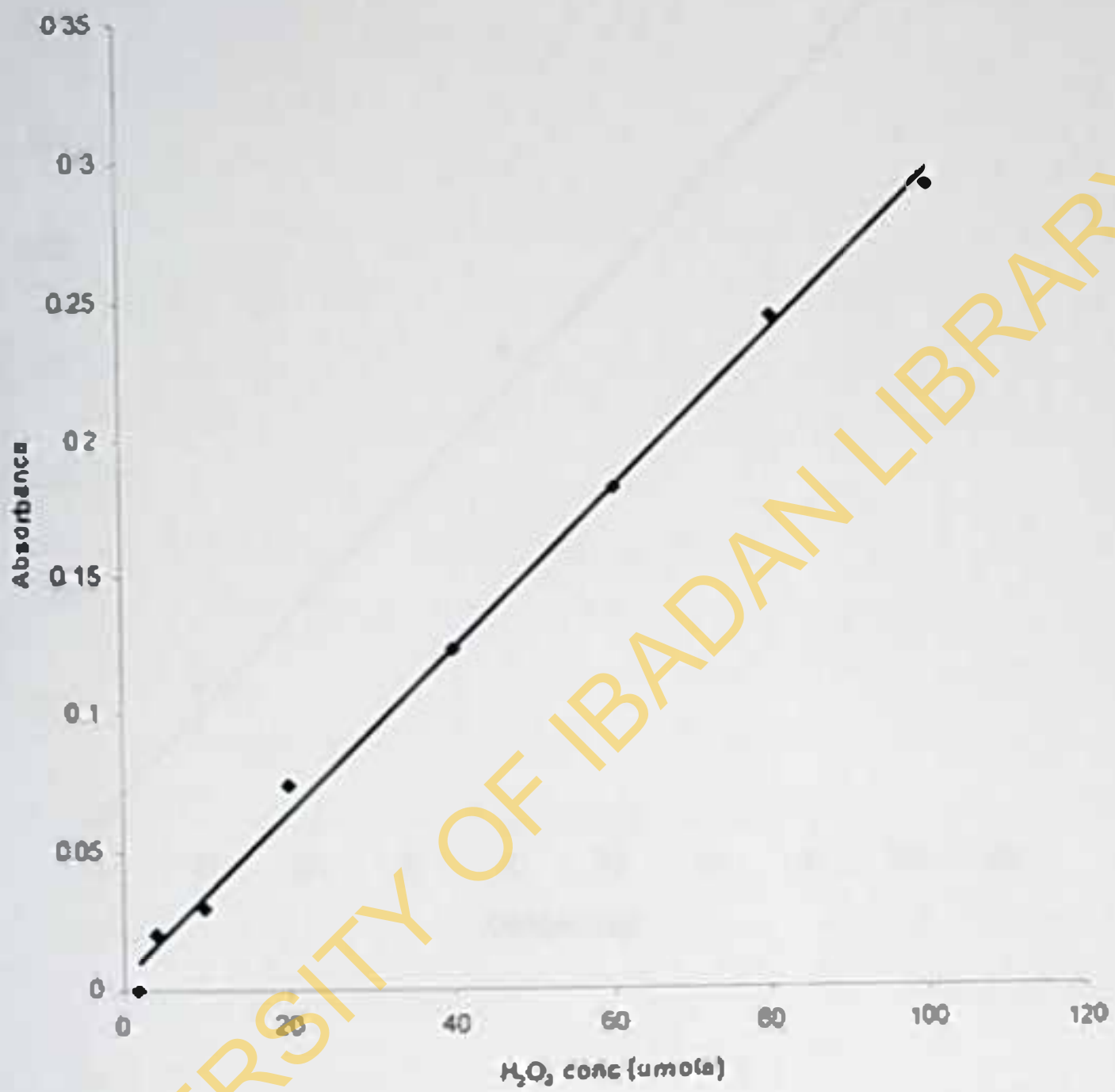
Table 3G: DPPH· radical scavenging activity (RSA) of methanolic extract of soybean (MESB).

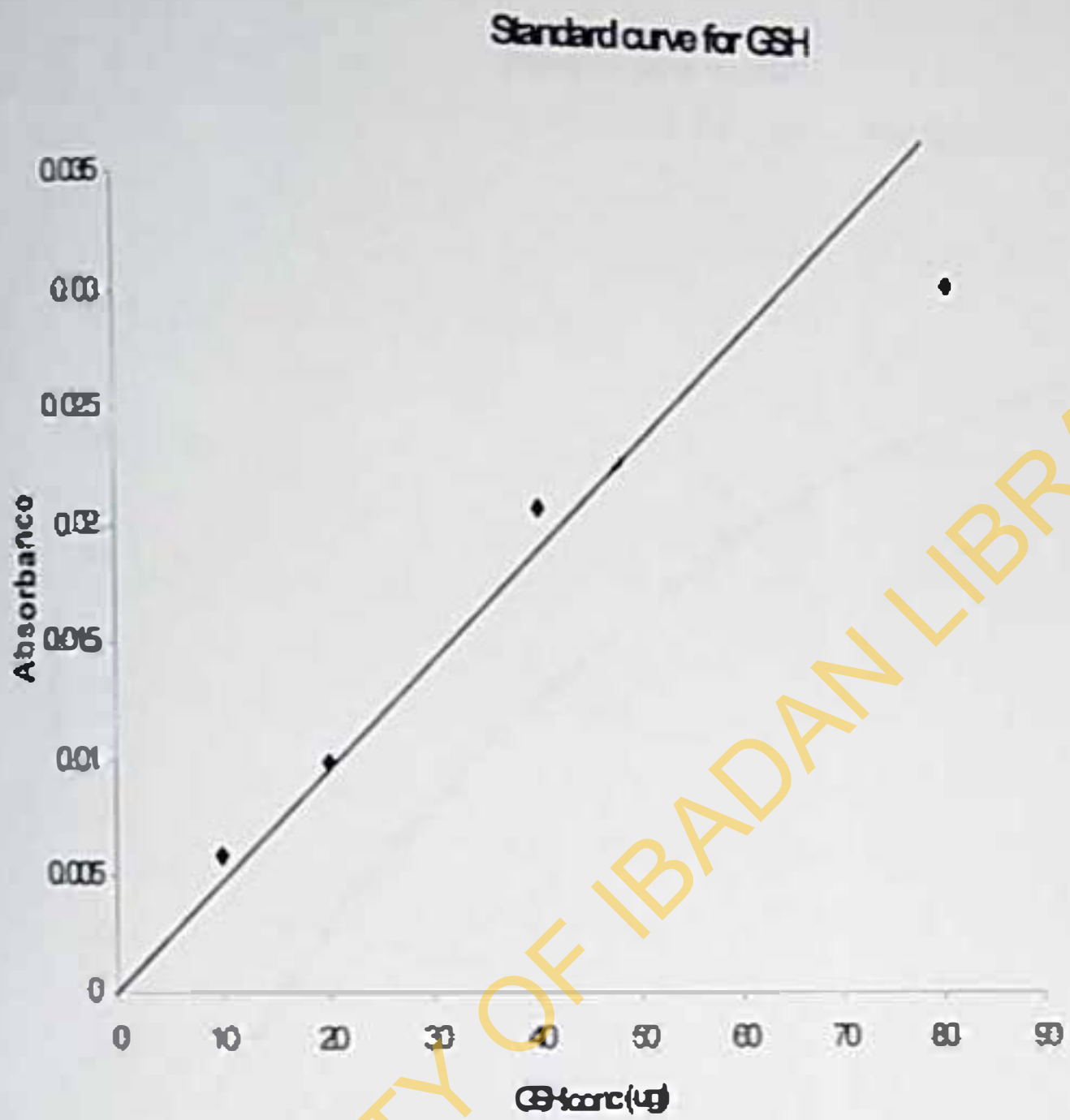
Concentration (µg)	Absorbance	% RSA
Control	0.86±0.004	-
MESB		
25	0.79±0.006*	8.1
50	0.76±0.062*	11.6
100	0.82±0.037	4.7
500	0.85±0.007	1.2
Catechin		
100	0.68±0.00**	20.9

Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates.

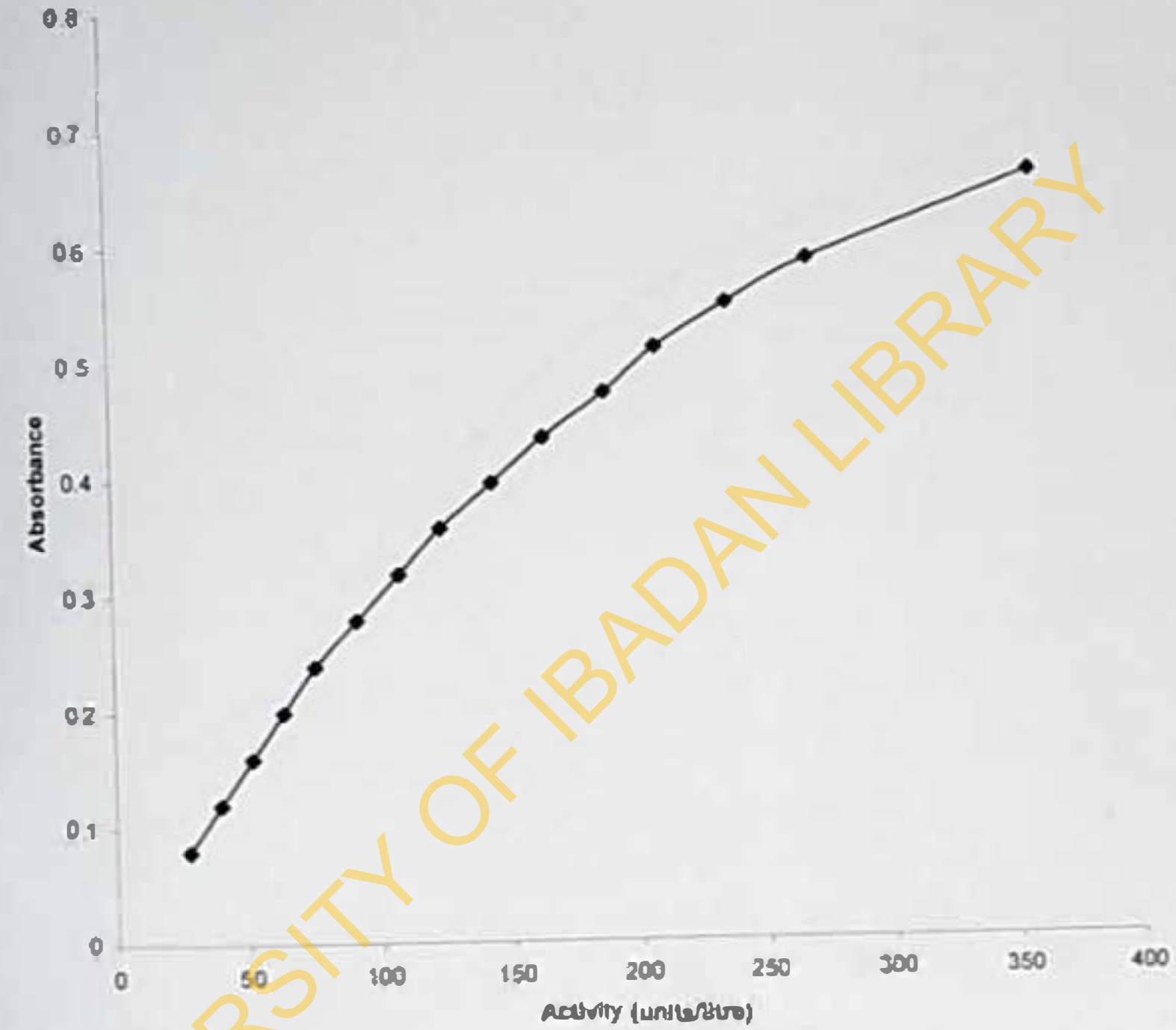
*p<0.05 and **p<0.001 when compared with control.

FIGURES





Standard curve for AST



Standard Curve for Total Phenolic Content