PROTECTIVE EFFECTS OF ETHANOLIC EXTRACT OF *PSIDIUM GUAJAVA* ON ADRIAMYCIN-INDUCED NEPHROTOXICITY AND GENOTOXICITY IN RATS

BY

ADESIDA, ADEBUKOLA (B.Sc.(Hons), M.Sc. (Ilorin) Biochemistry)

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DEDICATION

This work is dedicated to my lovely daughter Ayomiposi whose arrival has been a source of continuous joy unto me

CERTIFICATION

I certify that this work was carried out by Miss **ADESIDA**, Adebukola under my supervision in the Drug Metabolism and Toxicology Unit of the Department of Biochemistry, University of Ibadan, Nigeria.

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Ebenezer Olatunde Farombi, Ph.D (Ibadan),
MNYAS (USA), FRSC (UK),
Supervisor,
Professor of Biochemistry and Molecular Toxicology,
Dean, Faculty of Basic Medical Sciences,
Director, Drug Metabolism and Toxicology Unit,
Department of Biochemistry,
University of Ibadan,
Ibadan, Nigeria

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ABSTRACT

Adriamycin, a widely used anti-cancer drug, induces nephrotoxicity and genotoxicity in experimental animals through generation of free radicals. Phenolic phytochemicals in guava leaves possess antioxidant properties. Hence, they could play a protective role in adriamycin-induced nephrotoxicity and genotoxicity. The protective effects of Ethanolic Extract of *Psidium guajava* (EEPG) on adriamycin-induced nephrotoxicity and genotoxicity were investigated in rats.

Dried powdered guava leaves (2kg) were extracted with ethanol and concentrated in rotary evaporator to obtain the EEPG. Two hundred male Wistar rats (weighing180g-220g) were divided into control, adriamycin-treated (single dose-20mg/kg and cumulative dose-15mg/kg, i.p), and adriamycin and EEPG-treated (125, 250 and 500mg/kg p.o) animals in pre-, and co-treatments. Genotoxicity test involved bone marrow cytology to identify Micronucleated Polychromatophilic Erythrocytes (MPEs) 24 hrs after treatments. Animals were sacrificed by cervical dislocation and blood was obtained to determine blood urea nitrogen and creatinine. Post-mitochondrial fraction of kidney homogenate was used to evaluate Lipid Peroxidation (LPO), Cytochrome P450 3A4 (CYP3A4), antioxidant enzymes [Superoxide Dismutase (SOD), catalase, glutathione-S-transferase, Glutathione Peroxidase (GPx), Glutathione Reductase (GR)] activities, and glutathione level spectrophotometrically. Antioxidant activity of EEPG was assessed *in vitro* using 2-2-Azobis-2´ Amidinopropane-Hydrochloride (AAPH)-induced LPO model and Radical Scavenging Activities (RSA) against hydrogen peroxide (H_2O_2), hydroxyl, nitric oxide and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were determined spectrophotometrically. Histological changes in the kidney were assessed using a light microscope. The EEPG was fractionated by column chromatography over silica gel and sephadex LH-20 and its purified compound analysed for chemical structure by infrared and nuclear magnetic resonance spectroscopy. Data were analysed using ANOVA and Student's t-test at p=0.05.

About 500g of EEPG was obtained from 2kg of leaves. Renal dysfunction caused by 20mg/kg adriamycin (urea,-7.6µmol/L, creatinine,-11.8µmol/L) was prevented by pre-treatment with EEPG at 250mg/kg (4.9µmol/L, 7.9µmol/L) while antioxidant status was improved significantly by reducing LPO (0.5nmol) and increasing activities of SOD (3.6units), GPx (0.3nmol), catalase (0.1µmol), glutathione-S-transferase (0.3nmol), GR (0.6nmol) and

glutathione level (16.0µg/g) when compared with adriamycin-treated rats (0.9nmol, 1.7units, 0.1nmol, 0.04µmol, 0.2nmol, 0.4nmol and 13.9µg/g), respectively. Increase in creatinine by 15mg/kg adriamycin (1.0mg/dL) was reduced by co-treatment with 250 and 500mg/kg EEPG (0.4mg/L, 0.3mg/dL). This reduction was accompanied by increase in glutathione-S-transferase activity (0.11nmol, 0.09nmol) when compared with adriamycin (0.08nmol) and inhibition of CYP3A4 activity (7.9±0.3, 8.2±0.2) when compared with adriamycin (9.0±0.1). Toxicity was profound when adriamycin was administered as cumulative dose. The EEPG (125, 250 and 500mg/kg) decreased the frequency of MPE (11.8, 8.8 and 3.4/1000 MPEs respectively) when compared with 20mg/kg adriamycin (19.3/1000 MPEs). The EEPG showed significant antioxidant activities *in vitro* through reduction of AAPH-induced LPO-65.3%, RSA in H_2O_2-98.7%, nitric oxide-30.1%, DPPH·-70.4% and hydroxyl radicals-72.8% when compared with catechin (39.5%, 62.8%, 32.2%, 44.9% and 55.5%) respectively. Adriamycin induced renal tubular necrosis, while normal renal histology was maintained with EEPG at all doses. The purified compound from EEPG was found to be a triterpene.

Ethanolic extract of *Psidium guajava* protected rats against adriamycin-induced nephrotoxicity and genotoxicity. Free radical scavenging property and antioxidant activity of ethanolic extract of *Psidium guajava* may be responsible for this protective effect.

Key words: Nephrotoxicity, Genotoxicity, Adriamycin, Antioxidants, Guava extract.

Word count: 497

ABBREVIATIONS

A.A	Ascorbic acid
AAPH	2,2′-azobis (2-amidinopropane) hydrochloride
ABTS	2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt
ACE	Angiotensin-converting enzyme
ADH	Anti-diuretic hormone
ADR	Adriamycin
AMP	Adenosine monophosphate
ANOVA	One-way analysis of variance
ARBs	Angiotensin receptor blockers
ARF	Acute renal failure
ATP	Adenine triphosphate
BHA	Butylated hydroxyanisole
BSA	Bovine serum albumin
BUN	blood urea nitrogen
cAMP	Cyclic adenine monophosphate
CAT	Catalase
CC	Column chromatography
CDNB	1-chloro-2,4-dinitrobenzene
cNOS	Constitutive calcium-dependent isoforms
CNS	Central nervous system
COX-2	Cyclo-oxygenase 2
CREA	Creatinine
$CuSO_4$	Copper II tetraoxosulphate VI
DNA	Dinucleotide Adenine
$DPPH^{\cdot}$	Diphenyl picryl hydrazyl hydrate
DTNB	5',5'-dithiobis-2-nitrobenzoic acid
DXR	Doxorubicin
EDTA	Ethylenediamine tetraacetic acid

EEPG	Ethanolic extract of *Psidium guajava*
$FeSO_4$	Ferrous sulphate
FOX-1	Ferrous oxidation-xylenol orange
GE	Guava extract
GEF	Guava extract fraction
GFR	Glomerular filtration rate
G6Pase	Glucose-6-phosphatase
GMC	Glomerular messengial cells
GMP	Guanine monophosphate
GPx	Glutathione peroxidase
Gred	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
HCl	Hydrochloric acid
H&E	hematoxylin-eosin
H_2O_2	Hydrogen peroxide
H_2SO_4	Tetraoxosulphate (VI) acid
ICLs	Interstrand crosslinks
iNOS	Inducible nitric oxide synthase
IR	Infrared
KCl	Potassium chloride
$K_2Cr_2O_7$	Potassium dichromate
$K_3Fe(CN)_6$	Potassium ferricyanide
KH_2PO_4	Potassium dihydrogen phosphate
K_2 HPO_4	Dipotassium hydrogen phosphate
$K_2S_2O_6$	Potassium persulfate
LPO	Lipid peroxidation
LSD	Least significant difference
MDA	Malondialdehyde
MDR-1	Multidrug resistant

$MgCl_2$	Magnesium chloride
Na^+	Sodium ion
NaCl	Sodium chloride
NADPH	Reduced nicotinamide adenine dinucleotide
$NaHCO_3$	Sodium hydrogencarbonate
$NaNO_2$	Sodium nitrite
NAOH	Sodium hydroxide
NF-kB	Nuclear factor Kappa B
$NH_2C_6H_4SO_3H$	4-aminobenzenesulphonic acid
NMR	Nuclear magnetic resonance
$NO^{\cdot}$	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NSAIDs	Non-steroidal anti-inflammatory drugs
5′NTD	5'nucleotidase
O_2^-	Superoxide anion
OH	Hydroxyl
$ONOO^-$	Peroxylnitrite
PBS	Phosphate buffered saline
PG	Prostalglandin
PMF	Post-mitochondrial fraction
PO_4^{3-}	Phosphate group
PPAR	Peroxisome proliferator-activated receptor
RNA	Ribonucleotide adenine
RNS	Reactive nitrogen species
$ROO^{\cdot}$	alkylperoxyl radicals
ROS	Reactive oxygen species
RP	Reducing power
RSA	Radical scavenging activity
SEM	Standard error of mean
SOD	Superoxide dismutase

TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin Layer Chromatography
TNF-α	Tumor necrotic factor alpha
VLC	Vacuum Liquid Chromatography
UV	Ultraviolet
$ZnSO_4$	Zinc sulphate

CHAPTER ONE

1.1 INTRODUCTION

Adriamycin also known as doxorubicin is widely used to treat a variety of cancers including breast, ovarian, lung, uterine and cervical cancers, Hodgkin's disease, non-Hodgkin lymphoma, acute leukemia, soft tissue and primary bone sarcomas (Priestman, 2008). It is considered to be one of the most potent broad-spectrum antitumor anthracycline (Floyd *et al*., 2006). However, its use in clinical chemotherapy is limited on account of its lethal cytotoxic effects, among which are genotoxicity, testicular toxicity renal toxicity and cardiotoxicity (Singal *et al.*, 2000, Jung and Reszka, 2001; Mohamed and Amr, 2007: Carvalho *et al*., 2009). One common mechanism by which adriamycin induces nephrotoxicity and genotoxicity has been found to be the generation of free radicals. Excessive generation of free radicals are harmful to cells, mainly because they damage lipids, proteins, and nucleic acids, which leads to structural and functional impairments. Adriamycin has been demonstrated to have the potential for initiating genetic events in non-tumor cells in human and in animal systems. The induction of genotoxicity by adriamycin has been attributed to the trapping of topoisomerase II-DNA complex, or its ability to intercalate into DNA and to the generation of free radicals in the cell (Jung and Reszka, 2001). The nephrotoxic action of adriamycin is also considered to be via drug-induced free radical generation (Liu *et al*., 2007).

Nephrotoxicants (in form of therapeutic drugs, industrial or environmental chemicals) may account for approximately 50% of all cases of acute and chronic renal failure. In animal trials, adriamycin demonstrated nephrotoxic activity and produces chronic progressive glomerular disease (Injac *et al.*, 2008). Adriamycin-induced nephrosis provides a well characterized model of progressive renal damage. Nephrotoxicity can be defined as renal disorder or dysfunction that arises as a result of direct or indirect exposure to medicines, and industrial or environmental chemicals (WHO, 1991). It is well established that toxic nephropathies are not restricted to a single type of renal injury. Some chemicals target one discrete anatomical region of the kidney and may affect only one cell type. Chemical insult to the kidney may result in a spectrum of nephropathies that are indistinguishable from those that do not have a chemical etiology.

Over the last 20 years, it has become increasingly obvious that the kidney is adversely affected by an array of chemicals that man is exposed to in form of medicines, industrial and environmental chemicals, and a variety of naturally occurring substances. The level of exposure varies from minute quantities to very high doses. Exposure may be

over a long period of time 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 therapeutic necessity. Some chemicals cause an acute injury while others produce chronic renal changes that may lead to end-stage renal failure and renal malignancies.

The kidney has several features that allow nephrotoxicants to accumulate (Weinberg, 1993). It is highly vascular, receiving about 25% of the resting cardiac output (CO). The proximal renal tubule presents a large area for nephrotoxicant binding and transport into the renal epithelium. Reabsorption of the glomerular filtrate progressively increases intraluminal nephrotoxicant concentrations. 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 ((Perazella, 2005). In addition, its cells have the potential to convert chemicals and metabolically activate a variety of compounds.

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. 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 (de Mendonca *et al.*, 2000; Liano *et al.*, 1998). The mortality of acute renal failure is approximatley 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).

The evolving understanding of the pathophysiology of toxicant-mediated renal injury has implications for potential therapies and preventive measures. Great efforts have been expended trying to prevent or attenuate the side effects of adriamycin administration due to its great importance in cancer therapy. Accordingly, several approaches have been pursued in the 90's, such as dosage optimization, synthesis, and use of analogues or combined therapy. No helpful results have been found and the application of different adriamycin-analogues did not show better antineoplastic effect or lower toxicity than adriamycin (Barnerjee and Verma, 2008). Many different chemical agents have been examined to prevent adriamycin-induced cytotoxicity (Kocak *et al.*, 2003), and some of them showed promising results. These chemicals include natural products such as vitamins (A, C, carotenoids, E), coenzyme Q, flavonoids, polyphenols, selenium, and virgin olive oil (Quiles *et al.*, 2002). Curcumin has been reported to prevent adriamycin-induced nephrotoxicity in rats (Venkatesan *et al.*, 2000, Yilmaz *et al.*, 2006). Also, the protective effect of dihydropyridine calcium antagonists (nifedipine, nitrendipine,

amlodipine), lycopene, low molecular weight heparin, caffeic acid phenethyl ester and melatonin on adriamycin-induced toxicity has been described (Liu *et al*., 2007, Yilmaz *et al*., 2006, Deepa and Varalakshmi, 2005, Yagmurca *et al.*, 2004 and Dziegiel *et al.*, 2002). Additionally, polyhydroxylated fullerenes, have demonstrated high antioxidant activity *in vitro* that is comparable to or even higher than that of natural biological antioxidants like ascorbic acid and vitamin E. The high antioxidative potential of these compounds is proposed to be a consequence of their ability to react with highly reactive oxygen radical species, such as superoxide and hydroxyl radicals, which may damage biological macromolecules (Trajkovic *et al.*, 2007). *In vitro* and *in vivo* studies have shown that the water soluble fullerenol (FLR) C60(OH)24 has strong antioxidant activity. In animal models, it suppressed the cytotoxicity of adriamycin (Injac *et al.*, 2008). Algal extracts have demonstrated antioxidative and antitumoral activities on mammals (Funahashi *et al.*, 1999; Rupérez *et al.*, 2002) while aqeous fenugreek seed extract ameliorated adriamycin-induced cytotoxicity and testicular alterations in albino rats (Sakr *et al*., 2012).

In recent years, flavonoids and other phenolic compounds of plant origin have received increasing attention, especially in the field of pharmaceutical sciences and medicine, due to their potential to prevent a number of chronic and degenerative diseases including cancer and cardiovascular diseases (Boudet, 2007). Flavonoids are family of substances whose members have many interesting biological properties which include anticancer, antimicrobial, antiviral and anti-inflammatory activities (Havsteen, 2002). Studies indicated that an increase in the consumption of flavonoid-rich fruits and vegetables is associated with a decrease in the incidence of cardiovascular disease and different types of cancer (Gandini *et al*., 2000) and they provide benefits without the adverse effects of pharmacological agents used in clinical practice. The course of search for plants used in folk medicine to treat diverse human ailments led to the discovery that *Psidium guajava* commonly known as guava, contains as part of its chemical composition alkaloids, terpenols and flavonoids and has a long history of traditional use. The leaf decoction is used to treat indigestion associated with severe diarrhoea and cough (Heinrich *et al*., 1998). It is widely used in Mexico to treat gastrointestinal and respiratory disturbances and as an anti-inflammatory medicine (Aguilar *et al*., 1994). The roots, bark, leaves and immature fruits, are used in the treatment of gastroenteritis, diarrhoea and dysentery. Leaves or its flower infusion are used topically for wounds, ulcers and skin sores and for rheumatic pain, while they are chewed to relieve toothache (Heinrich et al., 1998). Combination of leaves decoction and bark is given to expel the placenta after childbirth (Martınez and Barajas, 1991). The water leaf extract is used to reduce blood

glucose level in people with diabetes (Aguilar *et al*., 1994). The leaf of *Psidium guajava* is used traditionally in South Africa to manage or control diabetes mellitus and hypertension (Ojewole, 2005). Latin America and the Caribbean use decoction of the leaves, roots, and bark of the plant to treat diarrhoea and stomach-aches due to indigestion (Mejıa and Rengifo, 2000; Mitchell and Ahmad, 2006a). It also has been used for dysentery in Panama and as an astringent in Venezuela. In Uruguay, a decoction of the leaves is used as a vagina and uterine wash (Conway, 2002). In Costa Rica, a decoction of the flower buds is considered an effective anti-inflammatory remedy (Pardo, 1999). In Peru, it is used in the form of gargles for gastroenteritis, dysentery, stomach pain (by acting on the pathogenic microorganisms of the intestine), indigestion, inflammations of the mouth and throat (Cabieses, 1993). The young leaves are used for treating inflammation of the kidney (Ticzon, 1997). The leaves are used as an ingredient in the preparation of fever "teas". They are also used as part of the pot herb used in treating malaria. Indeed, the main ethnotherapeutic use in Africa is said to be for malaria. *Psidium guajava* stem-bark extract contained anthraquinones, flavonoids, seccoirridoids and terpenoids and was found to be effective for the treatment of malaria (Nundkumar and Ojewole, 2002). The tender leaves are chewed to control toothaches by their weak sedative effect. It is also employed by the Indians of the Amazons for treating dysentery, sore throats, vomiting, stomach upsets, vertigo, and to regulate menstrual periods, mouth sores, bleeding gums, or used as a douche for vaginal discharge and to tighten and tone vaginal walls after childbirth. Its flowers can also be mashed and applied to the eye in conditions such as sun strain, conjunctivitis or eye injuries (Smith and Nigel, 1992). Guava jelly is tonic to the heart and constipation (Conway, 2002). In the Philippines, the astringent unripe fruit, the leaves, the cortex of the bark and the roots are used for washing ulcers and wounds, as an astringent, and for diarrhoea. The dust of the leaves is used in the treatment of rheumatism, epilepsy and cholera; and guava leaves is given to children suffering from convulsions (Morton, 1987). In Latin America, Central and West Africa, and Southeast Asia, guava is considered an astringent, drying agent and a diuretic. A decoction is also recommended as a gargle for sore throats, laryngitis and swelling of the mouth, and it is used externally for skin ulcers, vaginal irritation and discharge (Mejia and Rengifo, 2000). In Mozambique, the decoction of leaves is mixed with the leaves of *Abacateira cajueiro*, to alleviate flu, cough and pressed chest. In Mozambique, Argentina, Mexico and Nicaragua, guava leaves are applied externally for inflammatory diseases (Jansen and Mendez, 1990). *Psidium guajava* leaves are also used as an antiseptic (Teixeira *et al*., 2003). In Brazil, the fruit and leaves are used for treating anorexia, cholera, diarrhoea, digestive problems,

dysentery, gastric insufficiency, inflamed mucous membranes, laryngitis, mouth (swelling), skin problems, sore throat, ulcers and vaginal discharge (Holetz *et al*., 2002). In USA, guava leaf extracts are used in various herbal formulas for various purposes; from herbal antibiotic and diarrhoea formulas to bowel health and weight loss formulas (Smith and Nigel, 1992).

Extracts and phytochemicals isolated from *Psidium guajava* leaves have been shown to have multiple disease ameliorating effects caused by microbial pathogens. Scientific investigations on the medicinal properties of guava showed that, aqueous extracts of the leaf of *Psidium guajava* has anti-diarrhoea activity (Lin *et al*., 2002), *in vitro* anti-microbial activity against Escherichia coli, Salmonella typhi, Staphylococcus aureus, Proteus mirabilis, and Shigella dysenteria (Iwu, 1993). The tannin present in the leaves is reported to be responsible for its anti-microbial activity (Hernandez, 1980). Also, the ethanolic extract of guava leaves was shown to exhibit significant anti-inflammatory activity in rats (Muruganandan *et al.*, 2000) and to have potent antimicrobial activities against *Propionibacterium acnes* and may be beneficial in treating acne especially when they are known to have anti-inflammatory activities (Qadan *et al.*, 2005). Mukhtar *et al* (2006) evaluated anti-hyperglycaemic activity of the ethanol extract obtained from the stem bark of *Psidium guajava* on blood glucose levels of normal and alloxan-induced hyperglycaemic rats. The results showed that ethanol stem bark extract exhibited significant hypoglycaemic activity in alloxan-induced, hyperglycaemic rats but was devoid of significant hypoglycaemic effect in normal rats. Furthermore, Yamashiro *et al.*, 2003 reported that aqueous leaf extract of *Psidium guajava* exhibited cardioprotective effects against myocardial ischemia-reperfusion injury in isolated rat hearts.

On the basis of all the pharmacological activities and ethnomedicinal uses of *Psidium guajava* mentioned above, there is no doubt that the extract of this plant has immense potential. In the light of this, the present study was undertaken to evaluate the possible protective effect of guava extract (rich in flavonoids) on nephrotoxicity and genotoxicity induced by adriamycin.

CHAPTER TWO

LITERATURE REVIEW

2.1 THE KIDNEY STRUCTURE AND FUNCTION

2.1.1 RENAL ANATOMY

The two kidneys are situated retro-peritoneally, on either side of the vertebral column, and process 25% of the resting cardiac output via an arterial blood supply. Much of the fluid and most of the solutes in blood are filtered through the glomeruli into the proximal part of the nephron (the functional unit of the kidney) from which essential small molecules are reabsorbed. Numerous macro-molecules are reabsorbed into the tubular cells by an endocytotic process and are digested in tubular lysosomes. Many organic acids and bases (including many drugs) are secreted (and reabsorbed) by carrier-mediated processes located principally in the proximal tubule. There are some secretions, mainly of waste solutes, from the blood into the distal part of the nephron, and much of the water in which they are dissolved is subsequently reabsorbed.

Each kidney is made up of a large number of nephrons, groups of which unite to continue as collecting ducts or tubules, and these in turn combine to make up the ducts of Bellini, which exit around the papilla tip. The papilla opens into the calix, which is in continuity with the renal pelvis, a funnel-shaped area that narrows to the ureter. The continued production of urine, together with peristalsis of the ureter, carries excreted waste to the bladder. The morphophysiology of the kidney varies markedly between species. Therefore, a generalized description will be provided here.

2.1.2 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 specie 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).

2.1.2.1 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.

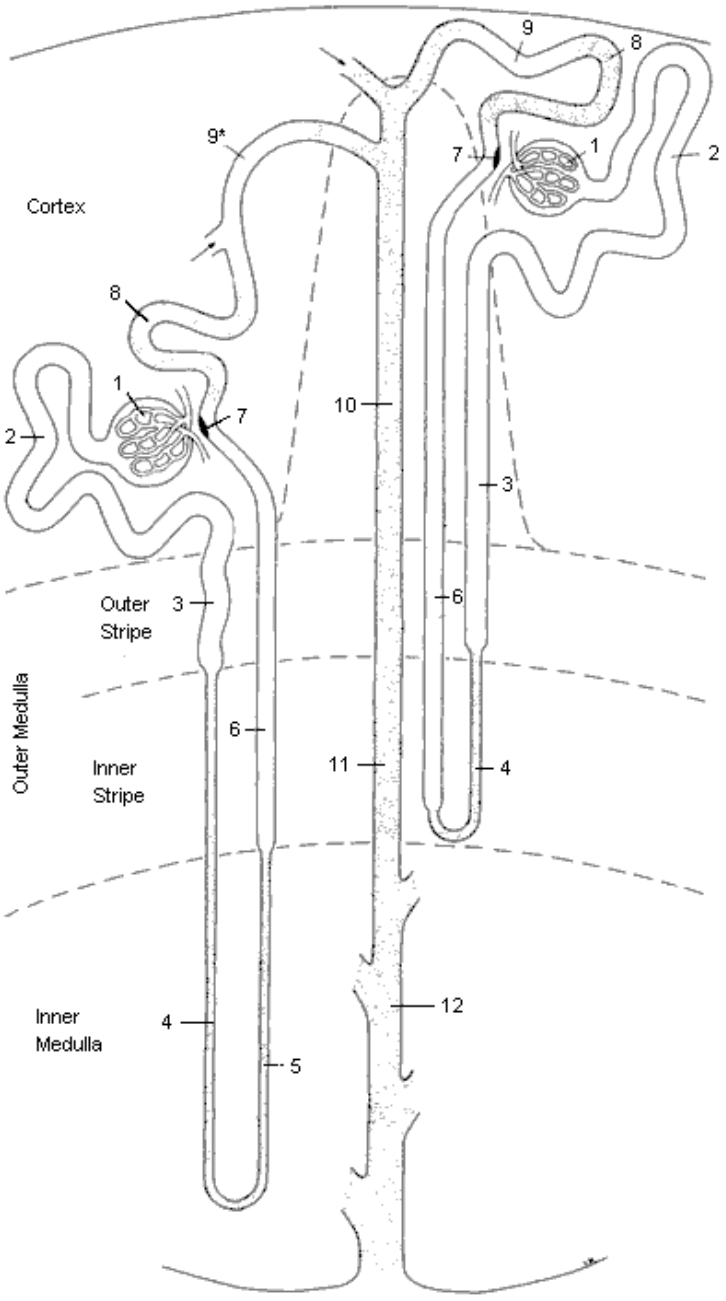

Figure 1: Scheme of nephron. This scheme depicts a short-looped and a long-looped nephron together with the collecting system. Within the cortex a medullary ray is delineated by a dashed line. 1 = renal corpuscle including Bowman's capsule and the glomerulus (glomerular tuft); 2 = proximal convoluted 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).

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).

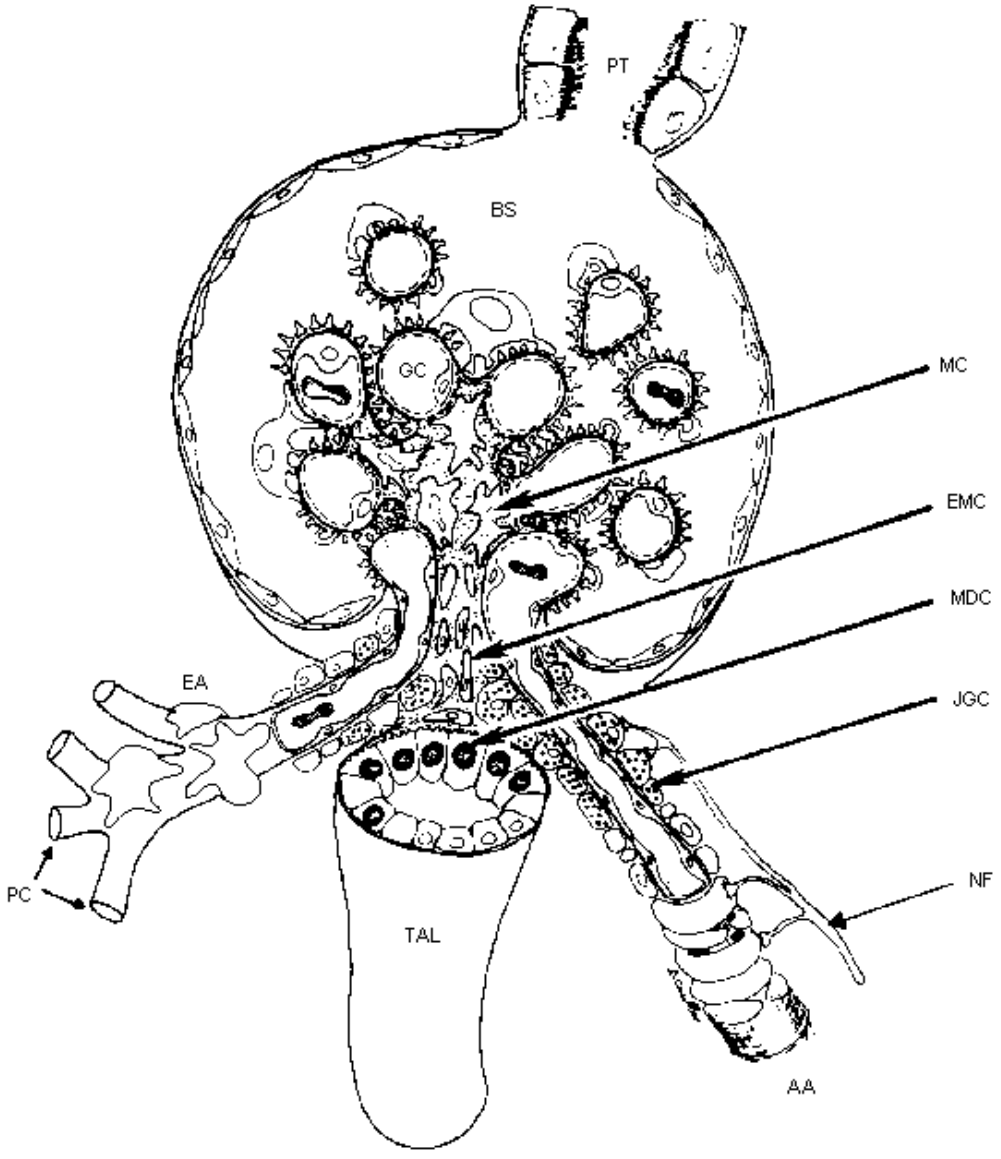

Figure 2: Glomerulus and juxtaglomerular complex, consisting of afferent arteriole (AA) with the granular cells (JGC) of the juxtaglomerular apparatus, the extraglomerular mesangial cells (EMC), the macula densa (MDC) segment of the ascending loop of Henle, and the efferent arteriole (EA). Also shown 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)

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.

2.1.2.2 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 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).

2.1.2.3 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).

2.1.2.4 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.

2.1.2.5 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.

2.1.2.6 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 in-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).

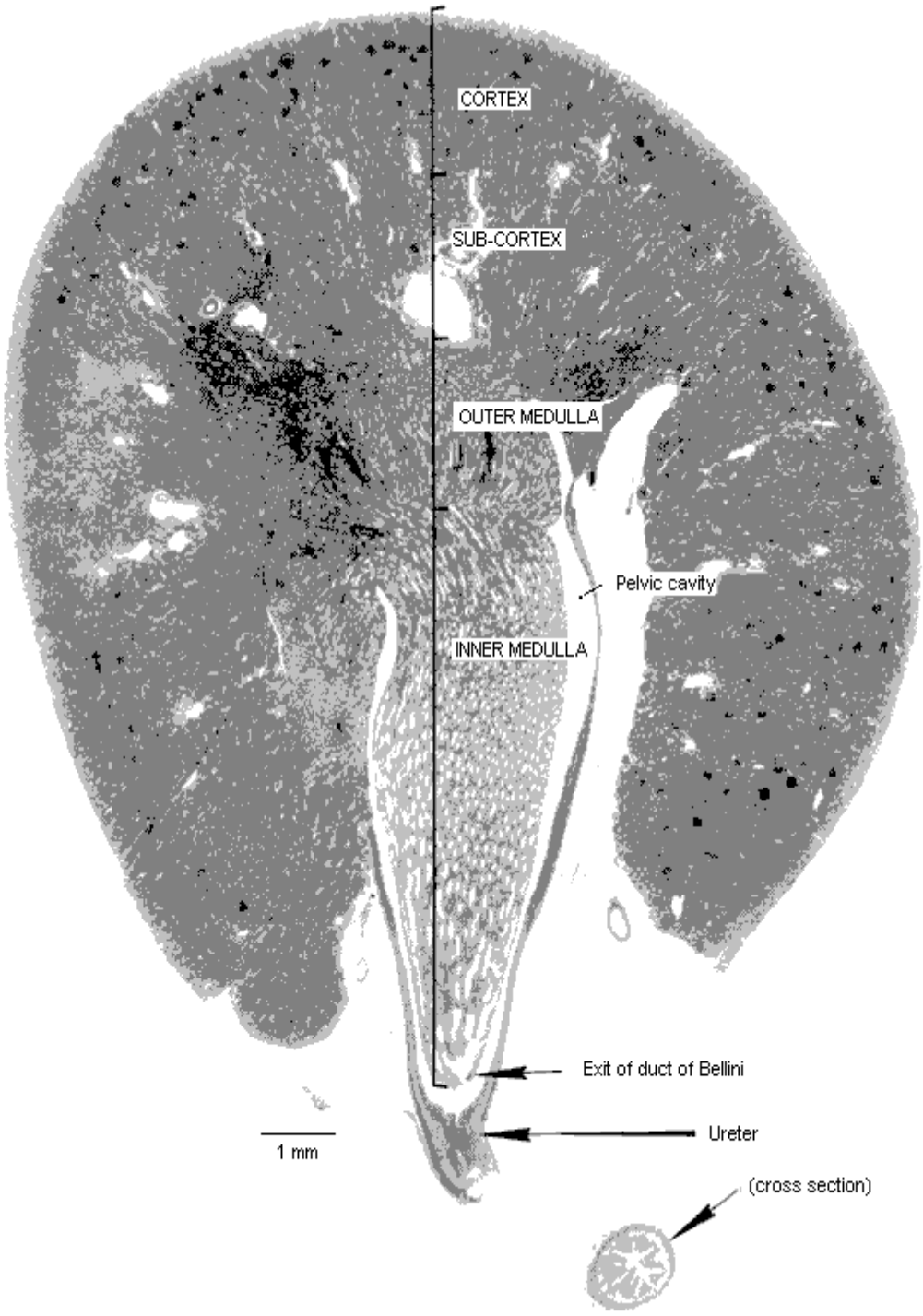

Figure 3: Coronal section through a normal rat kidney. Toluidine Blue; bar line = 1mm). From: Bach & Bridges (1985a).

2.1.2.7 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).

2.1.2.8 The interstitial 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).

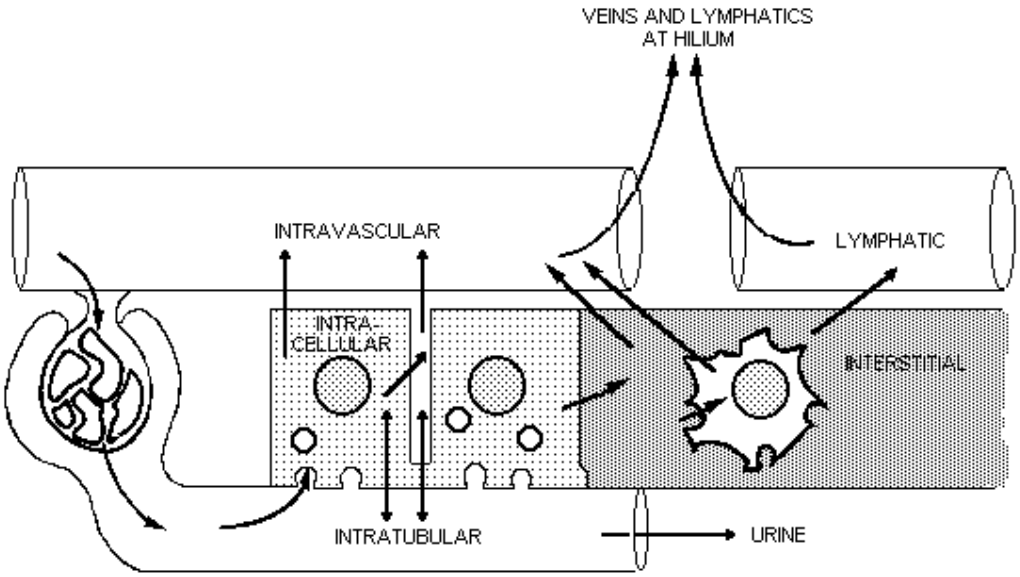

Figure 4: Schematic representation of the different cellular and extracellular components in the kidney. From: Moffat (1982).

2.1.3 DRUG-INDUCED NEPHROTOXICITY IN HUMANS

Drugs are common source of acute kidney injury. Acute renal failure (ARF) is abrupt deterioration of renal function sufficient to result in failure of urinary elimination of nitrogenous waste products (urea nitrogen and creatinine). This deterioration of renal function results in elevations of blood urea nitrogen and serum creatinine concentrations. Compared with 30 years ago, the average patient today is older, has more comorbidities, and is exposed to more diagnostic and therapeutic procedures with the potential to harm kidney function. Drugs shown to cause nephrotoxicity exert their toxic effects by one or more common pathogenic mechanisms. Drug-induced nephrotoxicity tends to be more common among certain patients and in specific clinical situations. Therefore, successful prevention requires knowledge of pathogenic mechanisms of renal injury, patient-related risk factors, drug-related risk factors, and preemptive measures, coupled with vigilance and early intervention. Knowledge of offending drugs and their particular pathogenic mechanisms of renal injury is critical to recognizing and preventing drug-induced renal impairment.

2.1.3.1 COMMON MECHANISMS OF DRUG-INDUCED NEPHROTOXICITY

Altered IntraGlomerular Hemodynamics

In an otherwise healthy young adult, approximately 120 mL of plasma is filtered under pressure through the glomerulus per minute, which corresponds to the glomerular filtration rate (GFR). The kidney maintains or autoregulates intraglomerular pressure by

modulating the afferent and efferent arterial tone to preserve GFR and urine output. For instance, in patients with volume depletion, renal perfusion depends on circulating prostaglandins to vasodilate the afferent arterioles, allowing more blood flow through the glomerulus. At the same time, intraglomerular pressure is sustained by the action of angiotensin-II–mediated vasoconstriction of the efferent arteriole. Drugs with antiprostaglandin activity (e.g., nonsteroidal anti-inflammatory drugs [NSAIDs]) or those with antiangiotensin-II activity (e.g., angiotensin-converting enzyme [ACE] inhibitors, angiotensin receptor blockers [ARBs] can interfere with the kidneys' ability to autoregulate glomerular pressure and decrease GFR (Schoolwerth *et al*., 2001 and Palmer, 2002). Other drugs, such as calcineurin inhibitors (e.g., cyclosporine [Neoral], tacrolimus [Prograf]), cause dose-dependent vasoconstriction of the afferent arterioles, leading to renal impairment in at-risk patients (Olyaei and de Mattos, 1999).

Tubular Cell Toxicity

Renal tubular cells, in particular proximal tubule cells, are vulnerable to the toxic effects of drugs because their role in concentrating and reabsorbing glomerular filtrate exposes them to high levels of circulating toxins (Perazella, 2005). Drugs that cause tubular cell toxicity do so by impairing mitochondrial function, interfering with tubular transport, increasing oxidative stress, or forming free radicals (Markowitz and Perazella 2005). Drugs associated with this pathogenic mechanism of injury include aminoglycosides, amphotericin B (Fungizone; brand not available in the United States), antiretrovirals (adefovir [Hepsera], cidofovir [Vistide], tenofovir [Viread]), cisplatin (Platinol), contrast dye, foscarnet (Foscavir), and zoledronate (Zometa) (Perazella, 2005).

Inflammation

Drugs can cause inflammatory changes in the glomerulus, renal tubular cells, and the surrounding interstitium, leading to fibrosis and renal scarring. Glomerulonephritis is an inflammatory condition caused primarily by immune mechanisms and is often associated with proteinuria in the nephritic range (Perazella, 2005). Medications such as gold therapy, hydralazine (Apresoline; brand not available in the United States), interferon-alfa (Intron A), lithium, NSAIDs, propylthiouracil, and pamidronate (Aredia; in high doses or prolonged courses) have been reported as causative agents (Markowitz and Perazella 2005). Acute interstitial nephritis, which can result from an allergic

response to a suspected drug, develops in an idiosyncratic, non–dose-dependent fashion (Rosert, 2001).

Medications that cause acute interstitial nephritis are thought to bind to antigens in the kidney or act as antigens that are then deposited into the interstitium, inducing an immune reaction (Rossert, 2001). However, classic symptoms of a hypersensitivity reaction (i.e., fever, rash, and eosinophilia) are not always observed (Markowitz and Perazella 2005). Numerous drugs have been implicated, including allopurinol (Zyloprim); antibiotics (especially beta lactams, quinolones, rifampin [Rifadin], sulfonamides, and vancomycin [Vancocin]); antivirals (especially acyclovir [Zovirax] and indinavir [Crixivan]); diuretics (loops, thiazides); NSAIDs; phenytoin (Dilantin); proton pump inhibitors (especially omeprazole [Prilosec], pantoprazole [Protonix], and lansoprazole [Prevacid]); and ranitidine (Zantac) (Geevasinga *et al*., 2006). Chronic interstitial nephritis is less likely than acute interstitial nephritis to be drug induced; it is also insidious in onset, and signs of hypersensitivity are often lacking (Appel, 2002). Drugs associated with this mechanism of nephrotoxicity include calcineurin inhibitors (e.g., cyclosporine, tacrolimus), certain chemotherapy agents, Chinese herbals containing aristocholic acid, and lithium (Isnard *et al.*, 2004). Chronic interstitial nephritis has been reported with analgesics such as acetaminophen, aspirin, and NSAIDs when used chronically in high dosages (i.e., more than 1 gram daily for more than two years) or in patients with preexisting kidney disease (Perneger *et al*., 1994). Early recognition is important because chronic interstitial nephritis has been known to progress to end-stage renal disease (Appel, 2002). Diagnosis may be difficult because most patients do not consider over-the-counter preparations to be medications and tend to underreport frequency of use.

Crystal Nephropathy

Renal impairment may result from the use of drugs that produce crystals that are insoluble in human urine. The crystals precipitate, usually within the distal tubular lumen, obstructing urine flow and eliciting an interstitial reaction (Markowitz and Perazella 2005). Commonly prescribed drugs associated with production of crystals include antibiotics (e.g., ampicillin, ciprofloxacin [Cipro], sulfonamides); antivirals (e.g., acyclovir, foscarnet, ganciclovir [Cytovene]); indinavir; methotrexate; and triamterene (Dyrenium) (Perazella, 1999). The likelihood of crystal precipitation depends on the concentration of the drug in the urine and the urinary pH (Perazella, 1999). Patients most at risk of crystal nephropathy are those with volume depletion and underlying renal

insufficiency (Perazella, 1999). Chemotherapy for lymphoproliferative disease, leading to tumor lysis syndrome with uric acid and calcium phosphate crystal deposition, has also been associated with renal failure (Davidson *et al.*, 2004).

Rhabdomyolysis

Rhabdomyolysis is a syndrome in which skeletal muscle injury leads to lysis of the myocyte, releasing intracellular contents including myoglobin and creatine kinase into the plasma. Myoglobin induces renal injury secondary to direct toxicity, tubular obstruction, and alterations in GFR (Coco and Klasner, 2004). Drugs may induce rhabdomyolysis directly secondary to a toxic effect on myocyte function, or indirectly by predisposing the myocyte to injury (Huerta-Alardín *et al.*, 2005). Clinical manifestations of rhabdomyolysis include weakness, myalgia, and tea-colored urine (Huerta-Alardín *et al.*, 2005). Statins are the most recognizable agents associated with rhabdomyolysis, but more than 150 medications and toxins have been implicated (Coco and Klasner, 2004). Rhabdomyolysis with statin monotherapy is rare, with an average reported incidence of 0.44 per 10,000 person-years of therapy (Graham *et al.*, 2004). Many drugs of abuse, such as cocaine, heroin, ketamine (Ketalar), methadone, and methamphetamine, have been reported to cause rhabdomyolysis (Coco and Klasner, 2004). Drugs and alcohol are causative factors in up to 81 percent of cases of rhabdomyolysis, and up to 50 percent of patients subsequently develop acute renal failure (Prendergast and George, 1993)

Thrombotic Microangiopathy

In thrombotic microangiopathy, organ damage is caused by platelet thrombi in the microcirculation, as in thrombotic thrombocytopenic purpura (Pisoni *et al.*, 2001). Mechanisms of renal injury secondary to drug-induced thrombotic microangiopathy include an immune-mediated reaction or direct endothelial toxicity (Pisoni *et al.*, 2001). Drugs most often associated with this pathogenic mechanism of nephrotoxicity include antiplatelet agents (e.g., clopidogrel [Plavix], ticlopidine [Ticlid]), cyclosporine, mitomycin-C (Mutamycin), and quinine (Qualaquin) (Manor *et al.*, 2004).

2.1.4 RECOGNITION AND EARLY INTERVENTION OF DRUG-INDUCED RENAL IMPAIRMENT

Most episodes of drug-induced renal impairment are reversible. Renal function generally returns to baseline provided the impairment is recognized early and the

offending medication is discontinued (Choudhury and Ahmed, 2006). Failure to act on available information relating to clinical findings or laboratory results was the most common monitoring error, occurring in 37 percent of preventable adverse drug events, including those affecting the kidney, in older ambulatory patients (Gurwitz *et al*., 2003). A decrease in renal function as evidenced by a rise in serum creatinine levels following the initiation of a drug signals the possibility of drug-induced renal injury. An exception to this is an increase in serum creatinine following the initiation of cimetidine (Tagamet) or trimethoprim (Proloprim), because they compete with creatinine for tubular secretion and are not associated with kidney damage or urine abnormalities (Choudhury and Ahmed, 2006).

Although there are no standard guidelines used to interpret changes in serum creatinine, a 50 percent rise from baseline, an increase of 0.5 mg per dL (40 µmol per L) or more when baseline serum creatinine is less than 2 mg per dL (180 µmol per L), or an increase of 1 mg per dL (90 µmol per L) or more if baseline creatinine is greater than 1.5 mg per dL have been used as biochemical criteria of acute renal failure (Nash *et al*., 2002) At the first sign of renal dysfunction, the patient's medication list should be reviewed to identify offending agents. If multiple medications are present and the patient is clinically stable, physicians should start by discontinuing the drug most recently added to the patient's medication regimen. Attention should then be directed at avoiding further renal insults by supporting blood pressure, maintaining adequate hydration, and temporarily discontinuing all other possible nephrotoxins (Fry and Farrington, 2006**).**

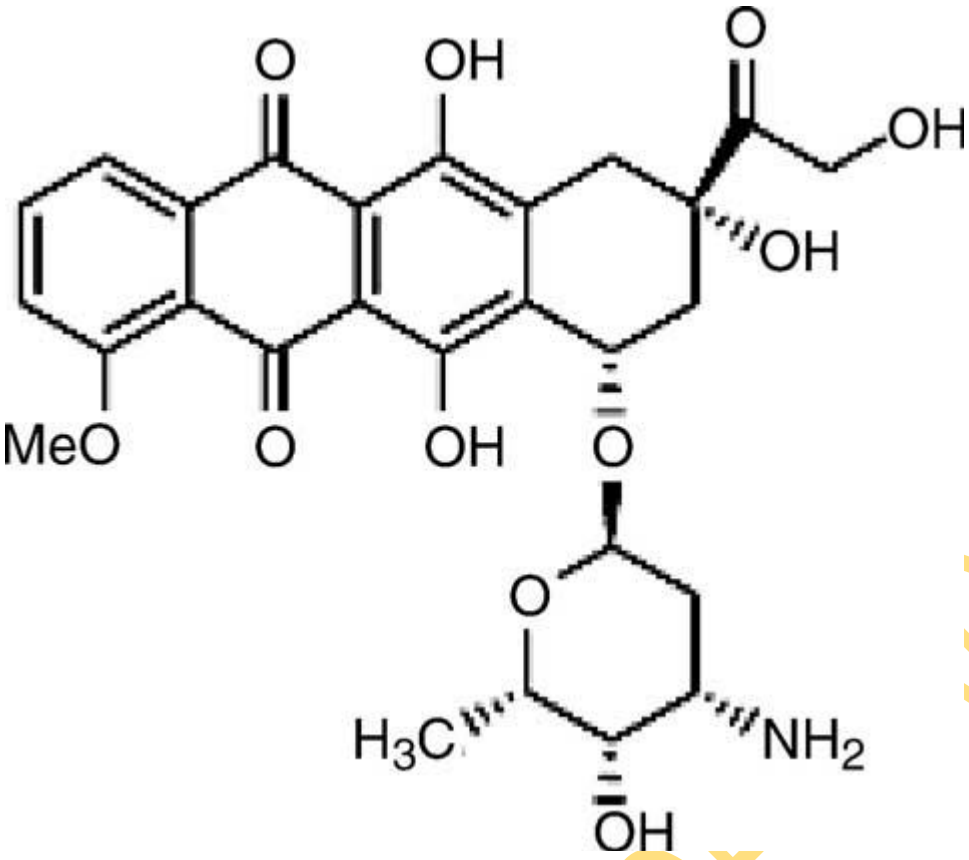

Figure 5: Structure of adriamycin (Yilmaz *et al.*, 2006)

2.2 HISTORY OF ADRIAMYCIN

Farmitalia Research Laboratories, an Italian research Company, in the 1950s, made an attempt to find anticancer compounds from soil-based microbes. A new strain of *Streptomyces peucetius* which produced a bright red pigment was isolated, from a soil sample in the area surrounding the Castel del Monte, a 13th century castle. The antibiotic produced from this bacterium was found to have good activity against murine tumors. Another group, who are French researchers also discovered the same compound at about the same time. The two teams named the compound daunorubicin, by combining the name *Dauni*, a pre-Roman tribe that occupied the area of Italy where the compound was isolated, with the French word for ruby, *rubis*, describing the colour (Weiss, 1992). In the 1960s, the drug was successfully used in treating acute leukemia and lymphoma. However, by 1967, it was discovered that daunorubicin could cause cardiac toxicity (Tan *et al.*, 1967). As a result of this, researchers at Farmitalia soon discovered that slight modification of its structure could result in changes in its biological activity. A strain of *Streptomyces* was mutated using N-nitroso-N-methyl

urethane and this new strain produced a different, red-coloured antibiotic. The new compound was named adriamycin, after the Adriatic Sea (Arcamone *et al*., 1969).

2.3 BIOSYNTHESIS OF ADRIAMYICIN

Adriamycin also known as doxorubicin (DXR) is a 14-hydroxylated version of daunorubicin, the immediate precursor of DXR in its biosynthetic pathway. Initially, Streptomyces peucetius was thought to be the only strain capable of producing doxorubicin (Lomovskaya, 1999). Later, Hutchinson's group showed that by genetic modifications, doxorubicin can be produced by other strains of streptomyces (Grimm, 1994). In 1996, the gene encoding the enzyme that converts daunorubicin into DXR called dox A, was discovered, isolated and characterized by Strohl's group (Dickens and Strohl, 1996). By 1999, recombinant Dox A, a Cytochrome P450 oxidase, was produced and found to participate in catalyzing multiple steps in DXR biosynthesis, including steps leading to daunorubicin (Walczak, 1999). Methods to improve the yield of DXR were developed by the Hutchinson's group aside from the fermentation process used in its commercial production. These include the introduction of Dox A encoding plasmids and deactivation of enzymes (by mutation) that shunt DXR precursors to less useful products, for example baumycin-like glycosides (Lomovskaya, 1999). Some triple mutants, that also over-expressed Dox A, were able to double the yield of DXR.

2.4 PHARMACOKINETICS OF ADRIAMYCIN

Distribution: It is distributed to many body tissues, particularly liver, spleen, kidney, lung, heart

Binding to plasma proteins: 70%

Metabolism: Enzymatic reduction at its 7 position and cleavage of the daunosamine sugar yields aglycones which are accompanied by free radical formation.

Plasma Clearance: Clearance is predominantly by metabolism and biliary excretion.

2.5 PROPERTIES

Adriamycin is the hydrochloride of doxorubicin (14-hydroxydaunomycin). It is a red, crystalline solid with a melting point of 24^{o}C. It is soluble in water and aqueous alcohols, moderately soluble in anhydrous methanol, and insoluble in nonpolar organic solvents. Neutral aqueous solutions of adriamycin are stable when stored at 5^{o}C (IARC, 1976).

2.6 CLINICAL USES AND SIDE EFFECTS.

Adriamycin is commonly used to treat a variety of cancers including breast, ovarian, lung, uterine and cervical cancers, Hodgkin's disease, non-Hodgkin lymphoma, acute leukemia, soft tissue and primary bone sarcomas (Priestman, 2008). Acute side-effects of adriamycin can include nausea, vomiting, and heart arrhythmias. It can also cause neutropenia (a decrease in white blood cells), as well as complete alopecia (hair loss). When the cumulative dose of adriamycin reaches 550 mg/m², the risks of developing cardiac side effects, including congestive heart failure, dilated cardiomyopathy, and death, dramatically increase.

2.7 MECHANISMS OF ACTION OF ADRIAMYCIN.

The mode of action of the anti-cancer drug, adriamycin, has been examined extensively over the past 20 years. Several studies reported the interaction of the inherently reactive drug with cell membranes, DNA, proteins, metal ions, and molecular oxygen, leading to an apparently complex interplay of the mechanism of antitumor action, the major determinants of which may differ according to the properties of target cancer cells (Myers *et al*., 1988). One possible physiological action in tumor cells involves the altered regulations of DNA-binding proteins in actively transcribed DNA (*i.e.* the open DNA regions in nuclear matrix attachment sites (Ciejek *et al*., 1983). Covalent attachment of the drug chromophore to specific DNA consensus sequences required for recognition by DNA-binding proteins may lead to altered levels and modes of binding by these proteins. The majority of adriamycin administered to sensitive tumor cells is known to rapidly localize in the nucleus (Gigli *et al*., 1988). It is well known that intercalation is the immediate form of interaction, and there is an extensive body of evidence to show that one of the first cellular responses is the inhibition of topoisomerase II activity (Capranico and Zunino, l992; Sinha, l995). The drug has a high affinity for DNA, thus providing the driving force for further nuclear uptake. Once a DNA-drug adduct is formed, it is widely accepted that the nature of the interaction

impedes cellular functions that involve DNA (*i.e.* replication and transcription), particularly in the event of damage to both strands by the formation of an interstrand cross-link (Hopkins *et al*., 1991). It follows that DNA-binding proteins would be affected to varying degrees by their modified substrate. If these adducts prevent binding of transcription factors to DNA in tumor cells, then the sequence selectivity of the particular drug will determine which transcription factors are affected, and hence which genes are inhibited. The net effect of this process is that gene-specific inhibition may occur; depending on the sequence specificity of the particular drug adducts (Suzanne *et al*., 1996).

2.7.1 Formation of covalent adducts

Of the approximately 50 drugs used in chemotherapy regimens, adriamycin has found one of the widest applications because of its activity against a broad range of malignancies (Weiss, 1992). Since adriamycin exhibits a wide range of cellular effects, it is likely that no single mechanism of action will account for all of the observed clinical and cellular responses (Weiss, 1992). There is a large body of evidence to show that the dominant cellular target is DNA (Phillips, and Cullinane, 1999), resulting in two major types of DNA damage: DNA adducts (Myers, 1992)) and protein-associated single- and double-strand DNA breaks (Holm *et al*., 1991).

Recently, the cytotoxicity of a range of derivatives of adriamycin was found to be proportional to the extent of formation of DNA interstrand cross-links (ICLs) in HeLa cells (Skladanowski and Konopa, 1994). This result therefore raises the possibility that a major mechanism of action involves the formation of cross-links with DNA. *In vitro* transcription analysis indicated that, adriamycin can form covalent adducts with DNA (Phillips *et al*., 1989). These occurred almost exclusively at GpC sequences and were found to behave much as classical interstrand DNA cross–links in their ability to block RNA polymerase (Cullinane and Phillips, 1990; Cullinane *et al*., 1994). It has since been shown that adduct formation has an absolute requirement for the 2-NH_2 of guanine (Cutts and Phillips, 1995). X-ray analysis has indicated that this group participates in one side of formaldehyde (CH_2O)-mediated aminal bridge, of which the other side is the 3′-NH_2 of the adriamycin sugar (Wang *et al.,* 1991., Zhen *et al*., 1993). The importance of the 3′-NH_2 has also been demonstrated in solution studies using modified adriamycin derivatives lacking this moiety.

Furthermore, adriamycin appears to be covalently linked to only one strand of the DNA (c-strand) in the manner described above and stabilizes the duplex via

uncommonly strong noncovalent interactions to the opposite strand (n-strand) (Figure 6). The ability of adriamycin to form adducts of this nature with DNA correlates strongly to its cytotoxicity in certain cell lines, suggesting that the therapeutic activity of this drug may derive from an ability to initiate such covalent complexes with DNA in patients (Skladanowski and Konopa, 1994).

The rate of formation of adducts *in vitro* was the same as the rate of formation of ICLs (Cutts and Phillips, 1995)) suggesting that the adducts are in fact ICLs. It has been confirmed that the adducts and ICLs are one and the same lesion (Taatjes *et al*., 1997). Electrospray mass spectral studies of oligonucleotides containing multiple GpC drug binding sites have revealed that the cross-links are mediated by formaldehyde which forms slowly under the reaction conditions employed for cross-link formation (Taatjes *et al*., 1997). Formaldehyde reacts with the amino group of adriamycin to form a Schiff base which then reacts with the N2 of guanine to form a monoadduct (Taatjes *et al*., 1997. The formaldehyde-conjugated complex is the active form of the drug (Fenick *et al*., 1997). The structure has been characterised by 2D NMR which has revealed that the drug intercalates adjacent to the GC site, and that the single adduct stabilises the duplex by the equivalent of 40 intercalated drug molecules or, alternatively, by an additional 12 hydrogen bonds (Zeman *et al*.,1998). The structure of the lesion was found to be essentially identical to the X-ray structure of the adduct discovered by accident some years ago as reported by Wang *et al* (1991). Taatjes *et al* (1997) reported that the term virtual cross-link has been used to describe this lesion because the monoadduct has the functionality of an interstrand cross-link. The adriamycin cross-links are both heat and alkali labile (van Rosmalen *et al.,* 1995), consistent with the known lability of the formaldehyde-mediated aminal link (N–C–N), and the absence of a second covalent link to complete the cross-link.

2.7.1.1 Significance of Adducts

In order for transcription to proceed, transcription factors and RNA polymerase must be able to recognize and bind to their target sequences, and additional processes that involve large regions of DNA being bent bring distant components of the transcription machinery together (van der Vliet and Verrijzer, 1993). Therefore it is conceivable that adducts could impair gene transcription at three different levels:

(1) By inhibiting DNA-binding proteins through steric constraints, rendering DNA unrecognizable due to adduct induced conformational change by bending, or through

subsequent mutation of the DNA bases required in the consensus sequence (Broggini and D'Incalci, 1994)

(2) By inhibiting the potential of the DNA to bend into the required conformation to bring distant regions together and initiate transcription (van der Vlet and Verrijzer, 1993)

(3) By posing a direct blockage to the path of RNA polymerase (Cullinane and Phillips, 1990)

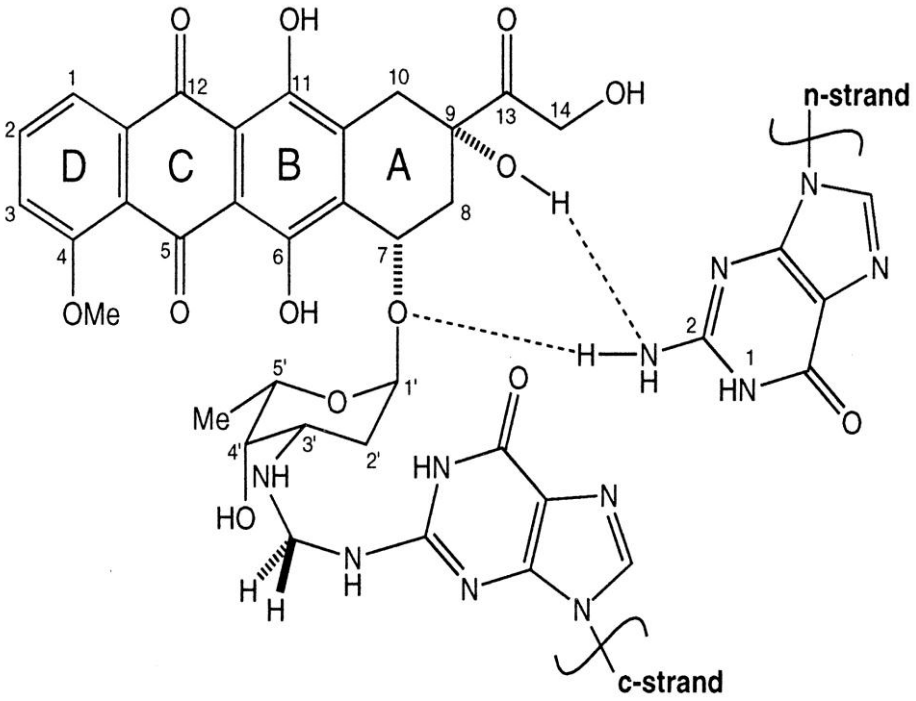

Figure 6: Structure of adriamycin showing its covalent and noncovalent bonding array to the c-strand and the n-strand of DNA. C-complementary, n-normal (Qu *et al*., 2001)

2.8 MECHANISMS OF ADRIAMYCIN-INDUCED NEPHROTOXICITY

Despite extensive studies on adriamycin nephrotoxicity, the mechanism(s) of the nephrotoxicity is uncertain. Although several hypotheses have been made, the biochemical mechanisms for this nephrotoxicity are not understood**.**

A lot of factors have been suggested to play a role in adriamycin-induced apoptosis ranging from reactive oxygen species (ROS) (Singal *et al.*, 2000), immune-mediated processes (Okuda, 1986; Amore *et al*., 1996), to cell-signaling pathways (Li *et al*., 2006).

2.8.1 An immune-system-mediated toxic effect

A previously unsuspected involvement of the immune system following adriamycin administration has been shown, with an increased production of IL1 by resident glomerular macrophages in rats receiving adriamycin (Bricio *et al*., 1992). This finding has led to the hypothesis that adriamycin induces the release of local mediators, by itself or via an early transient influx of T lymphocytes, which could modulate the la expression of resident macrophages and glomerular damage. A close relationship between glomerular TNF-α production and proteinuria has been demonstrated in rats treated with adriamycin (Gomez-Charri *et al.*, 1994).

The immune-mediated process might originate from adriamycin toxicity on the immune competent cells. Adriamycin intercalates itself between adjacent base pairs of DNA and it is likely that this phenomenon involves lymphocytes and monocytes or their precursors as well as resident renal cells. Adriamycin might react with the DNA of lympho-mononuclear cells in circulation modifying the activity of some regulatory genes resulting in the transcription and synthesis of cytokines which, following Couser's hypothesis (Narins, 1990), may induce proteinuria through a modification of epithelial cell integrin expression. This shows that adriamycin nephrotoxicity is at least in part mediated by immune system.

2.8.2 Programmed cell death (apoptosis)

A large number of drugs are known to induce renal cell apoptosis in cell culture or *in vivo* among which is adriamycin, and this is associated with renal dysfunction. Apoptosis plays a central role not only in the physiological processes of kidney growth and remodeling, but also in various human renal diseases and drug-induced nephrotoxicity. In the kidney, death through apoptosis is a physiological process in nephrogenesis as well as in maintenance of tissue homeostasis. When developing as a response to drug exposure, apoptosis may, however, become a double-edged weapon.

Induction of apoptosis is an important cytotoxic mechanism of adriamycin (Muller *et al*., 1998). The apoptosis of renal tubular cells has been reported in adriamycin-treated rats (Zhang *et al.*, 1996) and is believed to be a key feature of tubular atrophy, which is a hallmark of chronic renal diseases (Khan *et al.*, 1999).

MDR-1 (also termed P-glycoprotein, P-gp, and ABCB1) belongs to the ATP-binding cassette family of transporter molecules that require hydrolysis of ATP to run the transport mechanism. In normal kidney, MDR-1 is found in proximal tubules, mesangium, thick limb of Henle's loops, and collecting ducts (Ernest *et al*., 1997). It is likely that the MDR-1 function in kidney is to excrete toxic xenobiotics and metabolites into urine, preventing their accumulation in the body. Adriamycin was found to be one of the MDR-1 substrates (Kalyanaraman, 2002). Bello-Reuss and Ernest (1994) noted that in cultured human GMC (glomerular messengial cell) that were treated with adriamycin, the inhibition of MDR-1 resulted in increased cell damage. It therefore seems that in adriamycin nephrotoxicity, a pathologic change in glomeruli could be explained partially by adriamycin-mediated changes in GMC.

Studies have shown that, the overexpression of COX-2 leads to increased expression of multidrug resistance protein 1 (MDR-1) in rat GMC (glomerular

messengial cells), and this increase depends on COX-2 activity (Patel *et al*., 2002). This action rescued rat GMC from apoptosis induced by adriamycin, at least partially as a result of upregulation of MDR-1, causing efflux of adriamycin out of cells (Miller *et al*., 2006). This could be an illustration of a general mechanism by which COX-2 regulates drug efflux and exerts its antiapoptotic action in the GMC.

There are reports that, 1) expression of COX-2 has the ability to stimulate transcriptional upregulation of mdr1b promoter (Ziemann, 2002); 2) prostaglandins PGE_2 and $PGF_{2\alpha}$ have capacity to upregulate mdr1b gene expression in primary rat hepatocyte cultures (Ziemann, 2002); 3) prostaglandins PGE_2 and PGI_2 seem to be the principal PG metabolites that are induced by adenovirus- mediated transfer of COX-2 cDNA to rat GMC (Ishaque *et al.,* 2003); 4) both rat and human MDR-1 promoters could be regulated by products of COX activity. These findings raise the possibility that, administration of prostaglandins can protect from adriamycin-induced damage. Correspondingly, inhibition or suppression of COX-2, possibly via suppression of mdr1b promoter may have a role to play in mediating adriamycin-induced apoptosis.

In addition, cells that were induced to express MDR-1 were reported to maintain resistance to cell death that is induced by such death stimuli as FasL, glucocorticoid hormones, TNF (tumor necrosis factor) (Johnstone *et al.,* 2000). Accordingly, COX-2 expression and activity could be protective against apoptosis that is induced by these agents *via* upregulation of MDR-1. In other words, one can suggest that these agents would mediate adriamycin-induced renal apoptosis via downregulation of mdr1b promoter of MDR-1.

Also in human normal mammary epithelial cells, COX-2 protection from ADR-induced apoptosis was linked to COX-2 interaction with p53 *in vitro* and *in vivo*, resulting in regulation of its transcriptional activity as evidenced by suppression of p53 target gene induction by COX-2 co-transfection (Choi *et al*., 2005). Increase in apoptosis was found to be associated with an increase in p53 mRNA (Morrissey *et al.*, 1996). Consequently, increase in p53 mRNA may have a role in the development of apoptosis in the kidney following adriamycin administration.

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2.9 The role of CYP3A4 in drug metabolism

Cytochrome P450 (CYP) enzymes belong to heme-containing monooxygenases (Nelson *et al*., 1996). CYPs are categorized into families and subfamilies by their sequence similarities. Humans have 18 families of cytochrome P450 genes and 44 subfamilies. The CYP enzymes in families 1-3 i.e CYP1, CYP2 and CYP3 are active in the metabolism of a wide variety of xenobiotics including drugs (Pelkonen *et al*., 2008). Of these, the CYP3A sub-family represents perhaps the most significant group due to two facts: First, CYP3A enzymes are the most abundant CYPs in human liver, comprising between 30-50 % of total CYP content, and hence represent the bulk of the CYP enzymes that a chemical is likely to be exposed to (Watkins, 1994). Second, a large active site results in substrate promiscuity, meaning that up to 60 % of therapeutics in use today that are subject to metabolism are substrates for CYP3A subfamily members (Guengerish, 1997). Additionally, X-ray crystallography studies demonstrated that CYP3A4 has a very large and flexible active site, allowing it to oxidize either large substrates or multiple smaller ligands (Scott and Halpert, 2005). Taken together it can be seen that for the majority of xenobiotics, CYP3A plays some role in their metabolism in humans. It is widely known that concomitant oral administration of several foods and herbs affects drug metabolism in humans by inhibiting CYP3A4 activity. Among them, the inhibition by grapefruit juice has been well studied and it is reported that concomitant intake of the juice alters the pharmacokinetics of various drugs, including cyclosporin (Ducharme *et al*., 1995).

CYP3A4 is induced primarily in liver by glucocorticoids, drugs, pesticides, and carcinogens and encoded by human CYP3A4 Gene (Cytochrome P450 IIIA Subfamily). Cytochrome P450 3A4 is a 502-amino acid 57.3-kD membrane-bound ER heme-thiolate monooxygenase. CYP3A4 is involved in an NADPH-dependent electron transport pathway and phase I oxidative metabolism of carcinogens, steroids, fatty acids, and xenobiotics, as well as bioactivation of aflatoxin B(1) to a genotoxic derivative. Activity seems a key predictor of drug responsiveness and toxicity. CYP3A4 is a highly inducible enzyme with modulators that belong to a chemically diverse group of compounds consisting of drugs, steroids, and various nutraceuticals including herbal preparations (Desai *et al*., 2002). It has been reported that altered expression of this enzyme may be of clinical significance (Moore and Kliewer, 2000; Plant and Gibson, 2003). Induction or inhibition of CYP3A4 will affect the bioavailability of corresponding metabolised drug. For instance, Induction of the enzyme would result in drug metabolism which could result

in increased toxicity due to accumulation of toxic metabolites, drug side effects, or by altering the therapeutic efficacy of the administered drug.

Adriamycin and other drugs like cyclophosphamide, etoposide, and vincristine have been found to be metabolized by CYP3A4 (Baumhakel *et al*., 2001). CYP3A4 catalyses many different types of chemical reactions including N-oxidation, C-oxidation, N-dealkylation, O-dealkylation, nitro-reduction, dehydration and C-hydroxylation (Chen and Raymond, 2006).

2.10 OXIDATIVE STRESS AND PROTECTIVE EFFECTS OF FLAVONOIDS

It has long been recognized that ROS are harmful to cells, mainly because they damage lipids, proteins and nucleic acids, which leads to structural and functional impairments (Mantle and Preedy, 1999). Oxidative stress is a term denoting an imbalance between the production of oxidants and the respective defense systems of an organism. Oxidants encompass oxygen free radicals, reactive nitrogen species, sulphur-centred radicals and various others (Abuja and Albertini, 2001). This imbalance results in the build up of oxidatively modified molecules (Azzi *et al.*, 2004). Generation of these reactive products and induction of oxidative stress play significant role in the toxicity of some drugs. Considerable experimental evidence has contributed to support a key role of ROS in the numerous mechanisms of seemingly unrelated nephropathies (Rodrigo and Rivera, 2002). However, Living organisms have developed complex antioxidant systems to counteract reactive species and to reduce their damage. These antioxidant systems include enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, reduced glutathione (GSH) and vitamin C.

Numerous interventions have been put forward to counteract the effects of ROS, by reinforcing the antioxidant defense systems. The role of dietary flavonoids in the prevention of several chronic diseases has been the subject of intense research interest. 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). Flavonoids are typical phenolic compounds and, therefore, act as potent metal chelators, free radical scavengers (Kandaswami and Middleton, 1994) and powerful chain-breaking antioxidants. Flavonoids are subclass of polyphenols which are large family of natural compounds widely distributed in plant foods and contribute to the beneficial health effects of vegetables and fruit. They are

primarily recognized as the pigments responsible for the autumnal burst of hues and the many shades of yellow, orange, and red in flowers and food (Brouillard and Cheminant, 1988), and are found in fruits, vegetables, nuts, seeds, herbs, spices, stems, flowers, as well as tea and red wine. They are prominent components of citrus fruits (Kefford and Chandler, 1970) and other food sources (Herrmann, 1976) and are consumed regularly with the human diet. Their contribution to the antioxidant capacity of the human diet is much larger than that of vitamins. Red wine, a rich source of polyphenol has been shown to upregulate eNOS (endothelial nitric oxide synthase), a protective enzyme in the cardiovascular system (Sahelian, 2005). Study also indicated that flavonols may exert significant vascular protection because of their antioxidant properties. For instance, short-term administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons (Sahelian, 2005). Also, adriamycin-induced nephrotoxicity was ameliorated with curcumin, known to be rich in polyphenols (Venkatesan *et al.*, 2000). 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.

2.11 FLAVONOIDS AS ANTIOXIDANTS

Body cells and tissues are continuously threatened by the damage caused by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous factors (Nishikawa, 2008). The increased production of reactive oxygen species accompany most forms of tissue injury, which have been implicated in a multitude of disease states ranging from inflammatory injury to myocardial infarction and cancer (Willcox *et al.*, 2004). The mechanisms and the sequence of events by which free radicals interfere with cellular functions are not fully understood, but some of the detrimental effects in biological systems include peroxidation of membrane lipids, oxidative damage to nucleic acids or carbohydrates and the oxidation of sulfhydryl and other susceptible groups in proteins (Halliwell, 1991). Flavonoids have powerful antioxidant activities, being able to scavenge a wide range of reactive oxygen, nitrogen, and chlorine species, such as superoxide, hydroxyl radical, peroxyl radicals, hypochlorous acid, and peroxynitrous acid. They can also chelate metal ions, often decreasing metal ion prooxidant activity (Silva *et al.*, 2002). The best-described property of almost every group of flavonoids is their capacity to acts as antioxidants (Abou Seif, 2012).

2.12 WORKING MECHANISMS OF FLAVONOIDS

2.12.1 Effect on reactive oxygen species production by phagocytic cells

Phagocytosis is an important physiological process accompanied by the production of superoxide anions. While reactive oxygen species (ROS) generated by phagocytes play an important physiological function, they can also cause cellular damage. The highly reactive oxygen species, along with other mediators generated by neutrophils and macrophages, can promote inflammation and cause tissue damage (de Groot and Rauen, 1998). Several flavonoids have been shown to be effective inhibitors of ROS production by activating human neutrophils (Jung *et al*., 2003).

2.12.2 Radical scavenging

Flavonoids are scavengers of a wide variety of reactive oxygen, nitrogen, and chlorine species such as superoxide, hydroxyl radical, peroxyl radicals, hypochlorous acid and peroxynitrous acid. They are thermodynamically able to reduce these oxidizing free radicals due to their low redox potentials (Jovanovic *et al*., 1994). They are oxidized by radicals, resulting in a more stable, less reactive radical (Korkina and Afanas'ev, 1997). By scavenging radicals, flavonoids can inhibit LDL (low density lipoprotein) oxidation *in vitro*, (Keery and Abbey, 1997) protecting the LDL particles. Such effect may have preventive actions against atherosclerosis. Recent evidence shows that flavonoids are capable of quenching H_2O_2 and H_2O_2-generated hydroxyl radical (Agati *et al.*, 2012). Dihydroxy B-ring-substituted flavonoid glycosides have a great capacity to complex Fe and Cu ions, which catalyzes the formation of hydroxyl radical in the presence of H_2O_2, through the well-known fenton reaction. Flavonoids have been shown to quench singlet oxygen *in vivo* (Agati *et al*., 2007). Flavonoids have been reported to break free radical chain reactions in lipids (by donating hydrogen atoms to lipids or lipid peroxyl radicals), with quercetin having similar reduction potential of ascorbate and much lower than α-tocopherol (Galleano *et al*., 2010). Rutin has significantly smaller capacity than quercetin, but greater ability than kaempferol aglycone to reduce lipid radicals. These findings corroborate previous suggestions on the crucial role of the catechol group in the B-ring of the flavonoid skeleton in conferring free radical quenching capacity to flavonoids (Williams *et al.*, 2004). During inflammation, high concentrations of nitric oxide produced by inducible nitric oxide synthase in macrophages can result in oxidative damage. In such circumstances, activated macrophages greatly increase the simultaneous production of both nitric oxide and superoxide anions. Nitric oxide reacts with free radicals, thereby producing the highly damaging peroxynitrite that can directly oxidize LDL, resulting in irreversible damage to

the cell membrane (Haenen *et al*., 1997). When flavonoids are used as antioxidants, free radicals are scavenged and, therefore, can no longer react with nitric oxide, resulting in less cellular damage. Also, nitric oxide can be viewed as a radical itself, and it has been reported that nitric oxide molecules are directly scavenged by flavonoids (van Acker *et al.*, 1995). The soybean isoflavones genistein and daidzein increase LDL resistance to peroxynitrite-mediated oxidation, *in vitro*, in a concentration-dependent fashion (Lai and Yen, 2002). *In vivo* experiments have demonstrated that oral administration of isoflavones and extracts from soy-based products decrease serum nitrite, nitrate and nitrotyrosine levels in LPS-induced rats (Yen and Lai, 2003). Thus, isoflavone supplementation may inhibit reactive nitrogen species-induced oxidation, helping to provide a protective effect against cardiovascular and chronic inflammatory diseases.

2.12.3 Structure-reactivity relationships

Kinetic studies of reactions of flavonoids with active free radicals in homogeneous solution using pulse radiolysis and laser flash photolysis combined with theoretical calculations have related the number and position of hydroxyl groups and the extension of conjugation to the efficiency of flavonoids as antioxidants (Apak *et al*., 2007). Three structural requirements seems important: (i) the *ortho*-dihydroxy (catechol) structure in the B-ring, increasing the stability of oxidized flavonoid radicals through H-bonding or electron delocalization; (ii) the 2,3-double bond, in conjugation with the 4-oxo function, enhancing electron transfer and radical scavenging through electron-delocalization; (iii) the presence of both 3- and 5-OH groups, enabling the formation of stable quinonic structures upon flavonoid oxidation. Quercetin has been described as a typical flavonoid which meets the above three criteria, showing the highest antioxidant capacity. Aside from these structural requirements, the number and position of hydroxyl substituents on the flavonoid molecule, the presence of glycosides, and the overall degree of conjugation are important in determining their activities.

2.12.4 Inhibition of pro-oxidant enzymes

The stimulation of macrophages by inflammatory cytokines results in increased expression of inducible nitric oxide synthase (iNOS). Subsequently, this would lead to production of large amount of nitric oxide capable of inducing oxidative injury. Flavonoids and other natural polyphenols can inhibit lipopolysaccharide-induced iNOS gene expression and iNOS activity in cultured macrophages (Sarkar and Bhaduri, 2001) by reducing the nitric oxide production and, subsequently, oxidative damage. Also,

Lipoxygenases, cyclooxygenases and the xanthine oxidase pathway have the potential for increasing oxidative lesion in some tissues. The xanthine oxidase pathway has been implicated as an important route in the oxidative injury to tissues, especially after ischemia-reperfusion (Sanhueza *et al.,* 1992). Both xanthine dehydrogenase and xanthine oxidase are involved in the metabolism of xanthine to uric acid. Xanthine dehydrogenase is the form of the enzyme present under physiologic conditions, but its configuration is changed to xanthine oxidase during ischemic conditions. Xanthine oxidase is a source of oxygen free radicals. In the reperfusion phase (ie, reoxygenation), xanthine oxidase reacts with molecular oxygen, thereby releasing superoxide free radicals. Quercetin and silibin have been found to be capable of inhibiting xanthine oxidase activity, thereby resulting in decreased oxidative injury (Shoskes, 1998). Cos *et al* (1998) carried out a study on structure-function relations in which luteolin (3′, 4′5, 7-tetrahydroxyflavone) was reported to be the most potent inhibitor of xanthine oxidase. Some flavonoids and other plant polyphenols have the ability to inhibit the activities of these enzymes resulting in decreased oxidative injury (Nagao and Seki, 1999).

2.12.5 Leukocyte immobilization

The immobilization and firm adhesion of leukocytes to the endothelial wall is another major mechanism responsible for the formation of oxygen-derived free radicals, and also for the release of cytotoxic oxidants and inflammatory mediators and further activation of the complement system. Under normal conditions, leukocytes move freely along the endothelial wall. However, during ischemia and inflammation, endothelium-derived mediators and complement factors may cause adhesion of the leukocytes to the endothelial wall, thereby immobilizing them and stimulating degranulation of the neutrophil. As a result, oxidants and inflammatory mediators are released, resulting in injury to tissues. Oral administration of a purified flavonoid fraction was reported to decrease the number of immobilized leukocytes during reperfusion (Friesenecker *et al*., 1995). This decrease in the number of immobilized leukocytes by flavonoids may be related to the decrease in total serum complement and is a protective mechanism against inflammation-like conditions associated with reperfusion injury (Friesenecker *et al*., 1995).

2.12.6 Interaction with other enzyme systems

Another possible mechanism by which flavonoids act is through interaction with various enzyme systems. When reactive oxygen species are in the presence of iron, lipid peroxidation results. Specific flavonoids are known to chelate iron (Ferrali *et al*., 1997), thereby inhibiting generation of free radicals. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties. Direct inhibition of lipid peroxidation is another protective measure. Selected flavonoids can reduce complement activation, thereby decreasing the adhesion of inflammatory cells to the endothelium (Friesenecker *et al*., 1995) and in general resulting in a diminished inflammatory response. Another feature of flavonoids is a reduction in the release of peroxidase. This reduction inhibits the production of reactive oxygen species by neutrophils by interfering with α_1-antitrypsin activation. A progressive inactivation of proteolytic enzymes was described in neutrophils (Middleton and Kandaswami, 1992). Another interesting effect of flavonoids on enzyme systems is the inhibition of the metabolism of arachidonic acid which is a starting point for a general inflammatory response (Ferrandiz and Alcaraz, 1991).

2.13 GUAVA (*Psidium guajava*)

Guava is a low evergreen tree or shrub 6 to 25 feet high, with wide-spreading branches and square, downy twigs, is a native of tropical America. It is a common vegetation cover by roads and in waste places in Hawaii. Guava is a tropical and semitropical plant. It is well known in the islands for its edible fruit. It is common in the backyards. The branches are crooked, bringing opposite leaves. The flowers are white, incurved petals, 2 or 3 in the leaf axils; they are fragrant, with four to six petals and yellow anthers. The fruit is small, 3 to 6 cm long, pear-shaped, reddish-yellow when ripe.

Psidium guajava belonging to the family of *Myrtaceae*, is a native of tropical America and has long been naturalized in Southeast Asia. The positive effects of guava extract on human ailments have been described (Lozoya, 1999). The pharmacological actions and the medicinal uses of aqueous extract of guava leaves in folk medicine include the treatment of various types of gastrointestinal disturbances such as vomiting, diarrhea, inhibition of the peristaltic reflex, gastroenteritis, spasmolytic activity, dysentery, abdominal distention, flatulence and gastric pain (Lutterodt, 1992; Aguilar *et al*., 1994; Lozoya *et al*., 1994). Bronchitis, asthma attacks, cough, pulmonary diseases could be also treated with guava teas (Batick, 1984; Khan and Ahmad, 1985) and could

also be useful as anti-inflammatory and hemostatic agent (Liu, 1988). Moreover, aqueous extract of guava leaves were described to be effective against a number of microbial strains: *Aeromonas hydrophila*, *Shigella* spp. and *Vibrio* spp. (Chulasiri *et al.*, 1986), *Staphylococus aureus* and *β-streptococcus* group A (Jaiarj *et al*., 1999), *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* (Abdelrahim *et al*., 2002). In addition, anti-rotavirus activity has also been reported to exist in these extract (Gonçalves *et al*., 2005).

Phytochemical studies have identified more than 20 compounds in guava extract (Osman *et al*., 1974; Begum *et al*., 2002). The major constituents of its leaves were

Figure 7: Guava tree

identified to be tannins, *β*-sitosterol, maslinic acid, essential oils, triterpenoids and flavonoids (Osman *et al*., 1974; Arima and Danno, 2002; Begum *et al*., 2004). The antioxidant activity of phenolic compounds could be used to prevent various chronic diseases such as heart-disease, diabetes, cancer, arterial thrombosis, cataract and may provide health-promoting effects (Kimura *et al*., 1985; Qian and Nihorimbere, 2004). Chemical analysis of guava extract has revealed the presence of essential oils, tannins, saponins, carotenoids, flavonoids and triterpenes (Mercadante *et al*., 1999; Arima and Danno, 2002; Begum *et al*., 2002). The different phenolic phytochemicals in guava

leaves have been described to possess antioxidant action and able to inhibit peroxidation reaction in living systems (Kimura *et al*., 1985; Van acker *et al*., 1995; Qian and Nihorimbere, 2004). Moreover, aqueous and ethanolic extract of guava leaves showed anti-microbial activity (Arima and Danno, 2002; Qa'dan *et al*., 2005). Flavonoid glycosides and flavonoids as guaijavarin and quercetin have been isolated and showed activity against *Salmonella enteritidis* and *Bacillus cereus* (Arima and Danno, 2002).

2.13.1 Chemical composition

2.13.1.1 The fruit

The fruits of guava are characterized by low content of carbohydrates (13.2%), fats (0.53%), proteins (0.88%) and by highwater content (84.9%), (Medina and Pagano, 2003). The food value per 100 g is: Calories 36–50 kcal, moisture content of 77–86 g, crude fibre 2.8–5.5 g, ash 0.43–0.7 g, calcium 9.1–17 mg, phosphorus (Conway, 2002), 17.8–30 mg, iron 0.30–0.70 mg (Iwu, 1993), vitamin A of 200–400 I.U., thiamine 0.046 mg, riboflavin 0.03–0.04 mg, niacin 0.6–1.068 mg, ascorbic acid 100 mg and vitamin B3 40 I.U. (Conway, 2002). Manganese is also present in the plant in combination with phosphoric, oxalic and malic acids (Nadkarni and Nadkarni, 1999). Hexanal (65.9%), butyrolactone (7.6%), (E)-2-hexenal (7.4%), (E,E)-2,4-hexadienal (2.2%), (Z)-3-hexenal (2%), (Z)-2-hexenal (1%), (Z)-3-hexenyl acetate (1.3%) and phenol (1.6%) were reported from fresh white-flesh guayaba fruit oil. 3-caryophyllene (24.1%), nerolidol (17.3%), 3-phenylpropyl acetate (5.3%) and caryophyllene oxide (5.1%) were isolated from essential oil extracted from the fruits (Paniandy *et al*., 2000). Subsequently, the active aromatic constituents in pink guava fruit which is the 3-penten-2-ol and 2-butenyl acetate were isolated (Jordan *et al.*, 2003). The fruit also contains glykosen 4.14%, saccharose 1.62%, and protein 0.3% (Hwang *et al.*, 2002). The unripe fruit is found to be indigestible, causes vomiting and feverishness. The activities of hydrolytic enzymes α-amylase and β-amylase were found to decrease significantly with ripening. Chlorophyll, cellulose, hemicellulose, and lignin content increase while carotenoid content decreases. The unripe fruit is high in tannins, is astringent and has a tendency to cause constipation, but it is sometimes employed in diarrhoea (Jain *et al*., 2003). Iwu (1993) reported that the fruits contain vitamin C (Hernandez, 1980), vitamin A, iron, calcium and phospohorus. Guavas are up to 5 times richer in vitamin C than oranges (Conway, 2001). Manganese is also present in the plant in combination with phosphoric, oxalic and malic acids (Nadkarni and Nadkarni, 1999). The fruit contains saponin combined with

oleanolic acid. Morin-3-O-α-L-lyxopyranoside and morin-3-O-α-L-arabopyranoside and flavonoids, guaijavarin and quercetin (Arima and Danno, 2002).

2.13.1.2 The fruit skin

Ascorbic acid is the main constituent of the skin, secondly in the firm flesh, and a little content in the central pulp varies from 56 mg to 600 mg and may range from 350 mg to 450 mg in nearly ripe fruit (Charles *et al*., 2006). Canning or other heat processing destroys about 50% of the ascorbic acid. The strong odour of the fruit is attributed to its carbonyl compounds (Dweck, 2001).

2.13.1.3 The leaves

The leaves contain essential oil with the main components being *α*-pinene, *β*-pinene limonene, menthol, terpenyl acetate, isopropyl alcohol, longicyclene, caryophyllene, *β*-bisabolene, caryophyllene oxide, *β*-copanene, farnesene, humulene, selinene, cardinene and curcumene (Zakaria and Mohd, 1994). The essential oil from the leaves has been shown to contain nerolidiol, *β*-sitosterol, ursolic, crategolic, and guayavolic acids (Iwu, 1993). Flavonoids, and saponins combined with oleanolic acid have been isolated from the leaves (Arima and Danno, 2002). In addition, the leaves contain triterpenic acids as well as flavonoids; avicularin and its 3-l-4-pyranoside with strong antibacterial action (Oliver-Bever, 1986), fixed oil 6%, 3.15% resin, and 8.5% tannin, and a number of other fixed substances, fat, cellulose, tannin, chlorophyll and mineral salts (Nadkarni and Nadkarni, 1999). Guavanoic acid, guavacoumaric acid, 2*α*-hydroxyursolic acid, jacoumaric acid, isoneriucoumaric acid, asiatic acid, ilelatifol d and *β* -sitosterol-3-*O*-*β*-dglucopyranoside have also been isolated from the leaves of Psidium guajava (Begum *et al*., 2002). In mature leaves, the greatest concentrations of flavonoids were found in July: Myricetin (208.44 mg kg−1), quercetin (2883.08 mg kg−1), luteolin (51.22 mg kg−1) and kaempferol (97.25 mg kg−1) (Vargas *et al.*, 2006). Two triterpenoids, 20*β*-acetoxy-2*α*3*β*-dihydroxyurs-12-en-28-oic acid (guavanoic acid), and 2*α*,3*β*-dihydroxy-24-*p*-*z*-coumaroyloxyurs-12-en-28-oic acid (guavacoumaric acid), along with six known compounds 2*α*-hydroxyursolic acid, jacoumaric acid, isoneriucoumaric acid, asiatic acid, ilelatifol d and *β*-sitosterol-3-*O*- *β* –d-glucopyranoside, have been isolated from the leaves of *Psidium guajava*. Guajavolide (2a-,3*β*-6*β*-,23-tetrahydroxyurs-12-en-28,20*β*-olide, and guavenoic acid, have also been isolated from fresh leaves of *Psidium guajava*.

2.13.1.4 The bark

The bark contains 12-30% of tannin and Burkill (1997) says it contains tannin 27.4%, or polyphenols, resin and crystals of calcium oxalate (Nadkarni and Nadkarni, 1999).

2.13.1.5 The root

The roots are also rich in tannin (Quisumbing, 1978). The plant also contains leukocyanidins, sterols, and gallic acid in the roots (Iwu, 1993). There is a high percentage of carbohydrates and salts

2.13.1.6 The seeds

The seeds are very small but abundant in the fruit and have been reported to contain 14% oil on dry weight, with 15% proteins and 13% starch (Burkill, 1997). Ten phenolic and flavonoid compounds including one new acylated flavonol glycoside were isolated. The structures of quercetin-3-O-β-D-(2''-O-galloyglucoside)-4'-O-vinylpropionate and of the known compounds were elucidated (Michael *et al*., 2002).

2.13.1.7 Floral bud: The guava buds have the highest concentrations of myricetin (256 mg kg−1), quercetin (3605 mg kg−1), luteolin (229 mg kg−1), kaempferol (229 mg kg−1) and apigenin (252 mg kg−1) (Vargas *et al*., 2006).

2.13.1.8 Twigs: Guava twigs contain calcium (0.30–1.00%), magnesium (0.06–0.30%), phosphorus (0.10–0.38%), potassium (0.21–0.39%), and sodium (0.03–0.20%). The concentration of fluoride ranged from 0.02 ppm to 0.11 ppm, copper (0.02–0.14 ppm), iron (2.86–5.14 ppm), zinc (0.31–0.57 ppm), manganese (0.00–0.26 ppm), and lead (0.00–0.11 ppm) (Okwu and Ekeke, 2003). It also contains flavonoid, sesqui-terpenes alcohols and acids triterpenoids (Hegnauer, 1969).

2.14 BIOLOGICAL ACTIVITY

2.14.1 Anti-diarrhoea

Ethanol and aqueous extracts of *Psidium guajava* at a concentration of 80 µg/ml in an organ bath, was found to exhibit more than 70% inhibition of acetylcholine and/or KCl solution-induced contractions of isolated guinea pig ileum. This was carried out by using the rates of propulsion in the small intestine in male Sprague–Dawley rats as a means of assessing antidiarrhoea activity of aqueous extracts of the leaf of *Psidium guajava* using morphine as the standard drug of reference measured (Tona *et al.*, 1999). A dose of 0.2

ml/kg fresh leaf extract produced 65% inhibition of propulsion which is equitable with 0.2 mg/kg of morphine sulphate. The antidiarrhoea action of the extract may be due, in part, to the inhibition of the increased watery secretions that occur commonly in all acute diarrhoeal diseases and cholera.

According to Lutterodt (1989), Quercetin and quercetin-3-arabinoside, extracted from the buds and leaves of *Psidium guajava* at concentrations of 1.6 g/ml showed a morphine-like inhibition of acetylcholine release in the coaxially stimulated ileum, together with an initial increase in muscular tone, followed by a gradual decrease. It is also reported that the asiatic acid extracted from the leaves, showed dose-dependent (10–500 µg/ml) spasmolytic activity in spontaneously contracting isolated rabbit jejunum preparations (Conde *et al*., 2003). Methanol extract from leaves (8µg/ml) of *Psidium guajava* showed activity against simian (SA-11) rotavirus (93.8% inhibition) as reported by Goncalves *et al* (2005). In addition, galactose-specific lectin in guayaba was shown to bind to *Escherichia coli* (a common diarrhoeacausing organism), preventing its adhesion to the intestinal wall and thus preventing infection resulting in diarrhoea (Coutino *et al*., 2001). A methanolic leaf extract (8 µg/ml) of *Psidium guajava* also showed activity against simian (SA-11) rotavirus (93.8% inhibition) (Goncalves *et al*., 2005).

2.14.2 Antimicrobial

Methanolic extract from ripe guava fruit has been described by Sato *et al* (2000) to have fungicidal action against *Arthrinium sacc*M001and *Chaetomium funicola*M002 strains while the aqeous and methanolic extracts of the leaves are effective inhibitors of growth spore formation, and enterotoxin production of *Clostridium perfringens* type A. *Psidium guajava* leaf and bark tinctures were subjected to *in vitro* sensitivity tests by serial dilution at concentrations ranging from 5% to 15% against six test dermatophytes, viz., *Trichophyton tonsurans, Trichophyton rubrum, Trichosporon beigelii, Microsporum fulvum, Microsporum gypseum* and *Candida albicans*. Results showed that bark tincture exhibited higher efficacy in controlling the mycelial growth of dermatophytes than the leaf tincture. The bark tincture showed fungicidal activity at different concentrations but exhibited only fungistatic property in case of *Candida albicans* (Dutta and Das, 2000). Ethanolic extract from the shell of ripe fruit showed activity on *Streptococcus mutans* and *Escherichia coli* (Neira and Ramirez, 2005). These results supported the utilization of *Psidium guajava* in traditional medicine for intestinal diseases produced by microorganisms.

2.14.3 Antimalaria effects

An *in vitro* antiplasmodial assay carried out using a chloroquine-sensitive strain of malarial parasite, *Plasmodium falciparum* D10 was shown to have antigiardiasic activity with guava stembark extract showing IC50 values of 10–20 µg/ml (Nundkumar and Ojewole, 2002). Another study on the leaves and stem bark of *Psidium guajava* showed inhibition of *Entamoeba histolytica* growth with MAC< 10µg/ml (Tona *et al.*, 1998).

2.14.4 Hepatoprotective effects

Roy *et al* (2006) studied the hepatoprotective effect of an aqueous leaf extract of *Psidium guajava* in liver damage induced by carbon tetrachloride in rats by monitoring serum transaminase (aspartate amino transferase and serum alanine amino transferase), alkaline phosphatase, serum cholesterol, serum total lipids and histopathological alterations. The leaf extract at doses of 500 mg/kg was found to produce significant hepatoprotection. Pretreatment with asiatic acid (a triterpenoid extracted from *Psidium guajava* leaves and fruit) at doses of 25 mg/kg, 50 mg/kg or 100 mg/kg significantly blocked the LPS (lipopolysaccharide) and (d-galactosamine) d-GalN-induced increases in both serum aspartate aminotransferase and serum alanine aminotransferase levels, showing improved nuclear condensation, ameliorated proliferation and less lipid deposition (Gao *et al*., 2006).

2.14.5 Antioxidant and free radical scavenging activities

According to Qian and Nihorimbere (2004), a remarkably high total phenolic content (575.3±15.5) was obtained from guava extract. This was determined spectrophotometrically according to Folin–Ciocalteu's phenol method and calculated as gallic acid equivalent (GAE). The antioxidant activity of the lyophilized leaf extracts was also determined using free radical DPPH (2, 2-diphenyl-1-picrylhydryzyl). The results obtained showed that ascorbic acid was a more powerful antioxidant than the extracts from guava leaf. These antioxidant properties of guava extract have been suggested to be associated with its phenolic compounds such as protocatechuic acid, ferulic acid, quercetin and guavin B (Thaipong *et al*., 2005), quercetin, ascorbic acid, gallic acid and caffeic acid (Jimenez *et al*., 2001).

2.14.6 Cardiovascular and hypotensive effects

The effect of an aqueous leaf extract of *Psidium guajava* on myocardial injury was studied in the model of global ischemia followed by reperfusion. High-energy phosphates

and malondialdehyde in the reperfused hearts were found to be significantly reduced with the plant extract (Conde *et al*., 2003). In another study, aqueous leaf extract of *Psidium guajava* exhibited cardioprotective effects against myocardial ischemia-reperfusion injury in isolated rat hearts (Yamashiro *et al*., 2003). Ojewole (2005) showed that the aqueous leaf extract caused hypotension in the experimental animal model used via cholinergic mechanisms while acute intravenous administrations of the leaf extract (50–800 mg/kg i.v.) produced dose-dependent, significant reductions in systemic arterial blood pressures and heart rates of hypertensive, Dahl salt-sensitive rats. Belemtougri *et al* (2006) found that aqueous and ethanolic leaf extracts of *Psidium guajava* inhibits intracellular calcium release. Guava leaf extract may therefore be beneficial for the prevention of cardiovascular diseases, and also since its traditional use in hypertension is well established.

2.14.7 Anti-hyperglycemic

The evaluation of anti-hyperglycemic activity of the ethanol extract obtained from the stem bark of *Psidium guajava* on blood glucose levels of normal, alloxan-induced hyperglycemic rats and normal glucose loaded rats by Mukhtar *et al* (2006) showed that ethanol stem bark extract exhibited significant hypoglycemic activity in alloxan-induced, hyperglycemic rats but was devoid of significant hypoglycemic effect in normal and normal glucose loaded rats. In another study, a decoction of *Psidium guajava* leaves was screened for hypoglycemic activity on alloxan-induced diabetic rats. In both acute and sub-acute tests, the water extract, at an oral dose of 250 mg/kg, showed significant hypoglycemic activity (Mukhtar *et al.,* 2004). The treatment with *Psidium guajava* aqueous leaf extract (0.01–0.625 mg/ml) showed significant inhibition on LDL glycation in a dose-dependent manner. Tannins, flavonoids, pentacyclic triterpenoids, guiajaverin, quercetin, and other chemical compounds present in the plant are speculated to account for the observed hypoglycemic and hypotensive effects of the leaf extract (Ojewole, 2005; Wang *et al.,* 2005).

2.14.8 Anti-inflammatory

The anti-inflammatory property of the aqueous leaf extract of guava was investigated in rats, using fresh egg albumin induced pedal (paw) oedema. *Psidium guajava* aqueous extract (50–800 mg/kg, i.p.) produced dose-dependent and significant inhibition of fresh egg albumin-induced acute inflammation (oedema) in rats. This was carried out by Ojewole (2006).

2.15 RATIONALE AND OBJECTIVES

Adriamycin is a widely used anti-cancer agent with efficacy in a broad range of malignancies. However, its use in clinical chemotherapy is limited on account of its lethal cytotoxic effects, among which which are genotoxicity, testicular toxicity renal toxicity and cardiotoxicity (Singal *et al.*, 2000, Jung and Reszka, 2001; Mohamed and Amr, 2007: Carvalho *et al.*, 2009). Generation of free radicals with subsequent lipid peroxidation are important mechanisms by which adriamycin induces genotoxicity (Jung and Reszka, 2001) and nephrotoxicity in rats (Liu *et al.*, 2007). The hypothesis was then proposed that, if adriamycin toxicity is related to free radical formation and lipid peroxidation, then antioxidant therapy may protect against it.

Different trials to preserve the renal functions that have been impaired by adriamycin have been done by various research groups (Yilmaz *et al.*, 2006). The positive effects of guava extract on human ailments have been described (Gutierrez *et al.*, 2008) and have received considerable attention in recent years due to its diverse pharmacological properties including cancer chemopreventive effects (Boudet, 2007).

Review of literature reveal that the possible protective effect of *Psidium guajava* leaves on pathological or drug-induced renal dysfunction and genotoxicity has not been explored and addressed to date. The rationale and objectives of this present study therefore are to

i Investigate the possible protective effect of concurrent administration of *Psidium guajava* using the ethanolic extract (EEPG), in a rat model of adriamycin-mediated renal dysfunction and injury. This is an attempt to further identify safe, practical and effective agents to ameliorate nephrotoxicity.

ii Asess the disruption of antioxidant defense system and activities of membrane-bound enzymes following treatment with adriamycin *in vivo*.

iii Assess the role of guava extract against the activity of CYP3A4, a phase 1 enzyme, in adriamycin-induced nephrotoxicity.

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

v Identify some known compounds and possibly isolate new compound(s) from leaves of guava extract.

vi Isolate and characterize the active phytochemical(s) with antioxidant activities from EEPG.

CHAPTER THREE

MATERIALS AND METHODS

3.1 CHEMICALS

Adriamycin (doxorubicin hydrochloride) was obtained from United Pharm. Inc., South Korea. Ketoconazole was purchased from Strides Arcolab limited, India. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\cdot+}$), 7 Benzoyloxy-4-trifluoromethyl coumarin (BFC), acetonitrile and greiss reagent were purchased from Sigma-Aldrich (Germany). 1-chloro-2,4-dinitrobenzene (CDNB), 5',5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), glucose-6-phosphate, adenosine monophosphate (AMP), hydrogen peroxide (H_2O_2), 1,1-Dipheny-2-picrylhydrazyl (DPPH) radical, Thiobarbituric acid (TBA), Glucose-6-phosphate (G6P), Glycine, Catechin, [NADPH, glutathione reductase units and oxidized glutathione (GSSG) were obtained from Sigma Chemical Company, (USA)]. Metaphosphoric acid was obtained from MRS (U.K.). Adrenaline, was obtained from Surechem products Ltd (England). Xylenol orange sodium salt, and Cadmium were obtained from Alfa Aesar, Heyshan (Lancaster). Urea and Creatinine 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 bought from Ladokun feed, Mokola, Ibadan and tap water *ad libitum*.

3.3 ETHANOLIC EXTRACTION OF GUAVA LEAVES

Guava leaves were obtained from the University of Ibadan Campus and authenticated at the Department of Botany, University of Ibadan Herbarium (UIH) and deposited at the Herbarium of Forest Research Institute of Nigeria, Ibadan with Voucher

no 109518. About 2kg of leaves were air-dried and ground into powder. For each extraction run, 100g of the powdered material was subjected to continue hot extraction at 45^{o}C in a soxhlet apparatus using ethanol (95%) and the ethanolic extract evaporated in a rotary evaporator to obtain the crude extract. About 500g of EEPG was obtained as the solid extract from which a stock solution was prepared and administered to rats at a concentration of 200mg/ml.

3.4 EXPERIMENTAL DESIGN FOR PROTECTION AGAINST ADRIAMYCIN – INDUCED NEPHROTOXICITY AND GENOTOXICITY

Experiment I: Nephrotoxicity induced by a single dose of adriamycin (20mg/kg) and the effect of treatment of the ethanolic extract of *Psidium guajava*.

- Rats were divided into 6 groups of 5 animals.
- Group 1 (control) received normal saline (0.9% NaCl) by gavage.
- Group II received single intraperitoneal injection of adriamycin (ADR, 20mg/kg b.wt) on the 10th day.
- Groups III, IV and V received 125, 250 and 500mg/kg/day of guava extract *p.o.* respectively for 10 days before adriamycin injection.
- Group VI was treated with 500mg/kg/day of guava extract only.
- Rats were sacrificed on the 14th day by cervical dislocation (i.e. 96 hours after ADR injection).

Experiment II: Nephrotoxicity induced by repeated doses of adriamycin and the effect of concurrent administration of the ethanolic extract of *Psidium guajava.*

- Rats were divided into 6 groups of 5 animals.
- Group 1 (control) received normal saline by gavage.
- Group II received repeated doses of adriamycin (ADR) in 6 equal injections (2.5 mg/kg b.wt, i.p).
- Groups III, IV and V received 125, 250 and 500mg/kg/day of extract concurrently with ADR.
- Group VI was treated with 500mg/kg of guava extract only.
- All treatments lasted for 11 days. Rats were sacrificed by cervical dislocation 48 hours after the last treatment.

Experiment III: Nephrotoxicity induced by repeated doses of adriamycin and the effect of pretreatment of the ethanolic extract of *Psidium guajava.*

- Rats were divided into 6 groups of 5 animals.
- Group 1 (control) received normal saline by gavage.
- Group II received repeated doses of adriamycin (ADR) in 6 equal injections (2.5mg/kg b.wt, i.p).
- Groups III, IV and V received 125, 250 and 500mg/kg/day of extract for 7 days before ADR administration.
- Group VI was treated with 500mg/kg of guava extract only.
- All treatments lasted for 17 days. Rats were sacrificed by cervical dislocation 48 hours after the last treatment.

Experiment IV: Nephrotoxicity induced by repeated doses of adriamycin and the effect of posttreatment of the ethanolic extract of *Psidium guajava.*

- Rats were divided into 6 groups of 5 animals.
- Group 1 (control) received normal saline by gavage.
- Group II received repeated doses of adriamycin (ADR) in 6 equal injections (2.5mg/kg b.wt, i.p).
- Groups III, IV and V received 6 equal injections of adriamycin after which they were then administered with 125, 250 and 500mg/kg/day of extract for 5 days.
- Group VI was treated with 500mg/kg of guava extract only.
- All treatments lasted for 15 days. Rats were sacrificed by cervical dislocation 48 hours after the last treatment.

Experiment V: Effects of ethanolic extract of *Psidium guajava* on the CYP3A4-mediated dealkylation of 7-Benzoyloxy-4-trifluoromethyl coumarin (BFC)

- Rats were divided into 10 groups of 5 animals.
- Group 1 (control) received normal saline by gavage.
- Group II received repeated doses of adriamycin (ADR) in 6 equal injections (15mg/kg b.wt, i.p).
- Groups III, IV and V received 125, 250 and 500mg/kg of extract concurrently with ADR.
- Group VI received 400mg/kg of ketoconazole concurrently with ADR.
- Groups VII, VIII and IX received 125, 250 and 500mg/kg of guava extract only

respectively.

- Group X received 400mg/kg of ketoconazole only.
- All treatments lasted for 11 days. Rats were sacrificed by cervical dislocation 24 hours after the last treatment.

Experiment VI: Genotoxicity induced by adriamycin and the effect of ethanolic extract of *Psidium guajava*

- Thirty (30) male Wistar rats (aged 10-12 weeks) with body weight ranging between 80g-120g were used for this study.
- Rats were divided into 6 groups of 5 animals.
- Group 1(control) received normal saline.
- Group II received single intraperitoneal injection of adriamycin (ADR, 20mg/kg b.wt).
- Groups III, IV and V received 125, 250 and 500mg/kg/day of guava extract *p.o.* respectively for 7 days before adriamycin injection.
- Group VI was treated with 500mg/kg/day of guava extract only.
- Rats were sacrificed 24 hours after ADR injection.
- Bone marrow was flushed from both femurs of each rat using fetal calf serum and spread onto slides.
- Slides were coded and then air-dried, fixed with methanol and stained with maygrunword stain.
- Bone marrow cells were then examined microscopically and scored per animal for frequency of micronucleated cells in each of 5 animals per dose group

3.5 COLLECTION OF BLOOD SAMPLES AND PREPARATION OF POSTMITOCHONDRIAL (PMF) AND MICROSOMAL FRACTIONS OF KIDNEY SAMPLES

Rats were sacrificed by cervical dislocation. Blood samples were collected by cardiac puncture into heparinized tubes and kidneys immediately removed, rinsed in ice-cold 1.15% KCl, blotted 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^{o}C in the Department of Biochemistry, University of Ibadan and the supernatants, termed the postmitochondrial fractions (PMF) was taken to the Department of Virology

in International Institute of Tropical Agriculture (IITA) and recentrifuged at 100,000g for 1hr using the L5-50B ultracentrifuge Beckman to get the microsomal pellets which were resuspended in 0.25M sucrose solution. Aliquots of this suspension were stored at -20^{o}C and thawed before use.

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3.6 *IN VIVO* EVALUATION OF THE NEPHROPROTECTIVE EFFECTS OF ETHANOLIC EXTRACT OF *PSIDIUM GUAJAVA*

3.6.1 RENAL FUNCTION TESTS

3.6.1.1 PLASMA CREATININE ESTIMATION

PRINCIPLE

Creatinine reacts with alkaline picric acid to the form a red tautomer of creatinine picrate. Absorbance is proportional to creatinine concentration (Henry, 1974)

REAGENTS

1. **Saturated picric acid**.

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

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

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

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

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

PROCEDURE

3.5 mL of picric acid in a test tube was added 0.5 mL of plasma sample. The mixture was centrifuged for 5 minutes. To 3 mL of the supernatant was added 0.2 mL of 4N NaOH. This reaction mixture was incubated for 10 minutes and the absorbance was then read at 520 nm and the concentration of creatinine determined.

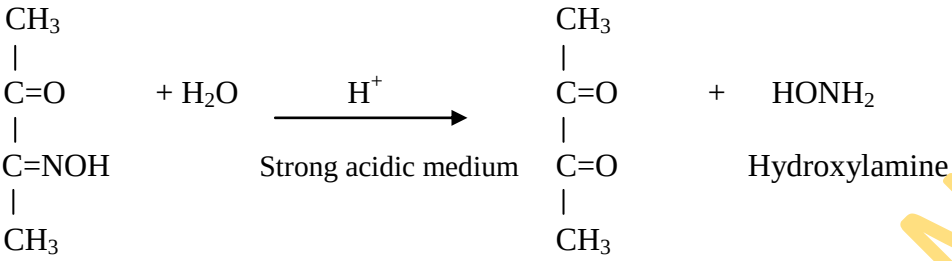
3.6.1.2 BLOOD UREA NITROGEN ESTIMATION

PRINCIPLE

THE FEARON REACTION

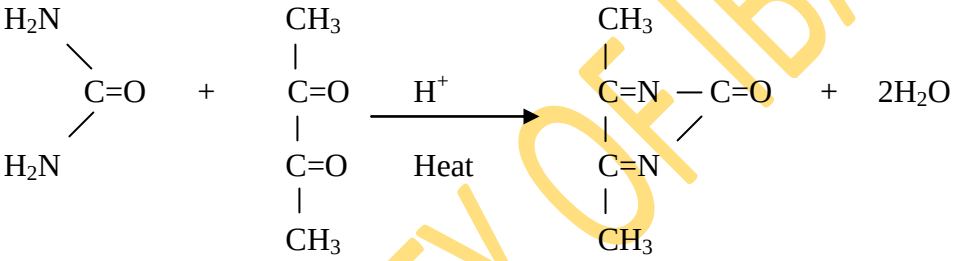
Plasma urea was determined according to the method of Weatherburn (1967) using diagnostic Randox Kit. The principle of this reaction is based on the condensation

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 540 nm. Thiosemicarbazide and Fe (III) are added to the system to enhance and stabilize the colour.

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 in duplicate, (0.1 mL) was added into a universal bottle containing 19.9 mL of distilled water and the mixture was shaken very well. Aliquot of the mixture was transferred into a test tube and to it was added 1 mL of colour reagent followed by 1 mL of acid 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/100 mL was then determined from a calibration curve (See Appendix).

3.6.2 DETERMINATION OF CATALASE ACTIVITY

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

PRINCIPLE

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured colorimetrically at 570-610 nm. 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)**

0.16ml of H_2O_2 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).

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 volumes of H_2O_2 (as shown below), 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)	0.0	0.1	0.2	0.3	0.4	0.5	0.6
Dichromate/acetic acid (mL)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Distilled water (mL)	1.0	0.9	0.8	0.7	0.6	0.5	0.4

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. 1 mL 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 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.

3.6.3 DETERMINATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY

The activity of superoxide dismutase was measured by the procedure of Misra and Fridovich (1972).

PRINCIPLE

This method is based on the inhibition, by SOD, of the spontaneous autoxidation of adrenaline to adrenochrome at pH 10.2 (Valerino and Mc Cormack, 1971). The reaction was performed at 30^{o}C in 1ml of 50nM sodium carbonate buffer, pH 10.2 containing 0.3mM adrenaline and 0.1mM EDTA. One unit of activity is defined as the amount of enzyme required to inhibit the change in absorbance at 480nm by 50%.

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 fresh.

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 blank contained distilled water, 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.

3.6.4 DETERMINATION OF REDUCED GLUTATHIONE LEVEL

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

PRINCIPLE

The reduced form of glutathione comprises in most instances the bulk of cellular non-protein 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 – nitro-5-thiobenzoic acid possesses a molar absorption at 412 nm.

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^oC, 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^oC. The EDTA was added to prevent difficulties that might arise where water supply contains appreciable concentration of metallic ions.

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 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 concentration was proportional to the absorbance at that wavelength and the estimate was obtained from the GSH standard.

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	-

4.5 mL of Ellman reagent was added to each test tube. GSH concentration was proportional to the absorbance at 412 nm. A graph of optical density against concentration was plotted.

3.6.5 ESTIMATION OF GLUTATHIONE-S-TRANSFERASE ACTIVITY

Glutathione-S-transferase 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-transferase 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 340nm 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_2 HPO_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 below and the reaction was allowed to run for 1 minute each time before the absorbance was read against the blank at 340 nm. 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	2.82mL	2.79 mL
PMF	-	30 µL

3.6.6 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 fatty acid membranes and food products react with the chromogenic reagent, 2-thiobarbituric acid to yield a pink coloured complex with maximum absorbance at 532 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^{o}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 x 10^{5} $M^{-1}cm^{-1}$.

3.6.7 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^{o}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)

Anhydrous 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

The reaction which consisted of glucose-6-phosphate (0.15 mL, 0.04 M), 0.2 mL of 1.0 M sodium acetate buffer (pH 5.8), 0.15 mL sample and 0.5 mL distilled water (final volume 1.0 mL) was incubated for 30 minutes at 37^{o}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.

3.6.8 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 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 or NaOH and made up to 1 litre with distilled water.

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 diluted with distilled water and the volume 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^oC.

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^{o}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.

3.6.9 DETERMINATION OF NITRATE/NITRITE (NITRIC OXIDE) LEVEL

Serum nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as indeces of nitric oxide ($NO^{\cdot}$) 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 oxyhaemoglobin 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^oC.

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^oC.

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 1.47mmol/L (0.43g/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.

3.6.10 DETERMINATION OF HYDROGEN PEROXIDE GENERATION

This was carried out according to the method of Nourooz-Zadeh *et al* (1994). The oxidation of ferrous to ferric ion in the presence of xylenol orange served as the basis for the determination of amount of hydrogen peroxide generated.

PROCEDURE

The ferrous oxidation with xylenol orange (FOX-1) reagent was prepared using 100 µMol/L xylenol orange, 250 µMol/L ammonium ferrous sulphate, 100 mmol/L sorbitol and 25 mmol/L H_2SO_4. 50 µL of sample was added to the reaction mixture and incubated at room temperature for a minimum of 30 minutes at which point the colour development was virtually complete. The absorbance was read at 560 nm and the standard was linear within a concentration range of 0-5 µMol/L. The amount of hydrogen peroxide generated was expressed as µmol/L/mg protein.

REAGENTS

1. Xylenol orange (100 µmol/L)

This was prepared by dissolving 0.0152 g of xylenol orange in 150 mL of distilled water and made up to 200 mL with same.

2. Ammonium ferrous sulfate (250 µmol/L)

This was prepared by dissolving 0.0196 g of ammonium ferrous sulphate in 150 mL of distilled water and made up to 200 mL with same.

3. Sorbitol (100 mmol/L)

3.64 g of sorbitol was dissolved in distilled in about 150 mL of distilled water and then made up to 200 mL with same.

4. H_2SO_4 (25 mmol/L)

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

3.6.11 DETERMINATION OF ASCORBIC ACID (VITAMIN C)

PRINCIPLE:

The ascorbic acid concentration in the postmitochondria fraction was determined according to the method of Jakota and Dani, 1982. This method was developed when it was discovered that ascorbic acid present in biological samples interferes with protein determination using the Folin-Ciocalteu method by Lowry *et al* (1951). This method is based on the fact that, ascorbic acid present in biological samples react with folin reagent, an oxidizing agent, to give a blue colour which has maximum spectrophotometric absorption at 760 nm.

REAGENTS

1. 10% Trichloroacetic acid (TCA)

10 g of TCA was dissolved with distilled water in a conical flask and made up to the 100 mL mark with same.

2. Folin-Ciocalteu reagent

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

3. Ascorbic acid standard solution (stock)

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

CALIBRATION OF ASCORBIC ACID STANDARD CURVE

PROCEDURE

A standard curve was prepared by taking varying concentrations of standard solutions of ascorbic acid in water, ranging from 0.05-0.7 mL. Then, 0.8 mL of 10% TCA was added to each tube. After vigorous shaking, the tubes were kept in an ice bath for 5 min and centrifuged at 3000 g for another 5 minutes. Supernatant of the same range (i.e 0.05-0.7 mL) were withdrawn and diluted to 2.0 mL using double-distilled, and after 0.2 mL of diluted folin's reagent was added, the tubes vigorously shaken. After 10 minutes, the absorbance of the blue colour developed was measured in a spectrophotometer at 760 nm.

Preparation of Vitamin C Standard Curve

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

PROCEDURE FOR DETERMINATION OF VITAMIN C IN TISSUE SAMPLES

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

3.6.12 DETERMINATION OF GLUTATHIONE PEROXIDASE ACTIVITY

Glutathione peroxidase was assayed according to the method of Paglia and Valentine (1967).

PRINCIPLE

The method is based on the fact that, in the presence of reduced glutathione (GSH) and NADPH, glutathione peroxidase detoxifies H_2O_2 and other organic hydroperoxides. In this reaction, GSH is oxidized to GSSG and the rate of formation of GSSG is followed spectrophotometrically as a measure of glutathione peroxidase activity at 340 nm.

REAGENTS

1. 0.05 M Phosphate buffer (pH 7)

3.48 g of K_2HPO_4 and 0.68 g of KH_2PO_4 dissolved in 450 mL of distilled and volume made up to 500 mL.

2. 0.5 units glutathione reductase

2.7 mg of glutathione reductase dissolved in 1 mL of distilled water.

3. Reduced glutathione (0.15 M)

0.092 g of reduced glutathione dissolved in distilled water and made up to 2 mL with same.

4. Sodium azide (1.125 M)

0.5548 g of sodium azide dissolved in distilled water and made up to 7.6 mL with same.

5. NADPH (8.4 mmol/L)

25 mg of NADPH dissolved in 3.5 mL of distilled water.

6. H_2O_2 (2.2 M)

7.48 g of H_2O_2 was mixed with distilled water in a 100 mL volumetric flask and the solution made up to the mark with same.

PROCEDURE

The assay mixture, consisting of 2.6 mL of 0.05 M phosphate buffer (pH 7) containing 1.125 M sodium azide, 0.5units of glutathione reductase, 0.1 mL of 0.15 M GSH, 0.1 mL of 8.4 mmol/L NADPH, and 0.1 mL homogenate of the tissue was allowed to equilibrate for 10 min at 37^{o}C. The reaction was initiated by adding 0.1 mL of 2.2 M H_2O_2. The increase in absorbance was calculated using a molar extinction coefficient of 6.1 mmol/L^{-1}cm^{-1}.

3.6.13 DETERMINATION OF GLUTATHIONE REDUCTASE ACTIVITY

Glutathione reductase was assayed according to the method of Racker (1955) as described by Carlberg and Mannervik, 1985.

PRINCIPLE

This assay is based on the principle that glutathione reductase maintains glutathione in its reduced form. Oxidized glutathione (GSSG) from glutathione peroxidase action is converted to reduced glutathione (GSH) using NADPH as source of reducing equivalent and the rate at which GSH is formed is followed spectrophotometrically as a measure of the enzyme activity at 340 nm.

REAGENTS

1. Tris buffer (0.1 M, pH 8)

Tris (1.211 g) was dissolved in distilled water and the volume made up to 100 mL.

2. EDTA (0.015 M)

EDTA (0.0175 g) dissolved and made up to 4 mL of distilled water.

3. Oxidized glutathione (GSSG) (0.05 M)

Oxidized glutathione (0.107 g) was dissolved in distilled water and made up to 3.5 mL with same.

4. NADPH (4 mmol/L)

NADPH (0.117 g) was dissolved in 3.5 mL of distilled water.

PROCEDURE

The assay mixture consisted of 2.5 mL of 0.1 M Tris buffer (pH 8), 0.1 mL NADPH (4 mmol/L), 0.1 mL EDTA (0.015 M), 0.1 mL GSSG (0.05 M), and 0.1 mL of homogenate. The decrease in absorbance at 340 nm was measured and enzyme activity was calculated using a molar extinction coefficient of 6.1 $mmol/L^{-1}cm^{-}$.

3.6.14 DETERMINATION OF CYP3A4 ACTIVITY

High-throughput fluorescence assay of cytochrome P450 3A4 was according to the method described by Cheng *et al*., 2009.

PRINCIPLE

The assay involves the oxidative debenzylation of a substituted coumarin, yielding an increase in fluorescence on reaction. 7-Benzoyloxy-4-trifluoromethyl coumarin (BFC) is used in this assay as the probe substrate and undergoes O-dealkylation to give the fluorescent product 7-hydroxy-4-trifluoromethyl coumarin (HFC). Rate of formation of HFC is followed spectrophotometrically as a measure of CYP3A4 activity at 425 nm. Activity was calculated as the rate of fluorescent metabolite production over the course of the reaction. Percent inhibition by test compound was calculated as the ratio of the activity observed in the presence of test compound compared with no inhibitor.

REAGENTS

1. Phosphate buffer (0.1 M, pH 7.4)

13.96 g dibasic potassium phosphate and 2.69 g monobasic potassium phosphate dissolved in 1 litre of distilled water (add phosphate salts to water, not vice-versa); remains stable for several months at $4^{o}C$.

2. Stop buffer

80% acetonitrile and 20% 0.5 M Tris-base (vol/vol), stored at an ambient temperature (23^{o}C); it remains stable for several months.

3. BFC (4 mM)

2.56 mg in 2 mL methanol, stored in a Teflon-sealed amber glass vial at 4^{o}C. This is stable for several months

4. 2xenzyme-substrate mix

Mix microsomal sample and BFC in 0.1 M potassium phosphate buffer so the final concentration of microsomal sample is 20 nM and BFC is 40 mM.

5. NADPH (1mmol/L)

25 mg of NADPH dissolved in 3.5 mL of distilled water.

PROCEDURE

Microsomal sample (20 nM) and BFC (40 mM) were mixed together in 0.1 M phosphate buffer to get a final volume of 2 mL. This was incubated at 37^{o}C for 5 minutes. 0.12 mL of NADPH was then added to the medium to initiate reaction. The resulting mixture was incubated at 37^{o}C for 15 minutes. The reaction was stopped by addition of 0.15 mL of stop buffer. Fluorescence reading was obtained at 425 nm using a fluorescent spectrophotometer.

CYP3A4 assay medium

	Blank	Test
Enzyme-substrate mix	-	2 ml
Phosphate buffer	2 ml	-
Stop buffer	0.15ml	-
Incubate at 37^{o}C	for 5 mins	
NADPH	0.12ml	0.12 ml
Incubate at 37^{o}C	for 15 mins	
Stop buffer	-	0.15 ml
Total	2.27ml	2.27 ml

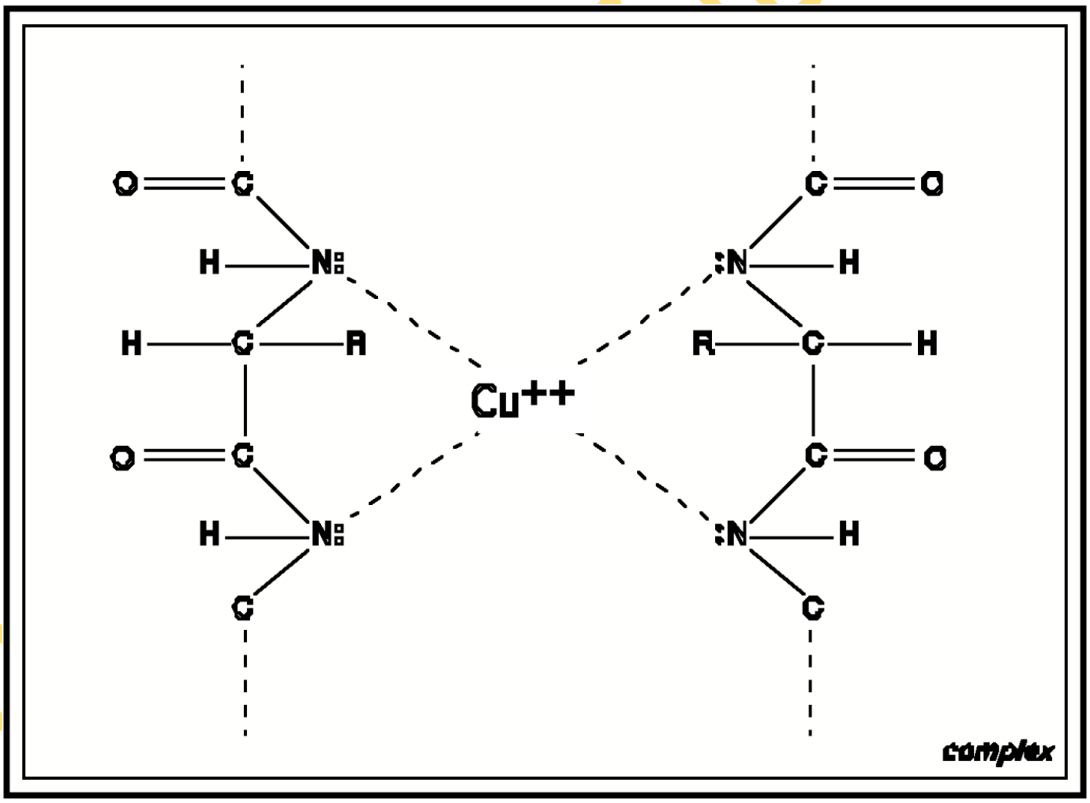
Calculation of the percentage of inactivated enzyme designated as I%

$$I\% = \frac{1- (\text{Mean of sample} - \text{mean of blank}) \times 100}{(\text{Mean of control} - \text{mean of blank})}$$

3.6.15 DETERMINATION OF TOTAL PROTEIN

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 Λ_{max} = 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^{o}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 0.2 M NaOH. Potassium iodide (100 mL) was added and the solution made up to a litre with 0.2 M 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.

3.7 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.

3.8 *IN VITRO* ANTIOXIDANT ACTIVITIES OF ETHANOLIC EXTRACT OF *PSIDIUM GUAJAVA*

3.8.1 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 Neergheen *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 (0.1 M)

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 $ABTS^{+}$ 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 $ABTS^{+}$ 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 $ABTS^{+}$ solution as shown in the table below:

Test tube	Trolox (µL)	Trolox (µmoL)	Buffer (mL)	$ABTS^{+}$ (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

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

The hydroxyl radical ($OH^{\cdot}$) scavenging potential of the extract was determined using the deoxyribose assay (Halliwell *et al.* 1987; Aruoma, 1994a, 1994b) as described by Neergheen *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). $OH^{\cdot}$ radicals are generated in a reaction mixture containing ascorbate, H_2O_2 and Fe^{3+} - EDTA at pH 7.4 (equations 1 and 2). The ascorbic acid greatly increases the rate of $OH^{\cdot}$ 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+} - - EDTA + ascorbate $\longrightarrow$ Fe^{2+} — EDTA + oxidised ascorbate —(1)

Fe^{2+} — EDTA + H_2O_2 $\longrightarrow$ Fe^{3+} — EDTA + $OH^{\cdot}$ + OH^{-} —(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^oC 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^oC 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.

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

The effect of the ethanolic extract of guava (EEPG) on 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) radical was estimated according to the method of Hatano *et al.* (1988).

PRINCIPLE

The relatively stable 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) free radical is used for antioxidant activity measurement of lipid soluble compounds. It is known that a freshly prepared $DPPH^{\cdot}$ 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 $DPPH^{\cdot}$ (By providing hydrogen atoms or by electron donation, conceivably via a free radical attack on the $DPPH^{\cdot}$ 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

EEPG (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 $DPPH^{\cdot}$ discolouration.

3.8.4 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).

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

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

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

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^{o}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.

3.8.5 DETERMINATION OF REDUCING POWER

The reducing power of EEPG was determined according to the method of Oyiazu (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 Prussian 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_2 HPO_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^{o}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.

3.8.6 DETERMINATION OF NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

The scavenging effect of EEPG 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 in distilled water and made up to 50 mL.

2. Griess reagent

This was prepared by dissolving 1 g of sulphanilic acid in 50 mL of distilled water containing 0.1 g of naphthylethylenediamine dihydrochloride 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

EEPG (10-400 µg) was added in the test tubes to 1ml of sodium nitroprusside solution (25 mM) and the tubes incubated at 37^{o}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).

3.8.7 DETERMINATION OF THE HYDROGEN PEROXIDE SCAVENGING ACTIVITY

The ability of EEPG to scavenge hydrogen peroxide was determined according to the method of Ruch *et al* (1989).

PROCEDURE

A solution of hydrogen peroxide (4 mM) was prepared in phosphate buffered saline (PBS), pH 7.4. Extract at (20-400ug) in 4 mL of distilled water was added to 0.6 mL of hydrogen peroxide solution. Absorbance of hydrogen peroxide at 230 nm was determined spectrophotometrically from absorption at 230 nm using the molar of absorptivity 81 $M^{-1}cm^{-1}$(Beers and Sizer, 1952).

REAGENTS

1. Phosphate buffer (0.2 M, pH 7.4)

This was prepared by dissolving 0.496 g of dipotassium hydrogen phosphate (K_2 HPO_4) and 0.973 g of potassium dihydrogen phosphate, (KH_2PO_4) in saline and the volume made up to 100 mL of same. The pH was adjusted to pH 7.4 with NaOH or HCl.

2. Saline (0.9%)

NaCl (1.8 g) was dissolved in distilled water and made up to 200 mL with same.

3. H_2O_2 (4 mM)

34 g of H_2O_2 was mixed with distilled water in a 100 mL volumetric flask and the solution made up to the mark with same.

3.8.8 DETERMINATION OF TOTAL PHENOLIC CONTENT

The amount of phenolic compound in the ethanolic extract of *Psidium guajava* was determined with Folin Ciocalteu reagent using the method of Spanos, 1990 as modified by Lister and Wilson (2001). To 0.5 mL of each sample (3 replicates) of plant extract solution (1 mg/mL) was added 2.5 mL of 10% Folin-Ciocalteu reagent and 2 mL of Na_2CO3 (2% w/v). The resulting mixture was incubated at 45°C with shaking for 15 min. The absorbance of the samples was measured at 765 nm using UV/visible light. Results were expressed as milligrams of gallic acid (0-0.5 mg/mL) dissolved in distilled water.

3.8.9 DETERMINATION OF TOTAL FLAVONOIDS

The aluminum chloride colorimetric method was used for flavonoid determination as described by Olayinka and Anthony (2009). One millilitre (1 mL) of sample was mixed with 3 mL of methanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water and remains at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm with UV visible spectrophotometer. The content was determined from extrapolation of calibration curve prepared with gallic acid solution (0–0.8 mg/mL) in distilled water. The concentration of flavonoid was expressed in terms of mg gallic acid equivalents/mL.

3.8.10 DETERMINATION OF TOTAL PROANTHOCYANIDINS

Total proanthocyanidins were determined based on the procedure of Sun *et al* 1998. A mixture of 3 mL of vanillin-methanol (4% v/v) and 1.5 mL of hydrochloric acid was added to 0.5 mL (1 mg/mL) of extract and vortexed. The resulting mixture was

allowed to stand for 15 min at room temperature followed by the measurement of the absorbance at 500 nm. Total proanthocyanidin content was expressed as gallic acid equivalent (mg/mL) from the standard curve.

3.9 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.

3.10 ISOLATION OF SECONDARY METABOLITE FROM *PSIDIUM GUAJAVA*

3.10.1 General Experimental Procedures

Solvents used for 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. 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 detected by the use of iodine, ferric chloride and DPPH spray reagents.

3.10.2 Antioxidant Test

3.10.2.1 TLC autographic analysis (Qualitative)

A small amount of the sample was dissolved in little amount of acetone 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 diphenyl picryl hydrazyl hydrate (DPPH). Only zones where the colour turned from purple to yellow within the first fifteen minutes after spraying were positive results established (i.e. possess antioxidant activity).

3.10.3 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.

3.10.4 Extraction and Solvent Fractionation of *Psidium guajava*

The dried powdered leaves (2 kg) were extracted with 3 litres of absolute ethanol and the pooled extract was concentrated to dryness *in vacuo* on a rotary evaporator (45ºC). The crude ethanolic extract was coded GE. About 20 g of this crude extract was mixed with 20 g of silica gel for adsorption of the extract into silical gel, allowed to dry before packing into Vacuum Liquid Chromatography (VLC). The VLC was wetted with *n*-hexane and gradient elution effected with the following solvent/ solvent mixtures:

Hexane (GEF1)	100%	500mL
Hexane - ethyl acetate(GEF2)	(90:10)	500mL
Hexane - ethyl acetate(GEF3)	(80:20)	500mL
Hexane - ethyl acetate(GEF4)	(70:30)	500mL
Hexane - ethyl acetate(GEF5)	(60:40)	500mL
Hexane - ethyl acetate(GEF6)	(50:50)	500mL
Hexane - ethylacetate(GEF7)	(40:60)	500mL
Hexane - ethylacetate(GEF8)	(30:70)	500mL
Hexane - ethylacetate (GEF9)	(20:80)	500mL
Hexane- ethylacetate(GEF10)	(10:90)	500mL
Ethylacetate (GEF11)	(100%)	500mL
EthylacetateMethanol(GEF12)	(95:5)	250mL
EthylacetateMethanol(GEF13)	(90:10)	250mL
EthylacetateMethanol(GEF14)	(85:15)	250mL
Ethylacetate-Methanol(GEF15)	(80:20)	250mL
EthylacetateMethanol(GEF16)	(75:25)	250mL
EthylacetateMethanol(GEF17)	(70:30)	250mL
EthylacetateMethanol(GEF18)	(65:35)	250mL
Ethylacetate - Methanol(GEF19)	(60:40)	250mL
EthylacetateMethanol(GEF20)	(55:45)	250mL
EthylacetateMethanol(GEF21)	(50:50)	250mL
Ethylacetate - Methanol (GEF22)	(45:55)	250mL

TLC Analysis

The resulting fractions coded GEF1-22 (guava extract fractions 1 to 22) were in turn concentrated to dryness *in vacuo* on a rotary evaporator. Each of the fractions was dissolved in small amount of ethylacetate and then analysed by TLC using Hexane:

Ethylacetate (3:1v/v). The resulting spots on TLC plates were visualized under UV light (254 nm) and detected by suspending in iodine tank. Fractions having the same TLC patterns were pooled together, and concentrated to dryness. GEF6, 7, and 8 were bulked together and coded GEFA; GEF10,11,12 and 13 were also bulked together and coded GEFB.

DPPH Spray Reagents

The active compound(s) were detected using DPPH spray reagents on the resulting spots. None of the fractions showed antioxidant property.

Phenolic Test

GEFB (i.e GEF10 to GEF13) when spotted on TLC plates were allowed to dry and then sprayed with $FeCl_3$ showed presence of phenolic compounds by turning the brown colur of $FeCl_3$ to blue-black.

3.10.5 Fractionation of GEFB on open column Chromatography

GEFB dissolved in *n*-hexane was adsorbed unto silica and allowed to dry before packing on to column. The column was wetted with *n*-hexane and gradient elution effected using the following solvent/ solvent mixtures:

Hexane	100%	100mL
Hexane - ethyl acetate	(90:10)	100mL
Hexane - ethyl acetate	(80:20)	100mL
Hexane - ethyl acetate	(75:25)	100mL
Hexane - ethyl acetate	(70:30)	100mL
Hexane - ethyl acetate	(65:35)	100mL
Hexane – ethylacetate	(60:40)	100mL
Hexane - ethylacetate	(55:45)	100mL
Hexane - ethylacetate	(50:50)	100mL
Hexane- ethylacetate	(45:55)	100mL
Hexane - ethylacetate	(40:60)	100mL
Hexane - ethylacetate	(35:65)	100mL
Hexane- ethylacetate	(30:70)	100mL
Hexane - ethylacetate	(25:75)	100mL
Hexane- ethylacetate	(20:80)	100mL

;Hexane - ethylacetate	(15:85)	100mL
Hexane - ethylacetate	(10:90)	100mL
Hexane- ethylacetate	(5:95)	100mL
Ethylacetate	(100%)	100mL
EthylacetateMethanol	(95:5)	100mL

Phenolic Test

170 Fractions were collected and thereafter subjected to test for presence of phenolic compounds using $FeCl_3$. All the fractions showed presence of phenolic compounds on TLC plates by turning the brown colour of $FeCl_3$ to black.

TLC Analysis

3 fractions from Hexane: Ethylacetate (35:65) coded GEFB1, GEFB2 and GEFB3 (from the above fractionation) were dissolved in little amount of ethylacetate and spotted on TLC plates using acetone: hexane (55:45) as solvent mixture. They were then pulled together based on similar TLC pattern and coded GEFBP.

Phenolic Test

The resulting spots were sprayed with $FeCl_3$ which turned blue-black showing the presence of phenolic compounds.

Antioxidant test

The resulting spots were sprayed with DPPH which turned from purple to yellow showing antioxidant property

3.10.6 Fractionation of GEFBP on Sephadex LH-20

GEFBP was dissolved in a minimum amount of acetone (100% v/v) and loaded on a Sephadex LH-20 column previously equilibrated with acetone: H_2O (90:10). Elution was carried out using the same solvent mixture.

TLC Analysis

13 fractions were collected and coded GEFBP1 to GEFBP13. GEFBP8 and GEFBP9 were bulked together based on having similar TLC pattern and coded GEFBP′.

INFRA RED (IR) AND HYDROGEN-NUCLEAR MAGNETIC RESONANCE (H-NMR) ANALYSES

The active purified principle present in GEFBP′ was analysed for its chemical structure by IR and H-NMR analyses.

CHAPTER FOUR

EXPERIMENTS AND RESULTS

EXPERIMENT 1: NEPHROTOXICITY INDUCED BY SINGLE DOSE OF ADRIAMYCIN AND THE EFFECT OF TREATMENT OF THE ETHANOLIC EXTRACT OF *PSIDIUM GUAJAVA*.

INTRODUCTION

Adriamycin (ADR) is an anti-neoplastic agent used in the treatment of a variety of human neoplasms. However, its clinical use is severely restricted by dose-dependent toxicity in various tissues, including the kidney. Experimental studies in animals showed that ADR caused renal toxicity and produced progressive glomerular injuries (Malarkodi *et al*., 2003; Deepa and Varalakshmi, 2005).

The exact mechanism of ADR-induced toxicity remains unclear. Some researchers proposed that ADR-induced toxicity is most likely mediated by the formation of an iron–anthracycline complex that generates reactive free radicals (ROS), which in turn, causes diverse oxidative damage on critical cellular components in the plasma membranes and mitochondria (Sazuka *et al*., 1989a, b; Lee *et al*., 1991). When metabolized, adriamycin (quinone structure) is reduced to semiquinone and further to hydroquinone by a two-electron reduction. Semiquinone reacts with molecular oxygen under aerobic conditions to form superoxide and hydrogen peroxide and consequently hydroxyl radical in the presence of transition metals (iron and copper) (Halliwell and Gutteridge, 1989). This view is well supported by the fact that antioxidants prevent the ADR-induced toxicity in experimental animals as well as in human (Mohamed *et al*., 2000). Life has evolved in a hostile environment. As a consequence, nature has devised defence mechanisms to protect cells against the various external pressures, such as chemical insult, oxidative damage and harmful radiation or limit their damaging effects.

Therapeutic strategies, designed to augment cellular endogenous defense systems have been identified as a promising approach to combat oxidative stress-associated disease conditions (Steare and Yellon, 1995). In recent years, emphasis of research has been on utilizing traditional medicines that have a long and proven history of treating various ailments. Guava extract, well known for its medicinal use since ancient times, has been reported to be used in tropical and subtropical countries to treat many disorders such as diarrhoea, cough and gastrointestinal disorders. It was reported that *Psidium guajava* leaf extract has a wide spectrum of biological activities such as anticough,

antibacterial, haemostasis (Jaiarj *et al*., 1999; 2000), antidiarrhoeal and narcotic properties (Lozoya *et al*., 1990), and antioxidant properties (Qian and Nihorimbere, 2004). Although, a lot of studies have been done on the guava plant, the possible protective effect on pathological or drug-induced renal dysfunction and injury has not been explored and addressed to date. In this regard, the present study was designed to investigate the effect of ethanolic extract of *Psidium guajava* on the nephrotoxic potential of single dose of adriamycin and also evaluate the possible participation of free radicals in its pathogenesis.

PROCEDURE

Albino rats of the Wistar strain weighing between 110 and 275 g were used in this study. Rats were randomly divided into six groups of five animals each. Group I (control) received normal saline (0.9% NaCl) orally (*p.o.*). Rats in group II received single intraperitoneal injection of adriamycin (ADR, 20 mg/kg b.wt) on the 10^{th} day. Groups III, IV and V received 125-, 250- and 500- mg/kg/day of EEPG *p.o.* respectively for 10 days before adriamycin injection. Group VI was treated with 500 mg/kg/day of EEPG only. On the 14^{th} day, after treatment, rats were sacrificed by cervical dislocation and dissected. Blood samples were collected by cardiac puncture into heparinized tubes for determination of plasma concentrations of creatinine, blood urea nitrogen (BUN). 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^{o}C and the supernatants, termed the post-mitochondrial fractions (PMF), were aliquoted and used for the enzyme assays.

RESULTS

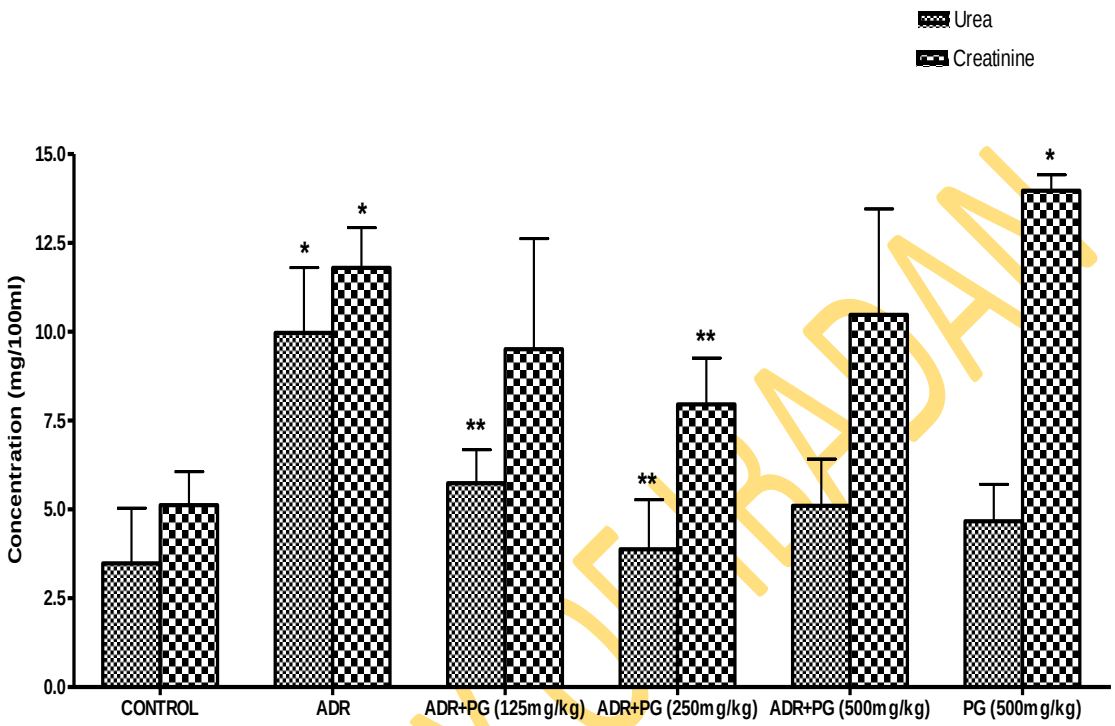


Figure 1A: Effect of ethanolic extract of *P. guajava* on changes in plasma urea and creatinine of normal and adriamycin (ADR)-treated rats. $^{*}p<0.05$ when compared with control; $^{**}p<0.05$ when compared with adriamycin-only group.

PG- *Psidium guajava*

Table 1A: Effect of treatment with ethanolic extract of *P.guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in reduced glutathione (GSH) level and glutathione-S-transferase (GST) activity.

Treatment group	GST (nmol/g tissue)	GSH (µg/g kidney wt)
Control (0.9% NaCl)	0.46±0.17	12.66±2.0
ADR (20 mg/kg)	0.16±0.04* (65.2)[a]	5.86±1.3* (53.7)[a]
EEPG (125 mg/kg) + ADR (20 mg/kg)	0.20±0.11 (20.0)[b]	9.43±2.0 (37.9)[b]
EEPG (250 mg/kg) + ADR (20 mg/kg)	0.28±0.05** (42.9)[b]	10.75±0.6** (45.5)[b]
EEPG (500 mg/kg) + ADR (20 mg/kg)	0.22±0.09 (27.3)[b]	9.07±1.7 (35.4)[b]
EEPG (500 mg/kg)	0.17±0.03* (63.0)[a]	11.68±0.9 (7.8)[a]

Values are expressed as mean±SEM for five rats in each group. *Significantly different from control ($p<0.05$); **Significantly different from ADR-treated rats ($p<0.05$). Values in parenthesis represent % change; [(a)] % change relative to control; [(b)] % change relative to ADR.

Table 1B: Effect of treatment with ethanolic extract of *P.guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase and glutathione reductase activities in rat.

Treatment group	CAT[x] H_2O_2 (µmole consumed/min)	SOD[y] (Units/mg protein)	Glutathione peroxidase[#]	Glutathione reductase[#]
Control (0.9% NaCl)	0.08±0.01	3.48±0.87	0.52±0.28	0.68±0.12
ADR (20 mg/kg)	0.04±0.00[*] (50.0)[a]	1.72±0.18[*] (50.6)[a]	0.15±0.04[*] (71.2)[a]	0.41±0.11[*] (39.7)[a]
EEPG (125 mg/kg) + ADR (20 mg/kg)	0.04±0.01 (0)[b]	3.05±0.54[**] (43.6)[b]	0.46±0.04[**] (67.4)[b]	0.43±0.08 (4.7)[b]
EEPG (250 mg/kg) + ADR (20 mg/kg)	0.06±0.03[**] (33.3)[b]	3.59±1.11[**] (52.1)[b]	0.27±0.08[**] (44.4)[b]	0.64±0.05[**] (35.9)[b]
EEPG (500 mg/kg) + ADR (20 mg/kg)	0.03±0.01 (25.0)[c]	3.22±0.68[**] (46.6)[b]	0.20±0.04 (25.0)[b]	0.43±0.12 (4.7)[b]
EEPG (500 mg/kg)	0.03±0.00[*] (62.5)[a]	2.26±0.46[*] (35.1)[a]	0.21±0.05[*] (59.6)[a]	0.40±0.10[*] (41.2)[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. [#]Activity expressed as nmoles product formed/mg protein/min. [*]Significantly different from control ($p<0.05$); [**]Significantly different from ADR-treated rats ($p<0.05$).Values in parenthesis represent % change; [(a)] % change relative to control; [(b,c)] % change relative to ADR.

Table 1C: Effect of treatment with ethanolic extract of *P. guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in vitamin C and malondialdehyde (MDA) levels

Treatment group	Vitamin C concentration (µg/mg protein)	MDA (nmol/mg protein)
Control (0.9% NaCl)	0.47±0.06	0.43±0.13
ADR (20 mg/kg)	0.21±0.02* (55.3)[a]	0.93±0.10* (53.8)[c]
EEPG (125 mg/kg) + ADR (20 mg/kg)	0.38±0.08** (44.7)[b]	0.56±0.07** (39.8)[d]
EEPG (250 mg/kg) + ADR (20 mg/kg)	0.42±0.07** (50.0)[b]	0.45±0.13** (51.6)[d]
EEPG (500 mg/kg) + ADR (20 mg/kg)	0.35±0.03** (40.0)[b]	0.82±0.09 (11.8)[d]
EEPG (500 mg/kg)	0.28±0.04* (40.4)[a]	0.71±0.24* (39.4)[c]

Values are expressed as mean±SEM for five rats in each group. *Significantly different from control ($p<0.05$); **Significantly different from ADR-treated rats ($p<0.05$). Values in parenthesis represent % change; [a,c] % change relative to control; [b,d] % change relative to ADR.

Table 1D: Effect of treatment with ethanolic extract of *P. guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in 5'nucleotidase (5′NTD) and glucose-6-phosphatase (G6Pase) activities in rat kidney.

Treatment group	5'-NTD (mmol/mg protein/min)	G6Pase (mmol/mg protein/min)
Control (0.9% NaCl)	177.28±22.6	23.08±4.0
ADR (20 mg/kg)	121.88±12.9* (31.3)[a]	14.56±1.8* (36.9)[a]
EEPG (125 mg/kg) + ADR (20 mg/kg)	136.75±11.6** (10.9)[b]	20.29±1.8** (28.2)[b]
EEPG (250 mg/kg) + ADR (20 mg/kg)	159.57±29.4** (23.6)[b]	22.66±4.2** (35.8)[b]
EEPG (500 mg/kg) + ADR (20 mg/kg)	131.55±7.9** (7.4)[b]	22.40±1.9** (35.0)[b]
EEPG (500 mg/kg)	103.64±5.0* (41.5)[a]	18.29±1.4* (20.4)[a]

Values are expressed as mean±SEM for five rats in each group. *Significantly different from control ($p<0.05$); **Significantly different from ADR-treated rats ($p<0.05$). Values in parenthesis represent % change; [(a)] % change relative to control; [(b)] % change relative to ADR.

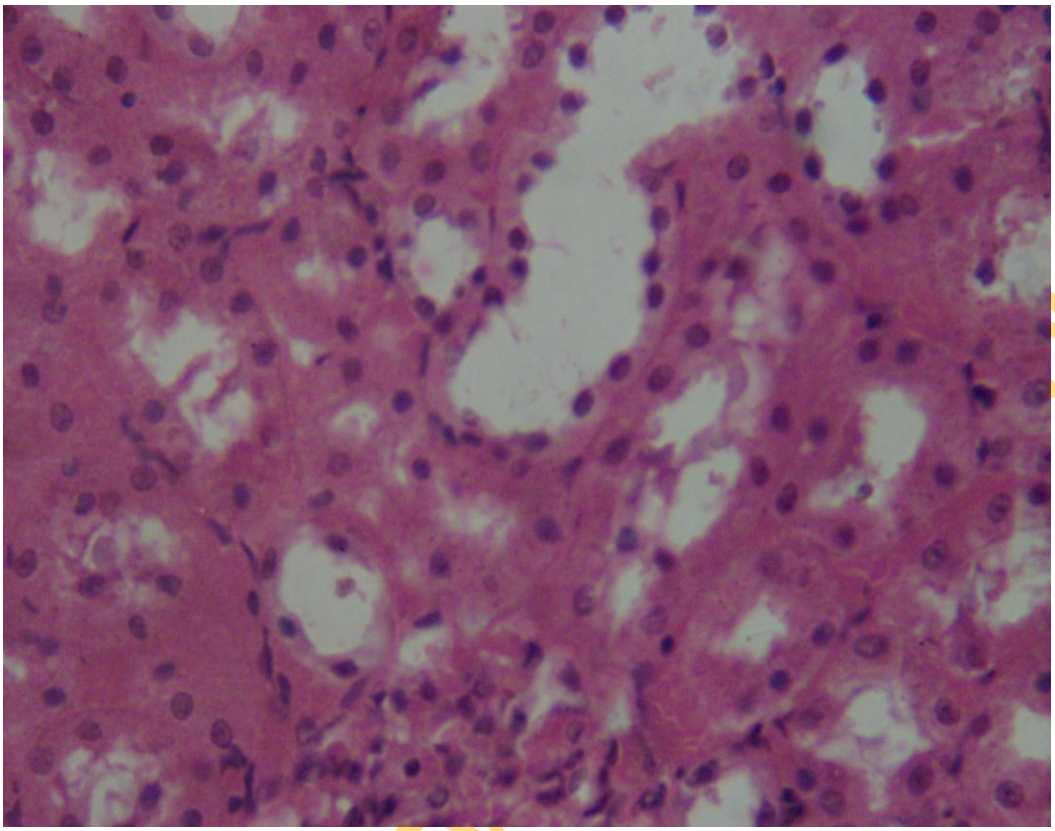


Figure 1B: Section of kidney from rat treated with normal saline (control).

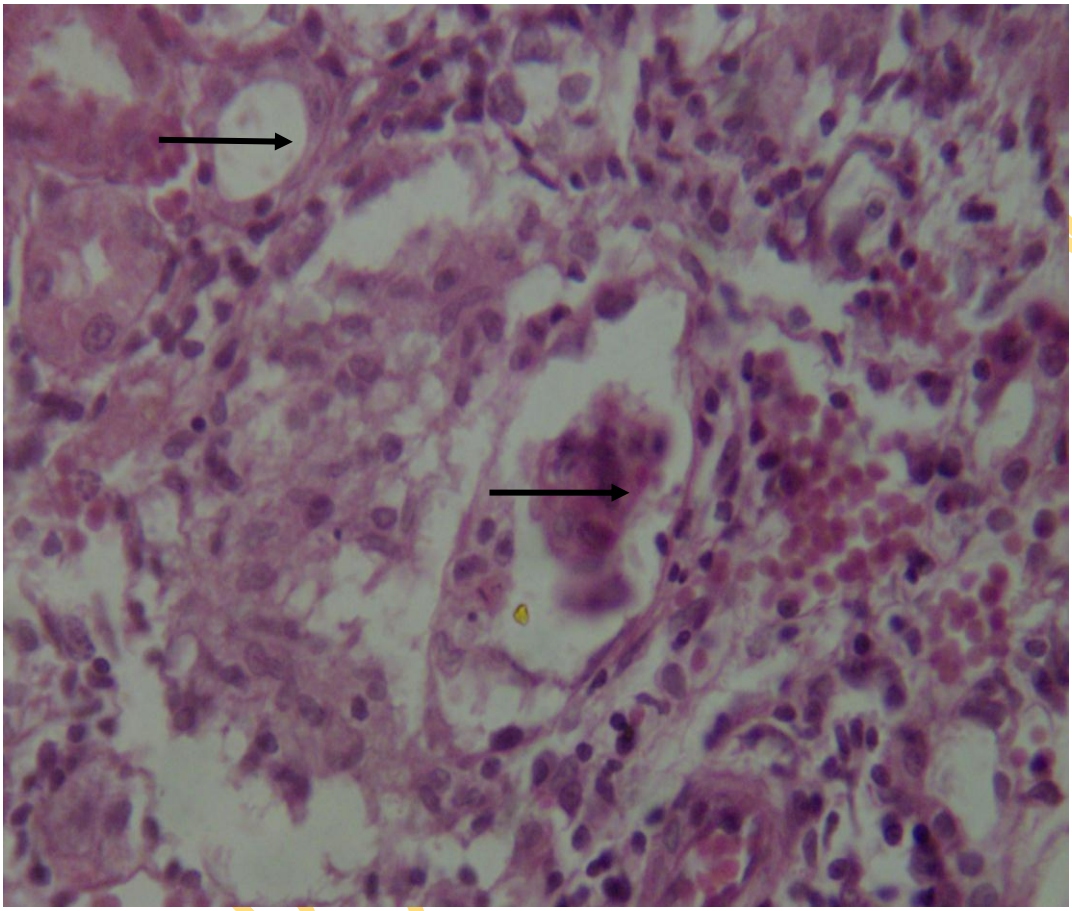


Figure 1C: Section of kidney from rat treated with adriamycin (20 mg/kg, i.p, single dose). Section shows foci of severe peritubular and periglomerular infiltration by macrophages and multifocal hemorrhage into the parenchyma (The black arrow shows this).

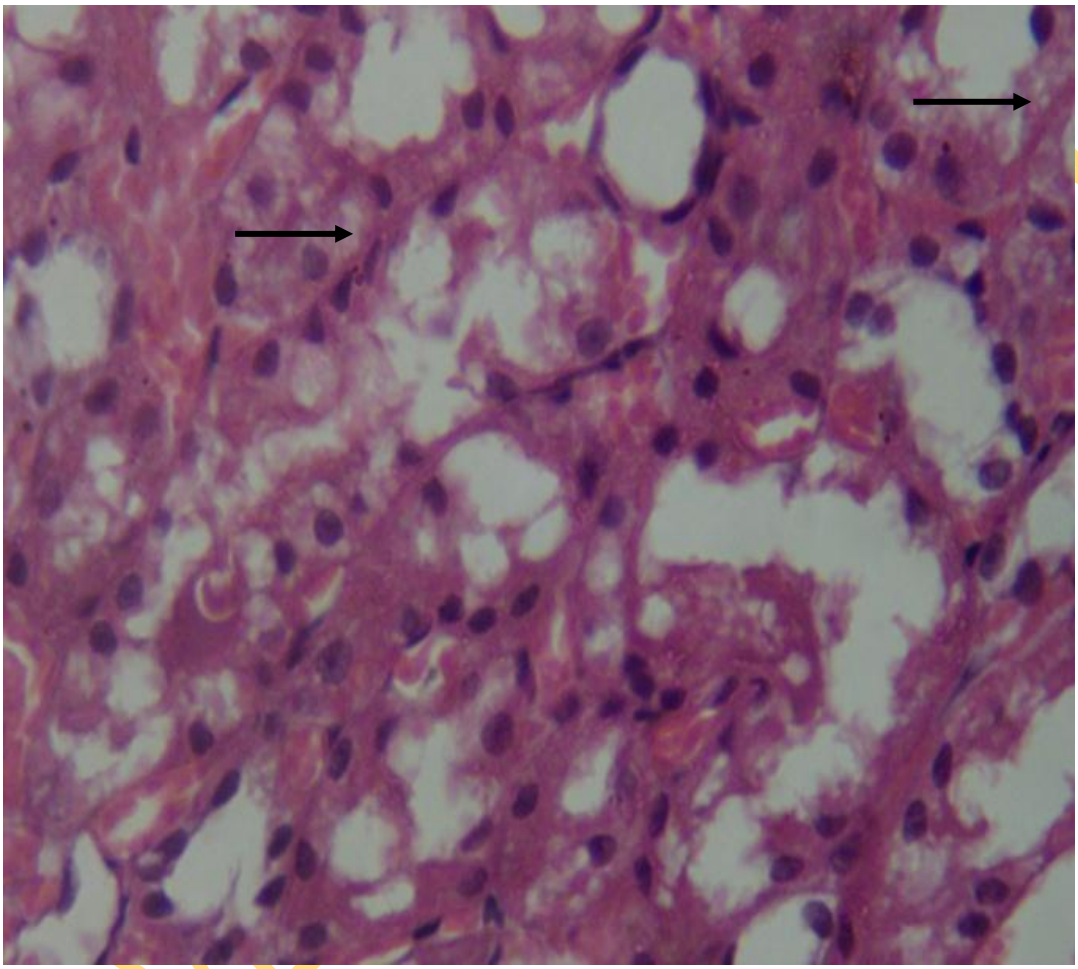


Figure 1D: Section of kidney from rat treated with ethanolic extract of *Psidium guajava* (125 mg/kg, p.o.) for 10 days before adriamycin injection (20 mg/kg, i.p.). Section shows mild tubular necrosis (red arrow) with evidence of hemorrhage into the parenchyma (the black arrow).

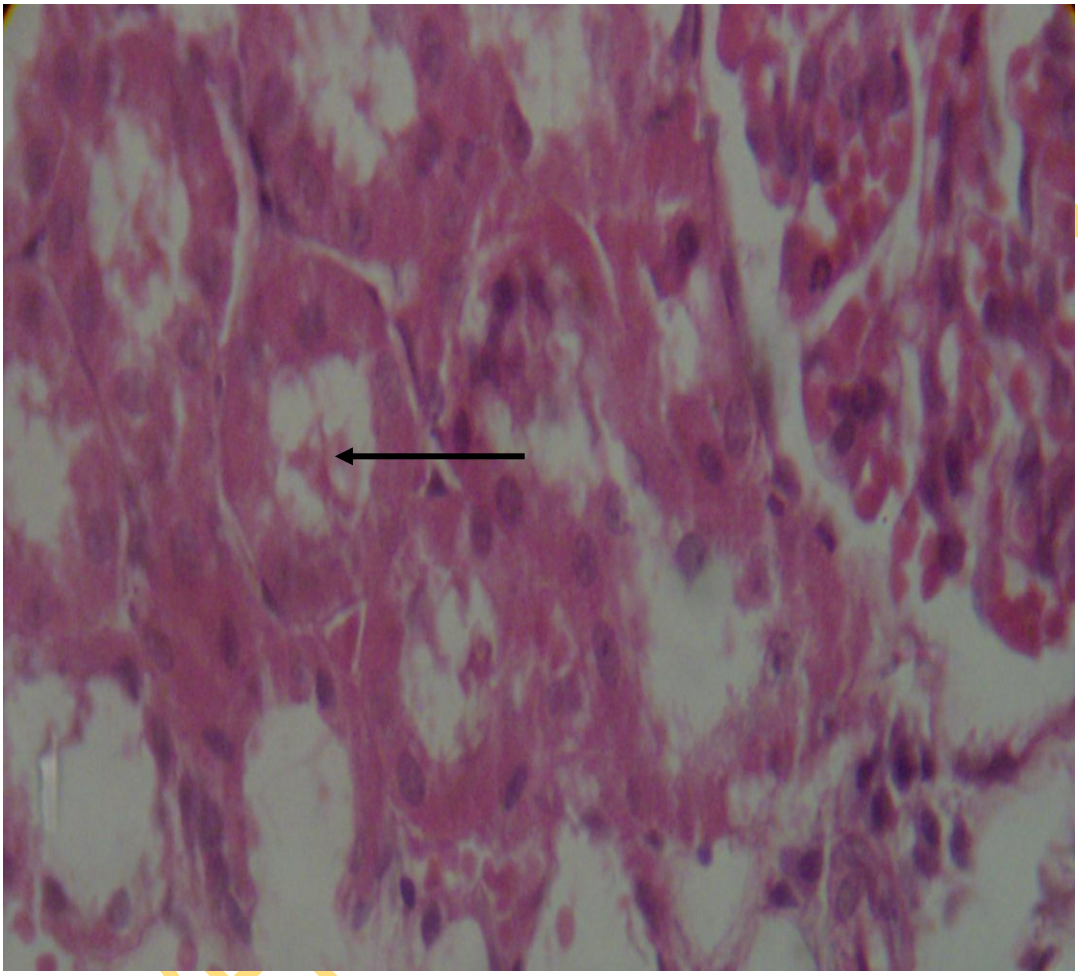


Figure 1E: Section of kidney from rat treated with ethanolic extract of *Psidium guajava* (250 mg/kg, p.o.) for 10 days before adriamycin injection (20 mg/kg, i.p.). Section shows mild congestion of renal blood vessels (black arrow).

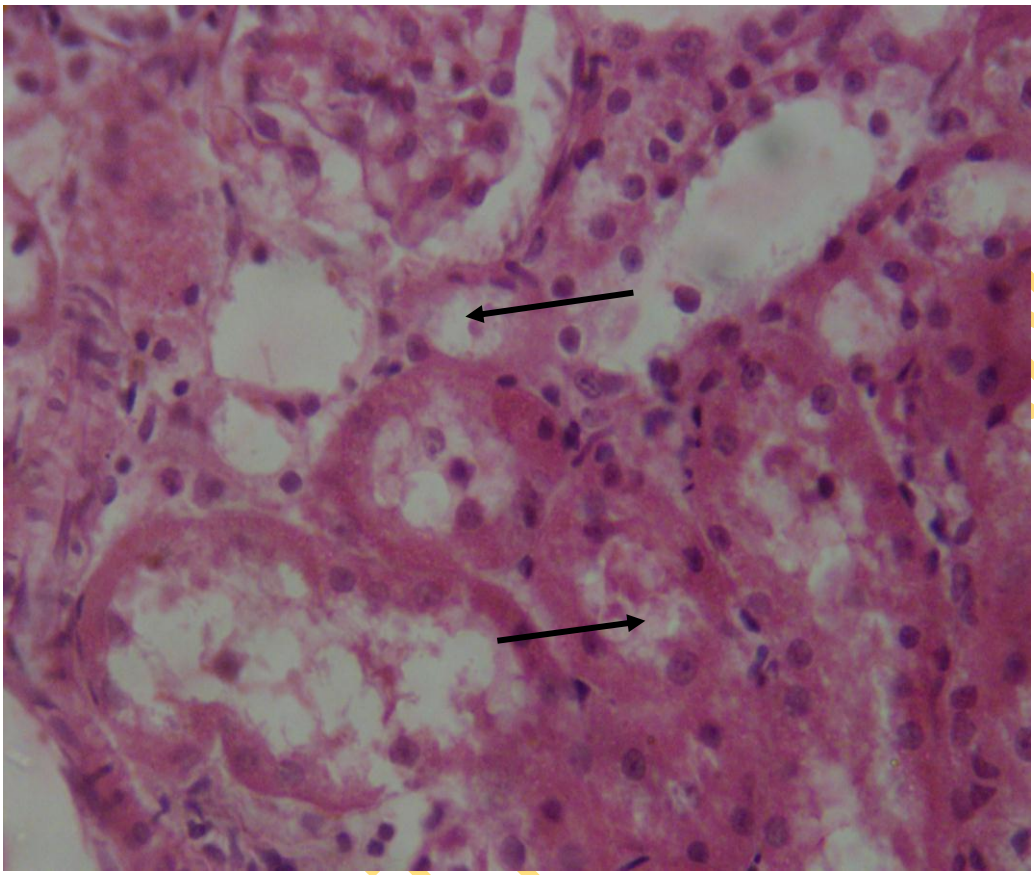


Figure 1F: Section of kidney from rat treated with ethanolic extract of *Psidium guajava* (500 mg/kg, p.o.) for 10 days before adriamycin injection (20 mg/kg, i.p.). Section shows moderate congestion of the renal vessels, with few foci of tubular erosions (black arrow).

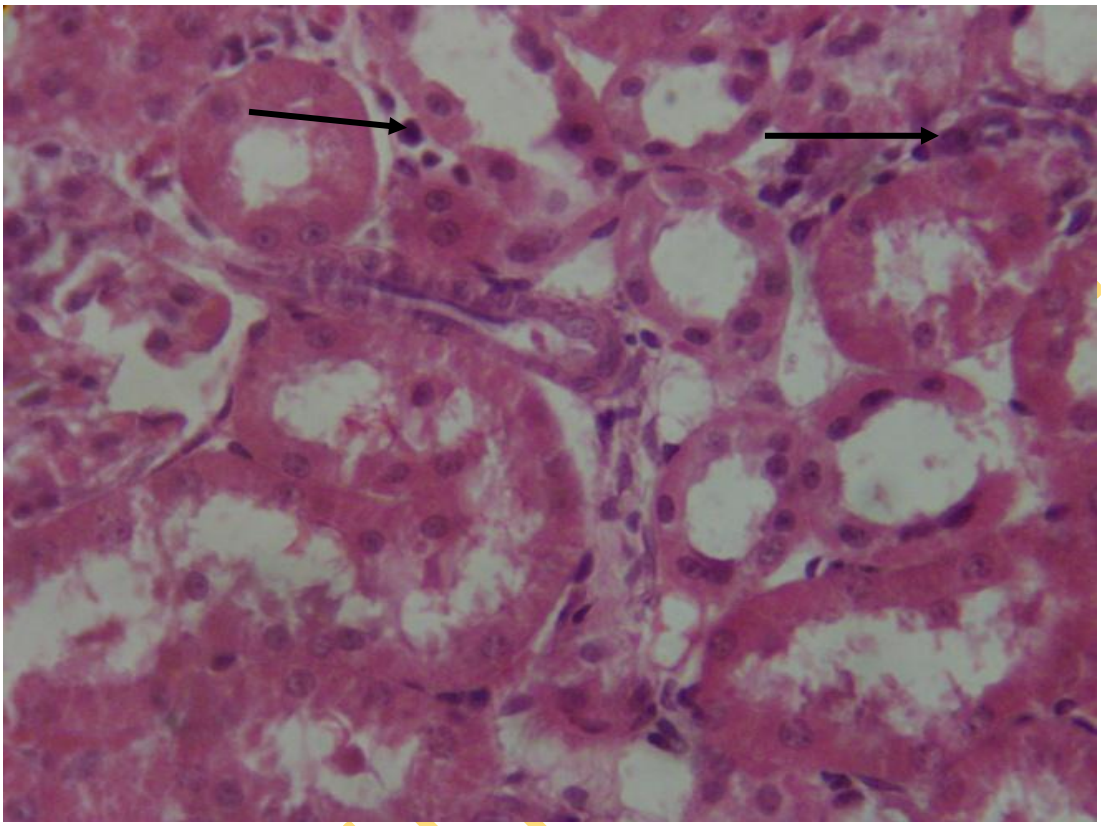
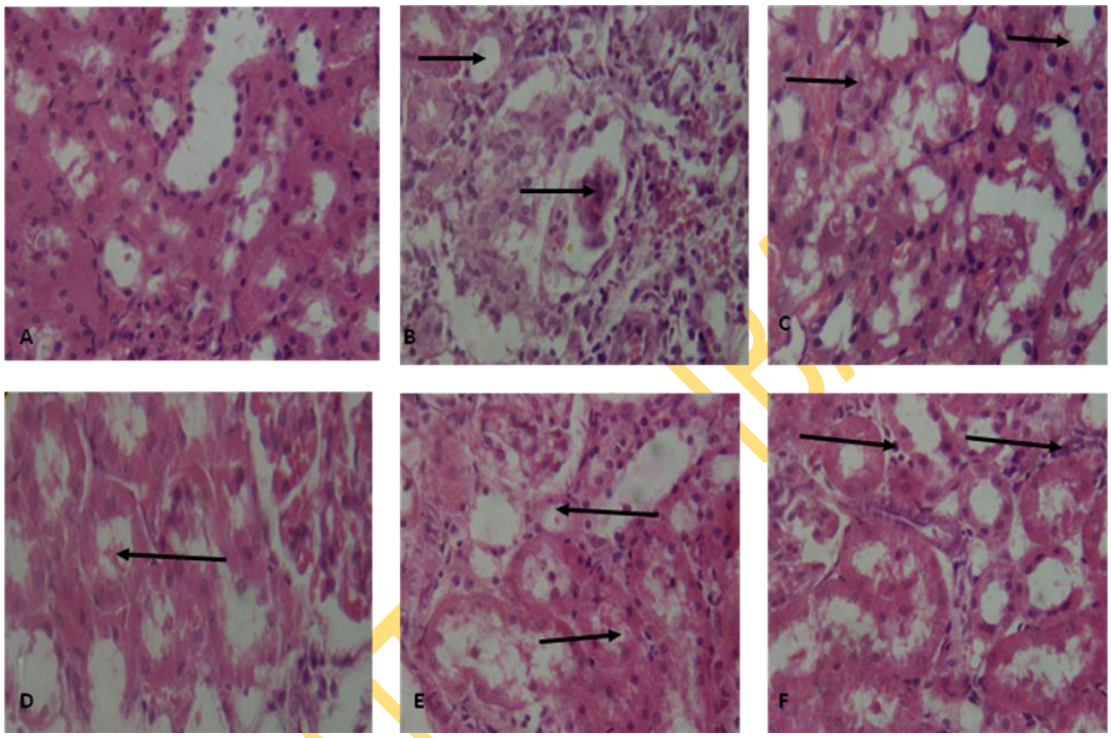


Figure 1G: Section of kidney from rat treated with 500 mg/kg oral dose of the ethanolic extract of *Psidium guajava* daily for 12 days. Section shows peritubular aggregation by macrophages.



Summary of photomicrograph of rat kidney section (x400). A. Control (normal saline) rat kidney Section. B. Adriamycin-treated (20 mg/kg) group showing severe peritubular and periglomerular infiltration by macrophage. C. Adriamycin (20 mg/kg) plus guava extract (125 mg/kg) treated group showing mild tubular necrosis. D. Adriamycin (20 mg/kg) plus guava extract (250 mg/kg) treated group showing mild congestion of renal blood vessels. E. Adriamycin (20 mg/kg) plus guava extract (500mg/kg) treated group showing moderate congestion of the renal vessels, with few foci of tubular erosions. F. Guava extract (500 mg/kg) treated group with peritubular aggregation by macrophages.

→ Indicate some of the tubules showing necrosis and congestion of renal blood vessels

The effect of adriamycin treatment on renal function test is illustrated in Figure 1A. Single administration of adriamycin at 20 mg/kg b.wt intraperitoneally resulted in a significant increase of 65.0% in level of blood urea nitrogen (BUN) when compared with that of control (untreated rats). Creatinine level was also significantly increased ($p<0.01$) when compared with the control. Treatment with the EEPG for 10 days before adriamycin injection at the dose of 125- and 250- mg/kg b.wt decreased the elevated concentrations of BUN by 42.5% and 61.1% respectively. The values were statistically significant when compared with those that received adriamycin injection only ($p<0.05$). Treatment with EEPG at the highest dose (i.e 500 mg/kg b.wt) also caused a significant decrease in the elevated concentration of BUN but not as pronounced as the 250 mg/kg dose of the extract. Although, there was slight increase in BUN level when extract alone was used at the highest dose, this was not significant ($p>0.05$) when compared with control. The adriamycin-induced increase in creatinine level was only suppressed significantly by the *Psidium guajava* treatment at the dose of 250 mg/kg b.wt when compared with the adriamycin-treated group. Treatment with the extract at the doses of 125- and 500- mg/kg b.wt only produced a slight and non-significant reduction in creatinine level when compared with adriamycin-treated group. EEPG alone at the highest dose (500 mg/kg) significantly ($p<0.05$) increased the plasma creatinine concentration by 62.7% when compared with the control-treated groups.

Table IA shows the effect of single intraperitoneal dose of adriamycin (20 mg/kg) on renal GSH level and GST activity. Administration of adriamycin produced a significant depletion of GSH ($p<0.05$) with a corresponding decrease in GST activity in the kidney when compared with control. Treatment with EEPG at various doses enhanced the activities of GST and GSH level. This effect was only significant at 250 mg/kg dose of the extract when compared with adriamycin-only treated group. Treatment with the extract only at 500 mg/kg produced a slight but non-significant decrease in GSH level (7.8%) when compared with control ($p>0.05$). The extract also at this dose caused a significant reduction of about 63.0% in GST activity when compared with control.

The changes observed in renal SOD and CAT activities are recorded in Table 1B. Administration of single dose of adriamycin (20 mg/kg) significantly ($p<0.05$) decreased CAT activity by 50%. EEPG (125 mg/kg) did not have any effect on the adriamycin-induced decrease in CAT activity when compared with adriamycin-treated group. However, activity of this enzyme increased slightly following treatment with 250 mg/kg

dose of EEPG and this was statistically significant when compared with adriamycin–treated rats. Superoxide dismutase (SOD) activity was significantly decreased by the single administration of adriamycin when compared with the control (untreated group). Treatment with EEPG at 125 mg/kg, 250 mg/kg and 500 mg/kg caused significant increases in SOD activity by 43.6%, 52.1%, and 46.6% respectively in the adriamycin-treated group. Treatment with EEPG (500 mg/kg b.wt) produced significant reductions in the activities of both enzymes.

The activities of glutathione peroxidase (GPx) and glutathione reductase (Gred) in the adriamycin-treated rats and the influence of EEPG are shown in Table 1B. Adriamycin treatment caused decrease in the renal activities of glutathione peroxidase (GPx) and glutathione reductase (Gred) and these effects were statistically significant ($p<0.5$) when compared with the control. The EEPG offered significant ($p<0.5$) protection at all doses against the adriamycin-induced decreases in GPx and Gred activities when compared with the adriamycin only group. EEPG treatment alone at the highest dose (500 mg/kg) decreased the activity of these enzymes when compared with the control group and this was statistically significant ($p<0.05$).

The result on vitamin C is shown in Table 1C. Adriamycin administration significantly ($p<0.05$) decreased vitamin C concentration by 55.3% when compared with the control rats. Treatment with EEPG at various doses prevented the adriamycin-induced reduction in vitamin C concentration, with the 250 mg/kg dose producing the greatest increase in vitamin C concentration. This effect was significant when compared with adriamycin-treated group. Extract alone at 500 mg/kg decreased vitamin C concentration when compared with control group.

The extent of lipid peroxidation was measured by assaying thiobarbituric acid reactive substances (TBARS) for all groups (Table 1C). Adriamycin significantly increased lipid peroxidation with 53.8% when compared with the control. Treatment with EEPG at 125 mg/kg and 250 mg/kg doses significantly ($p<0.05$) suppressed the increased in lipid peroxidation in the adriamycin-treated rats by 39.8% and 51.6% respectively. However, 500 mg/kg dose decreased lipid peroxidation by 11.8% ($p>0.05$). Administration of extract alone at 500 mg/kg significantly increased lipid peroxidation by 39.4%.

The activities of 5'-nucleotidase and glucose-6-phosphatase in the adriamycin-treated rats are shown in Table 1D. In the adriamycin group, the activities of 5' nucleotidase and glucose-6-phosphatase were decreased significantly by 31.3% and 36.9% respectively when compared with the control value. Treatment with EEPG at 125

mg/kg and 250 mg/kg resulted in significant increase in activity of 5'-nucleotidase when compared with the adriamycin-treated group. Treatment with 500 mg/kg dose of EEPG produced a slight increase (7.4%) which was not significant. Following treatment with EEPG at all doses, there was significant increase in the activity of glucose-6-phosphatase when compared with adriamycin group. There was significant decrease in activities of 5' nucleotidase and glucose-6-phosphatase enzymes by 41.5% and 20.4% respectively when EEPG alone (500 mg/kg) was used.

Photomicrographs showing renal morphology in all treatment groups are shown in Figures 1B to 1G. Kidney sections from rats in control group were essentially normal. Adriamycin, however, induced severe peritubular and periglomerular infiltration by macrophage (Figure 1C). Treatment with 125-, 250- and 500- mg/kg doses of EEPG reduced adriamycin- induced renal tubular damage with renal section in these groups of rats showing mild tubular necrosis to moderate congestion of renal blood vessels respectively (Figures 1D, 1E and 1F). Rats treated with 500 mg/kg EEPG alone appears not to show any better histological protection against the adriamycin- induced renal damage as there was evidence of peritubular aggregation by macrophages (1G).

CONCLUSION

The ethanolic extract of *Psidium guajava* (EEPG) at a relatively low dose protected against adriamycin-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 guava leaves. The extract, however, may also be exerting some toxicity when administered in large doses.

EXPERIMENT 2: NEPHROTOXICITY INDUCED BY REPEATED DOSES OF ADRIAMYCIN AND THE EFFECT OF CONCURRENT ADMINISTRATION OF ETHANOLIC EXTRACT OF *PSIDIUM GUAJAVA*

INTRODUCTION

Despite its side effects, adriamycin remains an important component in most chemotherapeutic regimens, because of its efficacy in treating a broad-spectrum of cancers. In this study, we used higher than usually administered single doses because experimental studies in animals showed that repeated administration of adriamycin led to substantial glomerular and tubular lesion and that chronic organ toxicity frequently develops upon its administration (Singal and IIiskovic, 1998).

This present study was aimed at examining the effect of repeated administration of adriamycin in a rat model in order to analyse toxicity that accumulate and also the effect of concurrent administration of EEPG as this will also shed more light on the mechanism(s) of protection of EEPG against toxicity induced by adriamycin.

PROCEDURE

Six groups of five rats per group weighing between 200 and 275 g were studied in this experiment; Group I (control) received 0.9% NaCl; Group II was treated with repeated doses of adriamycin (ADR) in 6 equal injections (2.5 mg/kg b.wt) intraperitoneally; Rats in groups III, IV and V were treated with 125-, 250- and 500-mg/kg oral doses of EEPG respectively concurrently with ADR. Group VI was treated with 500 mg/kg EEPG only. All the treatments were given for 11 days. Rats were sacrificed by cervical dislocation 48 hours after the last treatment. Blood samples were collected via cardiac puncture and plasma separated by centrifugation at room temperature. Kidney samples were immediately removed, homogenized and centrifuged at 4^{o}C to separate the postmitochondrial fraction (PMF) as in experiment 1. Renal function was assessed in plasma. Biomarkers of oxidative stress, plasma membrane and tubular damage were evaluated in the PMF. A portion of the kidney was removed before homogenizing and processed for histology.

RESULTS

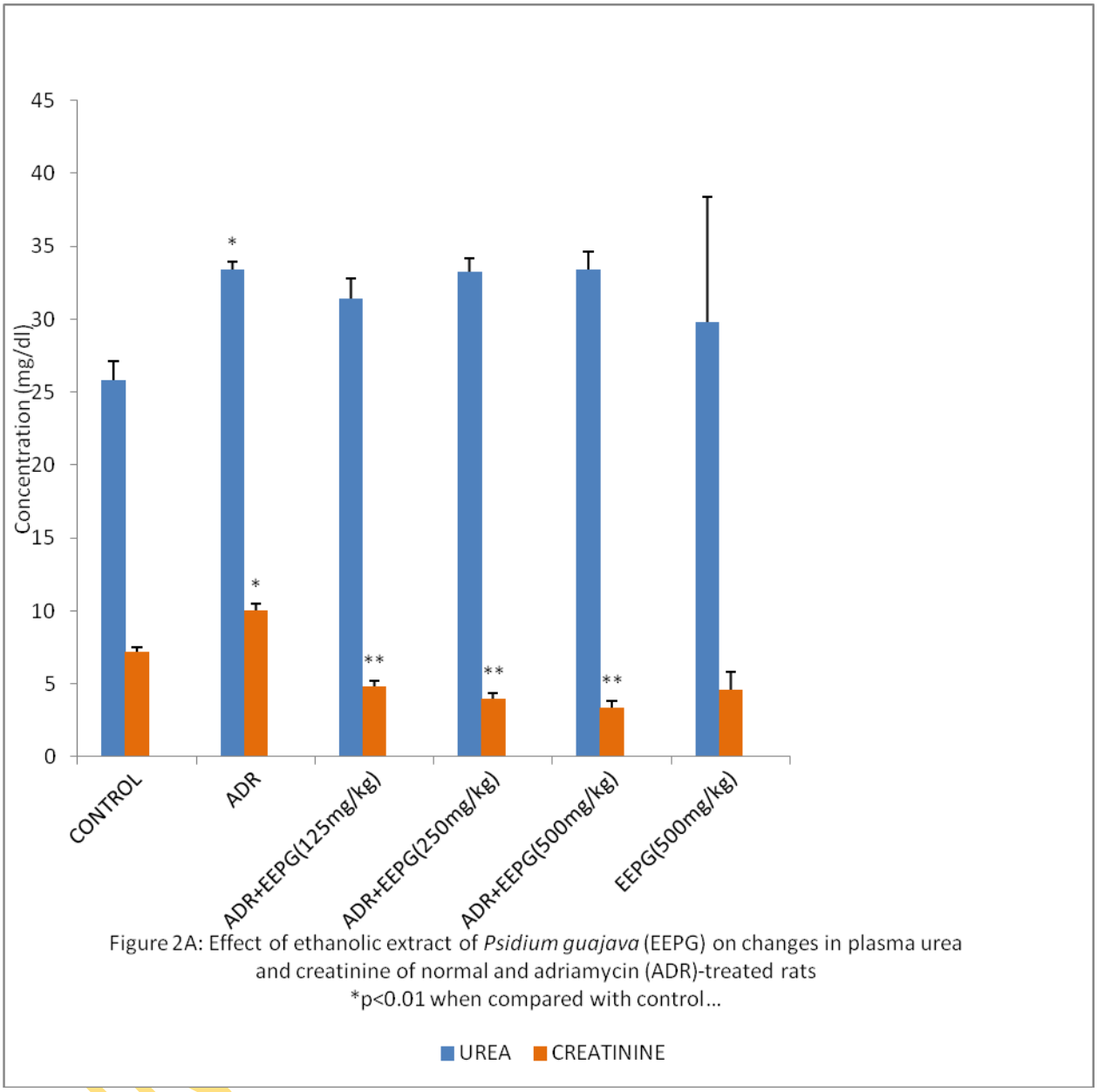


Figure 2A: Effect of ethanolic extract of *Psidium guajava* (EEPG) on changes in plasma urea and creatinine of normal and adriamycin (ADR)-treated rats
*p<0.01 when compared with control...

Table 2A: Effect of concurrent administration of ethanolic extract of *P. guajava* on the activity of glutathione-S-transferase (GST) and reduced glutathione (GSH) level in normal and adriamycin (ADR)–treated rats.

Treatment group	GST (nmol/min/mg protein)	GSH (μg/g tissue)
Control (0.9% NaCl)	0.10±0.01	17.19±4.2
ADR (2.5 mg/kg)	0.08±0.01 (20.6)[a]	14.58±0.83[*] (15.2)[a]
EEPG (125 mg/kg) + ADR (2.5 mg/kg)	0.13±0.01[**] (35.7)[b]	15.76±1.61 (7.5)[b]
EEPG (250 mg/kg) + ADR (2.5 mg/kg)	0.11±0.02[**] (28.3)[b]	15.42±1.41 (5.5)[b]
EEPG (500 mg/kg) + ADR (2.5 mg/kg)	0.10±0.01 (18.2)[b]	14.91±3.08 (2.2)[b]
EEPG (500 mg/kg)	0.12±0.01 (15.0)[c]	17.11±1.37 (0.5)[a]

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. [*]Significantly different from control ($p<0.05$); [**]Significantly different from ADR-treated rats ($p<0.05$). Values in parenthesis represent % change; [(a,c)] % change relative to control; [(b)] % change relative to ADR.

Table 2B: Effect of concurrent administration of ethanolic extract of *P. guajava* on catalase and superoxide dismutase activities and vitamin C and malondialdehyde levels in normal and adriamycin (ADR) – treated rats.

Treatment group	Catalase (µmole H_2O_2 consumed/min)	Superoxide dismutase (Unit/g tissue)	Vitamin C (µg/mg protein)	MDA (nmol/mgprotein)
Control (NaCl)	0.12±0.02	5.25±0.95	1.51±0.12	0.17±0.02
ADR (2.5 mg/kg)	0.09±0.02* (29.3)[a]	4.56±0.44 (13.1)[a]	0.85±0.13* (45.4)[a]	0.34±0.04* (58.7)[c]
EEPG (125 mg/kg) + ADR (2.5 mg/kg)	0.12±0.01** (27.5)[b]	5.80±1.36 (21.3)[b]	2.66±0.41** (71.1)[b]	0.27±0.03 (10.7)[d]
EEPG (250 mg/kg) + ADR (2.5 mg/kg)	0.12±0.00** (26.9)[b]	4.87±0.11 (0.8)[b]	2.24±0.25** (63.1)[b]	0.24±0.01** (38.7)[d]
EEPG (500 mg/kg) + ADR (2.5 mg/kg)	0.11±0.02** (22.3)[b]	4.35±0.35 (10.4)[c]	2.10±0.24** (58.7)[b]	0.22±0.02** (48.0)[d]
EEPG (500 mg/kg)	0.12±0.02 (3.3)[a]	5.08±0.89 (13.2)[a]	1.51±0.13 (0.3)[a]	0.18±0.02 (11.4)[c]

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. *Significantly different from control ($p<0.05$); **Significantly different from ADR-treated rats ($p<0.05$). Values in parenthesis represent % change; [(a)] % change relative to control; [(b,c)] % change relative to ADR.

Table 2C: Effect of concurrent administration with ethanolic extract of *P.guajava* on the activities of 5′ nucleotidase and glucose-6-phosphatase in normal and adriamycin (ADR) – treated rats.

Treatment group	5'-Nucleotidase[#]	Glucose-6-phosphatase[#]
Control (0.9% NaCl)	0.80±0.1	12.75±0.9
ADR (2.5 mg/kg)	0.26±0.1[*] (62.2)[a]	5.91±1.7[*] (66.1)[a]
EEPG (125 mg/kg) + ADR (2.5 mg/kg)	1.25±0.2[**] (79.0)[b]	19.69±2.6[**] (79.4)[b]
EEPG (250 mg/kg) + ADR (2.5 mg/kg)	0.87±0.1[**] (69.7)[b]	14.20±1.8[**] (69.5)[b]
EEPG (500 mg/kg) + ADR (2.5 mg/kg)	0.87±0.1[**] (69.8)[b]	14.56±1.6[**] (70.3)[b]
EEPG (500 mg/kg)	0.82±0.1 (2.2)[c]	12.47±0.4 (2.2)[a]

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. [*]Significantly different from control ($p<0.05$); [**]Significantly different from ADR-treated rats ($p<0.05$). Values in parenthesis represent % change; [(a,c)] % change relative to control; [(b)] % change relative to ADR. [#]Activity expressed as mmol/mg protein/min

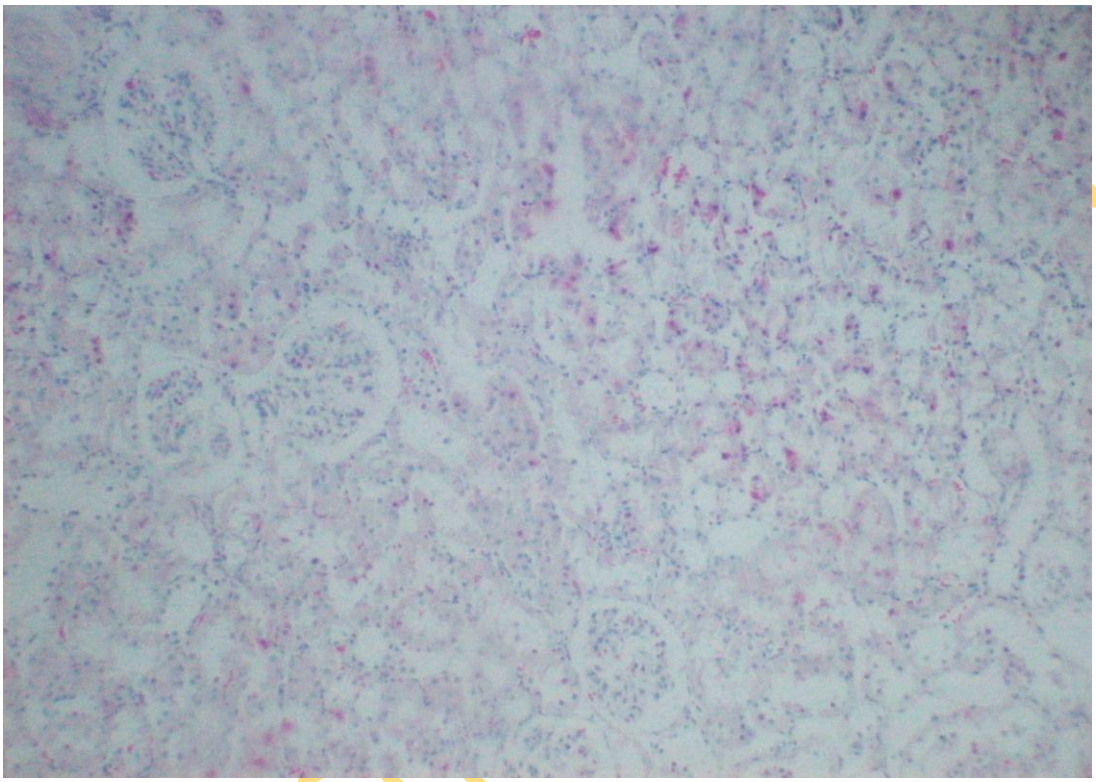


Figure 2B: Section of kidney from rat treated with normal saline (control). Renal morphology appears normal without any visible lesion.

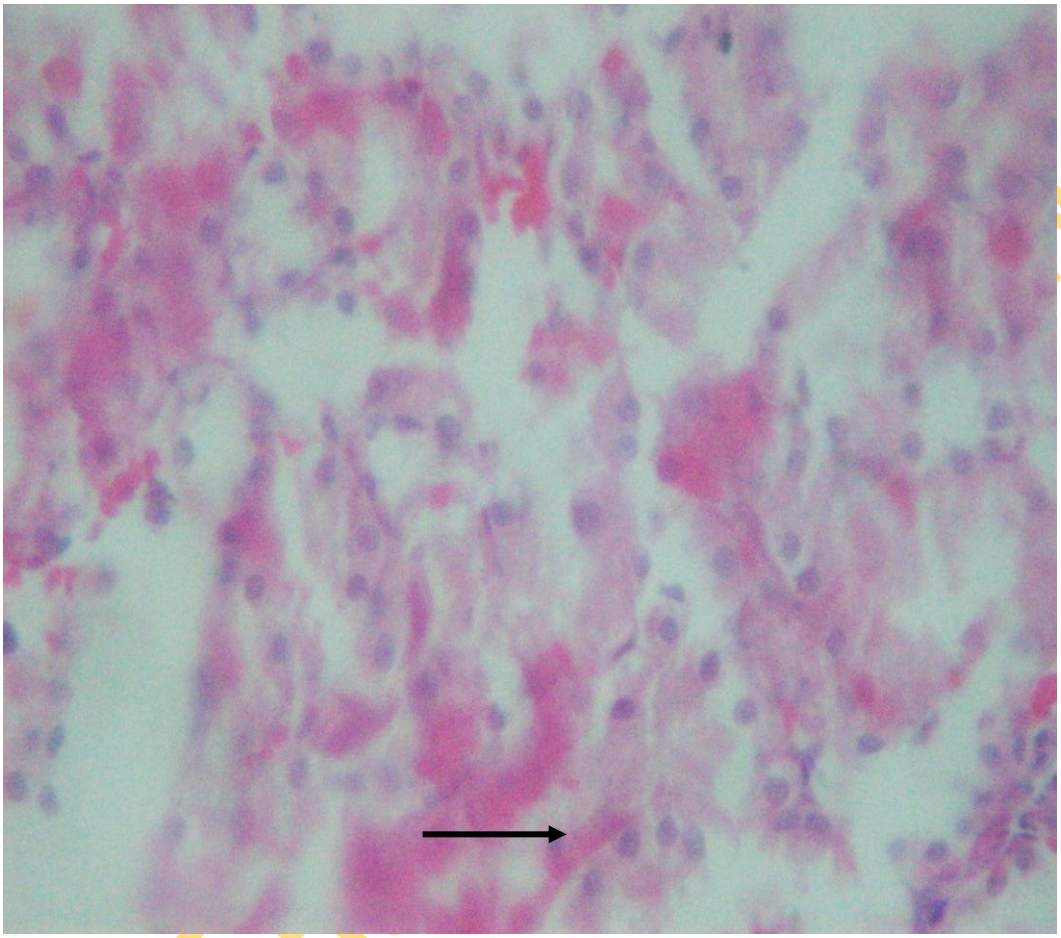


Figure 2C: Section of kidney from rat treated with adriamycin (2.5mg/kg, i.p.), 6 equal injections. Section shows severe periglomerular infiltration by macrophages. Eosinophilic material in the lumen (black arrow).

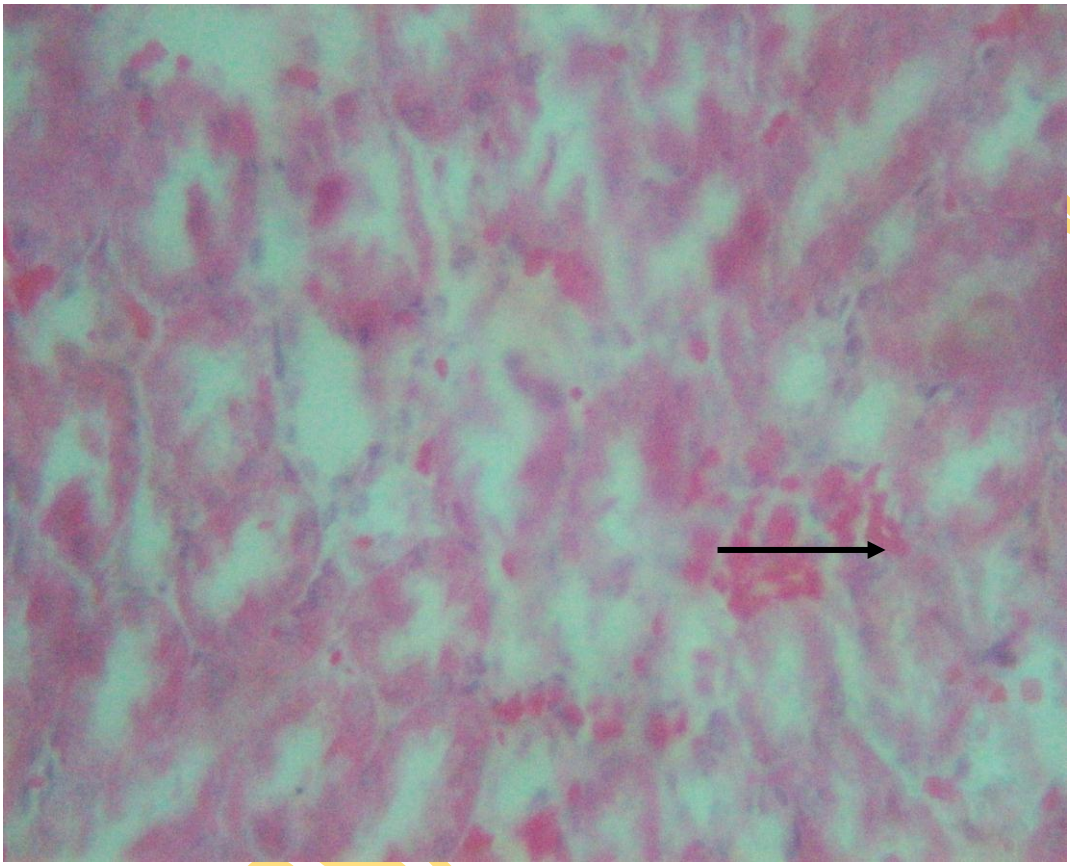


Figure 2D: Section of kidney from rat treated with ethanolic extract of *Psidium guajava* (125 mg/kg, *p.o.*) plus adriamycin (2.5 mg/kg, i.p.) in 6 equal injections. No visible lesions. There is evidence of hemorrhage (arrow)

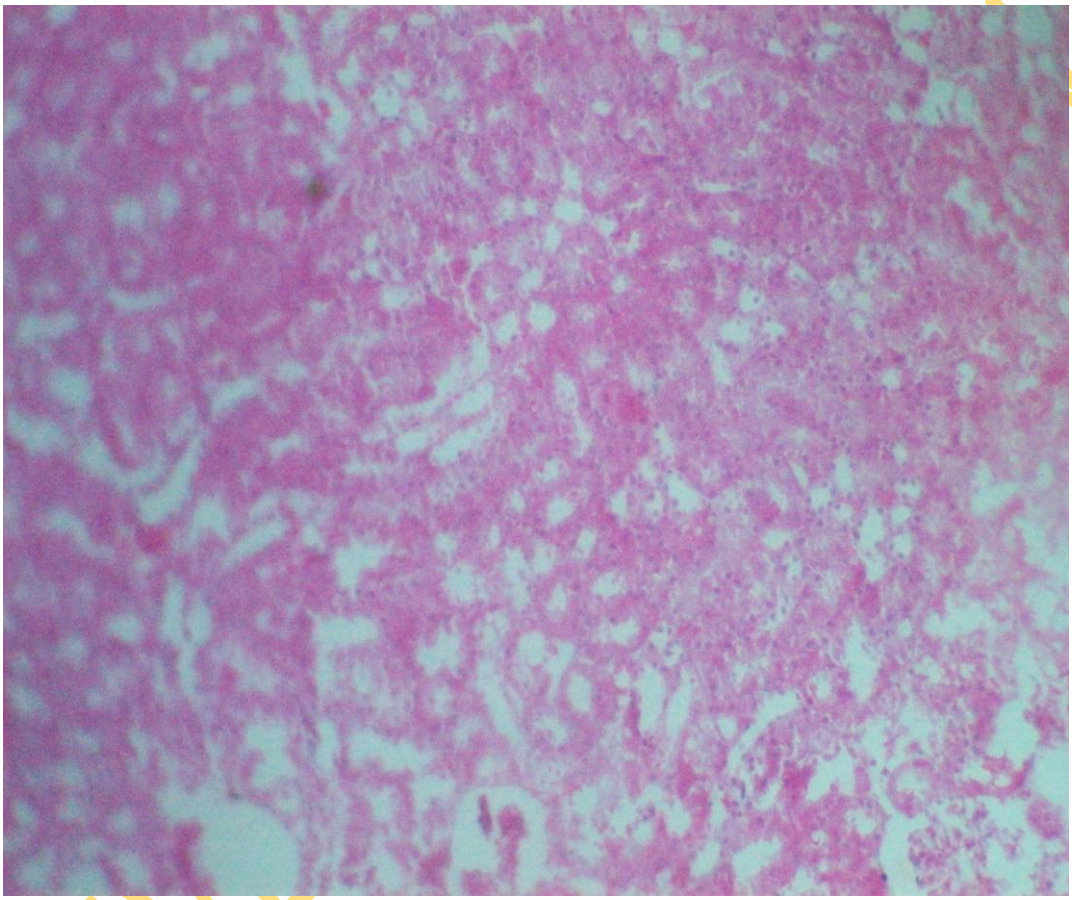


Figure 2E: Section of kidney from rat treated with ethanolic extract of *Psidium guajava* (250 mg/kg, *p.o.*) plus adriamycin (2.5 mg/kg, i.p.) in 6 equal injections. No visible lesions.

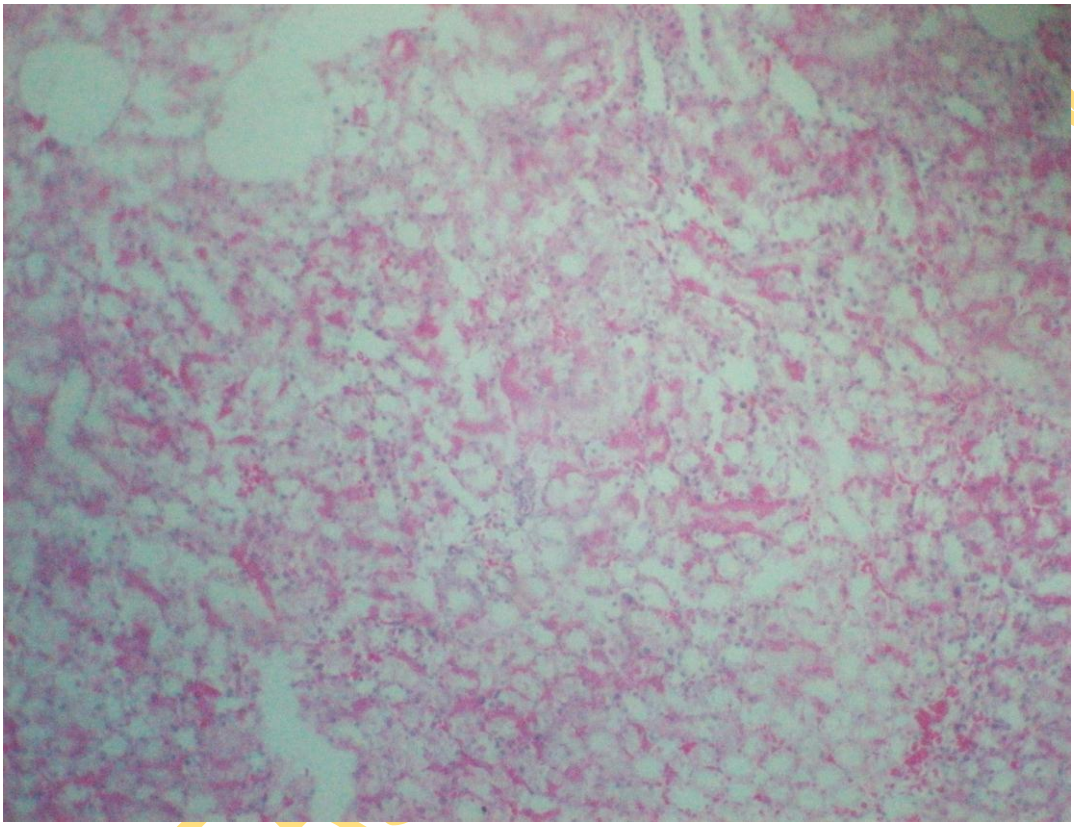


Figure 2F: Section of kidney from rat treated with ethanolic extract of *Psidium guajava* (500 mg/kg, *p.o.*) plus adriamycin (2.5 mg/kg, i.p.) in 6 equal injections. No visible lesions.

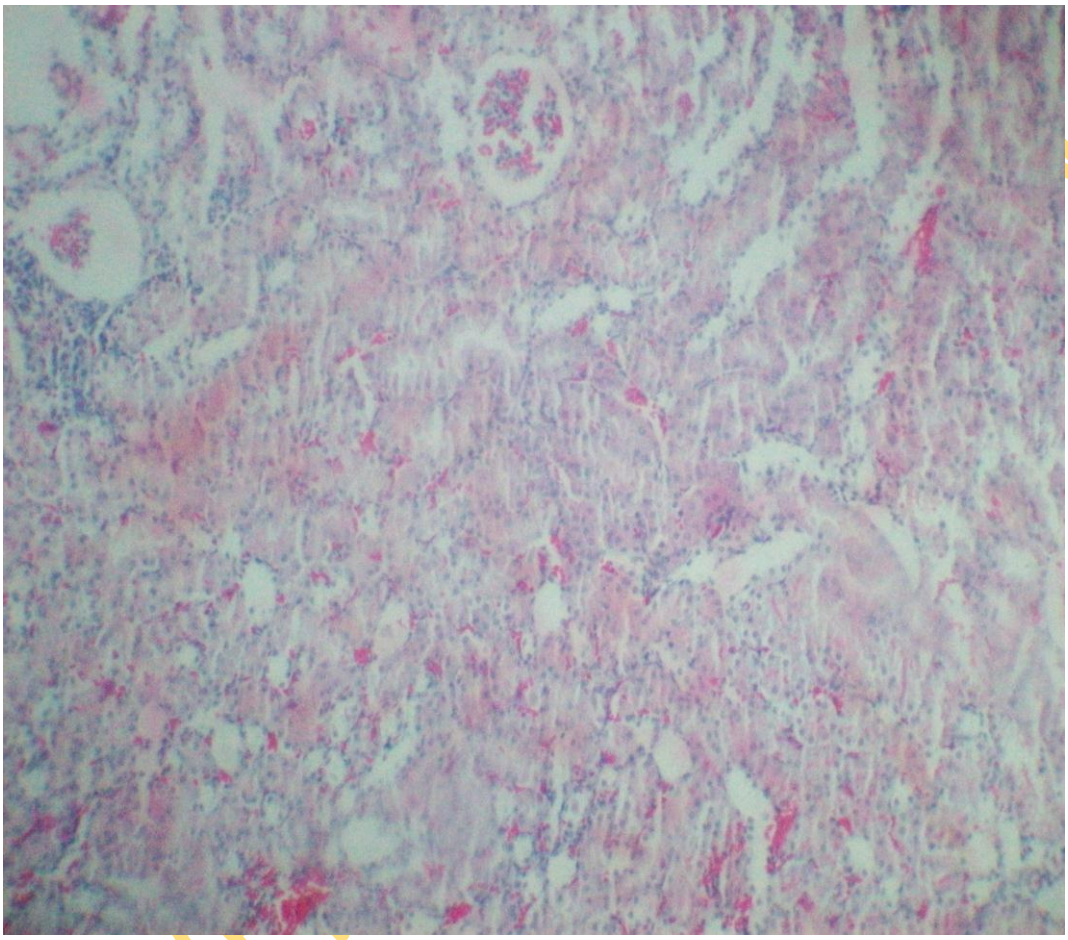
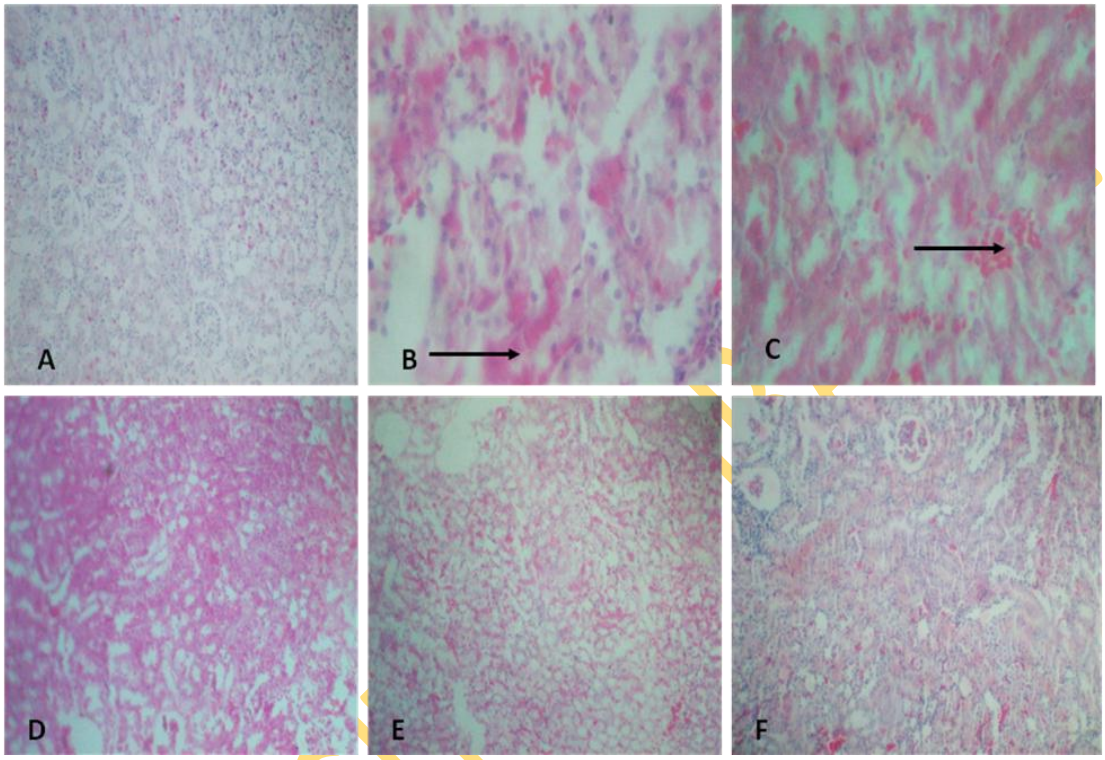


Figure 2G: Section of kidney from rat treated with ethanolic extract of *Psidium guajava* (500 mg/kg, *p.o.*). No visible lesions.



Summary of photomicrograph of rat kidney sections (X400)
Histological section of kidney from rat treated with A. normal saline; B. Adriamycin (2.5mg/kg); C. Adriamycin + EEPG (125mg/kg); D. Adriamycin+ EEPG (250mg/kg); E. Adriamycin + EEPG(500mg/kg); F. EEPG (500mg/kg).

→ Indicates evidence of hemorrhage and periglomerular infiltration.

Results for renal function tests is depicted in Figure 2A. Rats exposed to repeated doses of adriamycin (in 6 equal injections to give a total dose of 2.5 mg/kg body weight) produced a significant ($p<0.01$) increase in blood urea nitrogen level when compared with the group that received physiological saline only (control). EEPG at the doses of 125, 250 and 500 mg/kg body weight when concurrently administered with adriamycin did not produce any significant alteration in the concentration of blood urea nitrogen (BUN). Plasma creatinine also increased significantly ($p<0.01$) in the adriamycin-treated when compared with control rats. This increase in creatinine concentration was significantly ($p<0.001$) prevented by 51.6%, 62.1% and 65.9% following EEPG concurrent administration at the doses of 125, 250 and 500 mg/kg respectively. Levels of BUN and creatinine when compared with control was not significantly ($p>0.05$) affected by the extract alone at the highest dose of 500 mg/kg.

Adriamycin renal toxicity was associated with induction of oxidative stress in the rats. Table 2A shows the effect of EEPG administration on glutathione-S-transferase (GST) and glutathione (GSH) levels in ADR-treated rats. Repeated doses of adriamycin led to reduction in the activity of renal glutathione-S-transferase by 20.6% when compared with the untreated group (control), although this reduction was not statistically significant. The concurrent administration of EEPG with adriamycin at the doses of 125 and 250 mg/kg raised renal activity of glutathione-S-transferase significantly ($p<0.05$) by 35.7% and 28.3% respectively when compared with the group treated with adriamycin alone. Although, the 500 mg/kg of the extract also raised the glutathione-S-transferase activity (18.8%), this was not statistically significant ($p>0.05$). There was significant depletion of GSH content in adriamycin only-treated rats by 15.2% when compared with the control. EEPG when concurrently administered with adriamycin produced slight but insignificant increase in GSH level by 7.5%, 5.5% and 14.9% at 125, 250 and 500 mg/kg doses respectively. EEPG when administered alone at 500 mg/kg did not produce any significant change in GST activity as well as GSH level when compared with control.

The activity of catalase in the post mitochondrial fraction of rat kidney decreased significantly in the animals treated with adriamycin alone when compared with control animals as shown in Table 2B. The 125, 250 and 500 mg/kg of the extract when concurrently administered with adriamycin prevented this decrease in the activity of catalase in adriamycin-treated rats by 27.5%, 26.9% and 22.3% respectively. The activity of superoxide dismutase (Table 2B) decreased in the rats treated with adriamycin by 13.1% but this difference was not significant ($p>0.01$) when compared

with the control group. No significant change in the activity of superoxide dismutase was observed in all the groups that received the various doses of EEPG plus adriamycin. The highest dose of the extract had no significant effect on the activities of catalase and superoxide dismutase when compared with control ($p>0.05$).

ADR-induced decrease in antioxidant defense system resulted in significant increase in renal lipid peroxidation (LPO) and decrease in vitamin C concentration as indicated in Table 2B. There was a significant reduction in the concentration of ascorbic acid following the repeated doses of adriamycin ($p<0.01$). The 125, 250 and 500 mg/kg of EEPG when concurrently administered with adriamycin raised the concentration of ascorbic acid when compared with adriamycin only-treated group. This effect was statistically significant. Adriamycin treatment produced a significant increase in malondialdehyde level which is an index of lipid peroxidation. The concurrent administration of EEPG at 125, 250 and 500 mg/kg prevented this adriamycin-induced lipid peroxidation but significant reduction in MDA level was only seen at 250 and 500 mg/kg of the extract. Ethanolic extract of guava when administered alone at the highest dose had no significant alteration in the levels of ascorbic acid and malondialdehyde when compared with the control values ($P>0.05$).

The effect of EEPG on ADR-induced decreases in renal glucose-6-phosphatase (G6Pase) and 5′-nucleotidase (5′-NTD) are shown in Table 2C. Repeated doses of adriamycin decreased the renal activities of 5' nucleotidase and glucose-6-phosphatase significantly by 62.2% and 66.1% respectively when compared with the control values. *P.guajava* extract when concurrently administered with adriamycin at 125, 250 and 500mg/kg doses preserved the activities of 5' nucleotidase and glucose-6-phosphatase by raising their activities in the kidney and this was significant when compared with the group treated with repeated doses of adriamycin alone. No significant change in the activities of 5' nucleotidase and glucose-6-phosphatase was observed in the groups that received extract alone at 500 mg/kg.

Adverse histopathological change in form of eosinophilic material in the lumen and severe periglomerular infiltration by macrophages were observed following repeated doses of adriamycin to the experimental rats. Kidney sections from rats treated with 125-, 250-, and 500- mg/kg doses of EEPG and repeated doses of adriamycin showed no visible lesions. The ethanolic extract of guava, at all doses, was able to protect against histopathological change induced by adriamycin

CONCLUSION

So far, results of the present study showed the ability of repeated doses of adriamycin to induce oxidative stress in the kidney and thus proving the role of oxidative stress in its toxicity. Also, the protection offered by ethanolic extract of *Psidium guajava* (EEPG) against adriamycin-induced renal toxicity by enhancing antioxidant defense supports its proposed antioxidant and free-radical scavenging activity. Results of the present study indicate that administration of EEPG exerts a protective effect on adriamycin-induced nephrotoxicity.

EXPERIMENT 3: EFFECT OF TREATMENT SCHEDULE ON THE PROTECTIVE ACTION OF *PSIDIUM GUAJAVA* AGAINST NEPHROTOXICITY INDUCED BY REPEATED DOSES OF ADRIAMYCIN

INTRODUCTION

The currently observed rapid increase in consumption of herbal remedies worldwide has been stimulated by several factors, including the notion that all herbal products are safe and effective (Said *et al*., 2002). However, over the past decade, several news-catching episodes in developed communities indicated adverse effects, sometimes life-threatening, allegedly arising from taking herbal products or traditional medicines (Elvin-Lewis, 2001; Chan, 2003). In some cases, adulteration, inappropriate formulation, or lack of understanding of plant and drug interactions or uses had led to adverse reactions that were sometimes life-threatening or lethal to patients (Abu-Irmaileh and Afifi, 2003; Pak *et al.*, 2004; Saad *et al*., 2005).

This present study aimed at undertaking comparative evaluation of EEPG pretreatment and posttreatment schedules in adriamycin-treated rats.

Experiment 3a: Effect of pretreatment with the ethanolic extract of *Psidium guajava* on nephrotoxicity induced by repeated doses of adriamycin.

PROCEDURE

Six groups of five rats per group, weighing between 204 and 265 g, were studied in this experiment; Group I (control) received 0.9% NaCl; Group II was treated with cumulative dose of adriamycin (ADR, 2.5 mg/kg b.wt, i.p) in 6 equal injections. Rats in groups III, IV and V were treated with 125-, 250- and 500- mg/kg/day oral doses of EEPG respectively for 7 days before repeated doses of ADR. Group VI was treated with EEPG (500 mg/kg/day) only for 7 days. All the treatments lasted for 17 days. Rats were sacrificed by cervical dislocation 48 hours after the last injection of adriamycin. Blood samples were collected via cardiac puncture and plasma separated by centrifugation at 3000 *g* at room temperature. Kidney samples were immediately removed, homogenized and centrifuged at 4^{o}C to separate the postmitochondrial fraction (PMF) as in experiment 1. Renal function was assessed in plasma. Biomarkers of oxidative stress, plasma membrane and tubular damage were evaluated in the PMF. A portion of the kidney was removed before homogenizing and processed for histology.

RESULTS

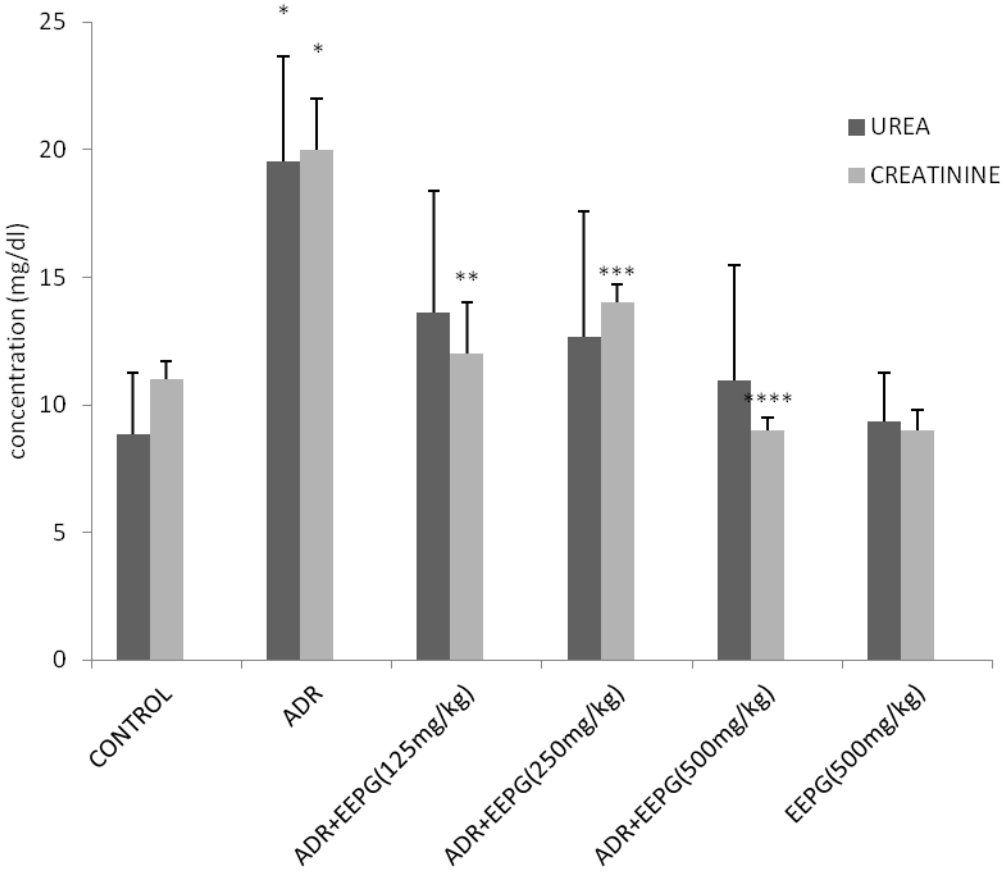


Figure 3A: Effect of pretreatment with ethanolic extract of *Psidium guajava* (EEPG) on changes in blood urea nitrogen and plasma creatinine of normal and adriamycin (ADR)-treated rats.
*p<0.01 when compared with control**p<0.05
***p<0.01 and ****p<0.001 wh

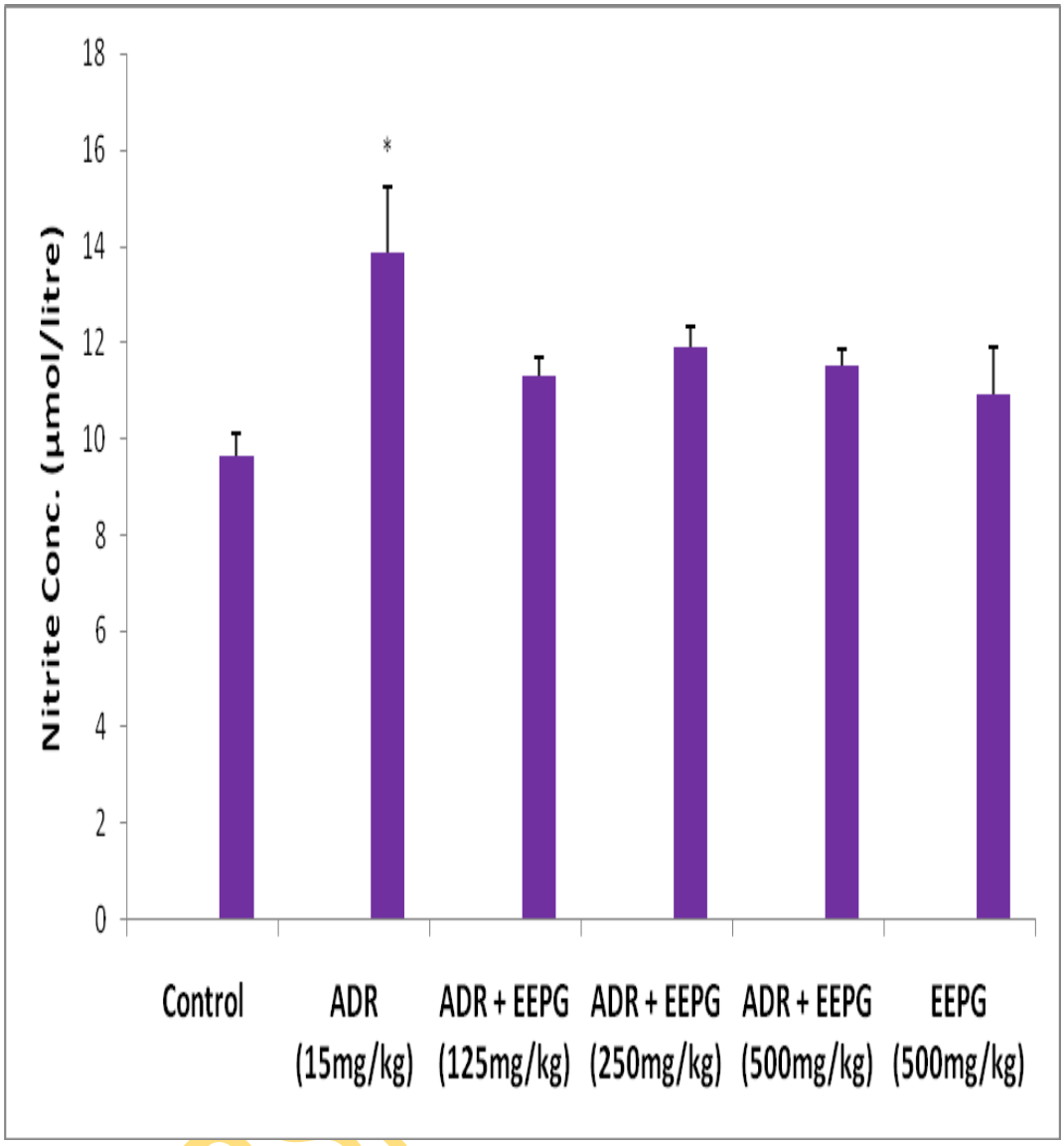


Figure 3B: Effect of ethanolic extract of *Psidium guajava* (EEPG) on nitrite concentration of normal and adriamycin (ADR)-treated rats.
*$p<0.01$ when compared with control.

Table 3A: Effect of pretreatment with ethanolic extract of *Psidium guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in reduced glutathione (GSH) level and glutathione-S-transferase (GST) activity

Treatment Group	GST activity (nmol/g tissue)	Renal GSH (µg/mg protein)
Control (saline)	0.50±0.09	1.25±0.15
ADR (2.5 mg/kg)	0.26±0.08$^{\#}$ (48.0)^{a}	0.74±0.15$^{\#}$ (40.80)^{a}
EEPG (125 mg/kg) + ADR (2.5 mg/kg)	0.55±0.07** (52.73)^{b}	1.07±0.28 (30.84)^{b}
EEPG (250 mg/kg) + ADR (2.5 mg/kg)	0.60±0.09** (56.67)^{b}	1.12±0.47 (33.93)^{b}
EEPG (500 mg/kg) + ADR (2.5 mg/kg)	0.42±0.07 (38.10)^{b}	1.34±0.08 (44.78)^{b}
EEPG (500 mg/kg)	0.44±0.02 (12.0)^{c}	1.35±0.18 (7.41)^{d}

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. $^{\#}p<0.05$ and $^{**}p<0.05$ significantly different from control and ADR-treated rats respectively. Values in parenthesis represent % change; $^{(a,c,d)}$ % change relative to control; $^{(b)}$ % change relative to ADR.

Table 3B: Effect of pretreatment with ethanolic extract of *Psidium guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in superoxide dismutase (SOD) and catalase (CAT) activities and H_2O_2 concentration.

Treatment Group	Catalase ($\mu moleH_2O_2$ consumed/min)	SOD (Units/mgprot.)	H_2O_2 generated ($\mu moleH_2O_2$ gener/min)
Control (saline)	0.008±0.002	0.510±0.13	4.10±0.31
ADR (15 mg/kg)	0.004±0.001$^{\#}$ (50.0)^{a}	0.395±0.08 (22.55)^{a}	7.27±0.82$^{\#}$ (43.60)^{e}
EEPG (125 mg/kg) + ADR (15 mg/kg)	0.009±0.002** (55.56%)^{b}	0.556±0.23 (28.96)^{b}	8.00±1.09 (9.13)^{f}
EEPG (250 mg/kg) + ADR (15 mg/kg)	0.005±0.001 (20.0)^{b}	0.479±0.11 (17.54)^{b}	5.17±1.15 (28.89)^{g}
EEPG (500 mg/kg) + ADR (15 mg/kg)	0.012±0.005 (66.67)^{b}	0.548±0.10 (27.92)^{b}	5.36±0.62 (26.27)^{g}
EEPG (500 mg/kg)	0.009±0.002 (11.11)^{c}	0.433±0.07* (15.10)^{d}	4.52±0.44 (9.29)^{e}

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. $^{\#}p<0.05$ and $^{**}p<0.05$ significantly different from control and ADR-treated rats respectively. Values in parenthesis represent % change; $^{(a,c,d,e)}$ % change relative to control; $^{(b,f,g)}$ % change relative to ADR.

Table 3C: Effect of pretreatment with ethanolic extract of *P.guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in vitamin C and malondialdehyde (MDA) levels

Treatment Group	Vitamin C (µg/g tissue)	MDA (µmol/mg protein)
Control (saline)	11.60±0.87	0.41±0.05
ADR (2.5 mg/kg)	10.35±0.66 (10.78)[a]	1.03±0.09[*] (60.19)[c]
EEPG (125 mg/kg) + ADR (2.5 mg/kg)	11.87±1.40 (12.81)[b]	0.87±0.18 (15.53)[d]
EEPG (250 mg/kg) + ADR (2.5 mg/kg)	12.63±0.97 (18.05)[b]	0.53±0.13[**] (48.54)[d]
EEPG (500 mg/kg) + ADR (2.5 mg/kg)	12.44±0.81 (16.80)[b]	0.56±0.10[**] (45.63)[d]
EEPG (500 mg/kg)	9.977±0.84 (13.99)[a]	0.43±0.07 (4.65)[c]

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. [*]Significantly different from control ($p<0.01$); [**]Significantly different from ADR-treated rats ($p<0.05$). Values in parenthesis represent % change; [(a,c)] % change relative to control; [(b,d)] % change relative to ADR.

Table 3D: Effect of pretreatment with ethanolic extract of *P.guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in 5' nucleotidase (5′NTD) and glucose-6-phosphatase (G6Pase) activities in rat kidney.

Treatment Group	5' nucleotidase (mmol/gtissue/min)	Glucose-6-phosphatase (mmol/gtissue/min)
Control (saline)	1.61±0.03	4.03±0.23
ADR (2.5 mg/kg)	1.37±0.05* (14.91)^{a}	2.86±0.26* (29.03)^{a}
EEPG (125 mg/kg) + ADR (2.5 mg/kg)	1.65±0.09** (16.97)^{b}	4.38±0.34** (34.70)^{b}
EEPG (250 mg/kg) + ADR (2.5 mg/kg)	1.66±0.04*** (17.47)^{b}	4.43±0.07** (36.79)^{b}
EPEG (500 mg/kg) + ADR (2.5 mg/kg)	1.53±0.12 (10.46)^{b}	4.13±0.12*** (30.75)^{b}
EEPG (500 mg/kg)	1.23±0.03$^{\#\#}$ (23.60)^{a}	3.04±0.06 (24.57)^{a}

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. *p<0.01 and $^{\#\#}$p<0.05 when compared with control; $^{\#}$p<0.05, **p<0.001 and ***p<0.01 when compared with ADR-treated rats. Values in parenthesis represent % change; $^{(a)}$ % change relative to control; $^{(b)}$ % change relative to ADR.

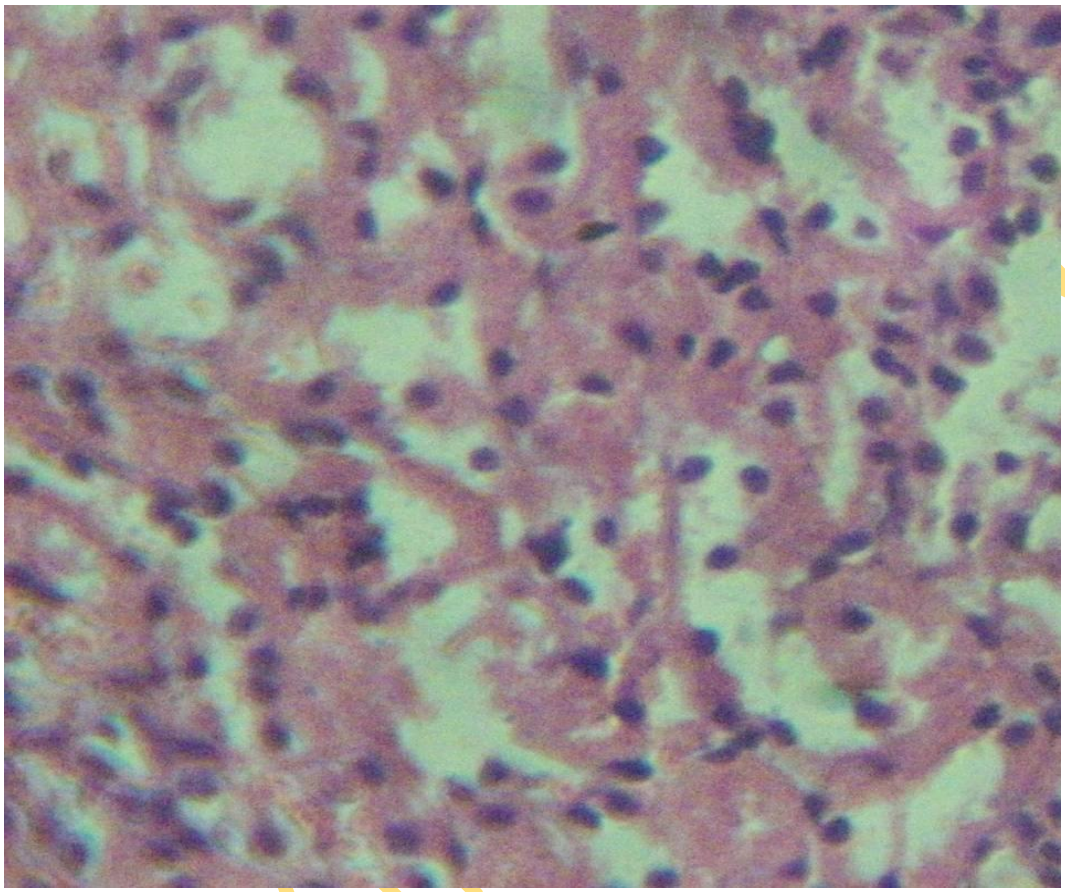


Figure 3C: Section of kidney from rat treated with normal saline (control). Renal morphology shows no visible lesion.

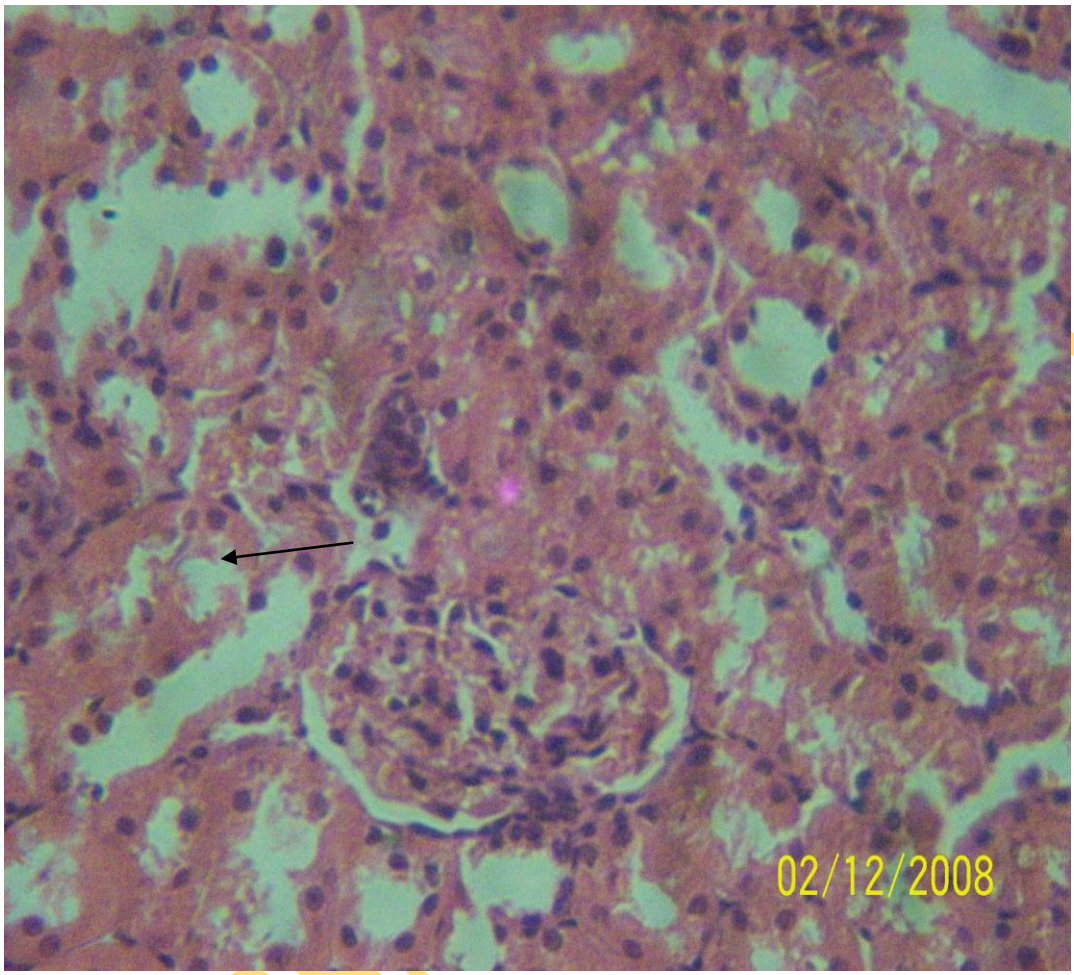


Figure 3D: Section of kidney from rat treated with adriamycin (2.5 mg/kg, i.p.) in 6 equal injections. Renal morphology shows tubular degeneration (black arrow).

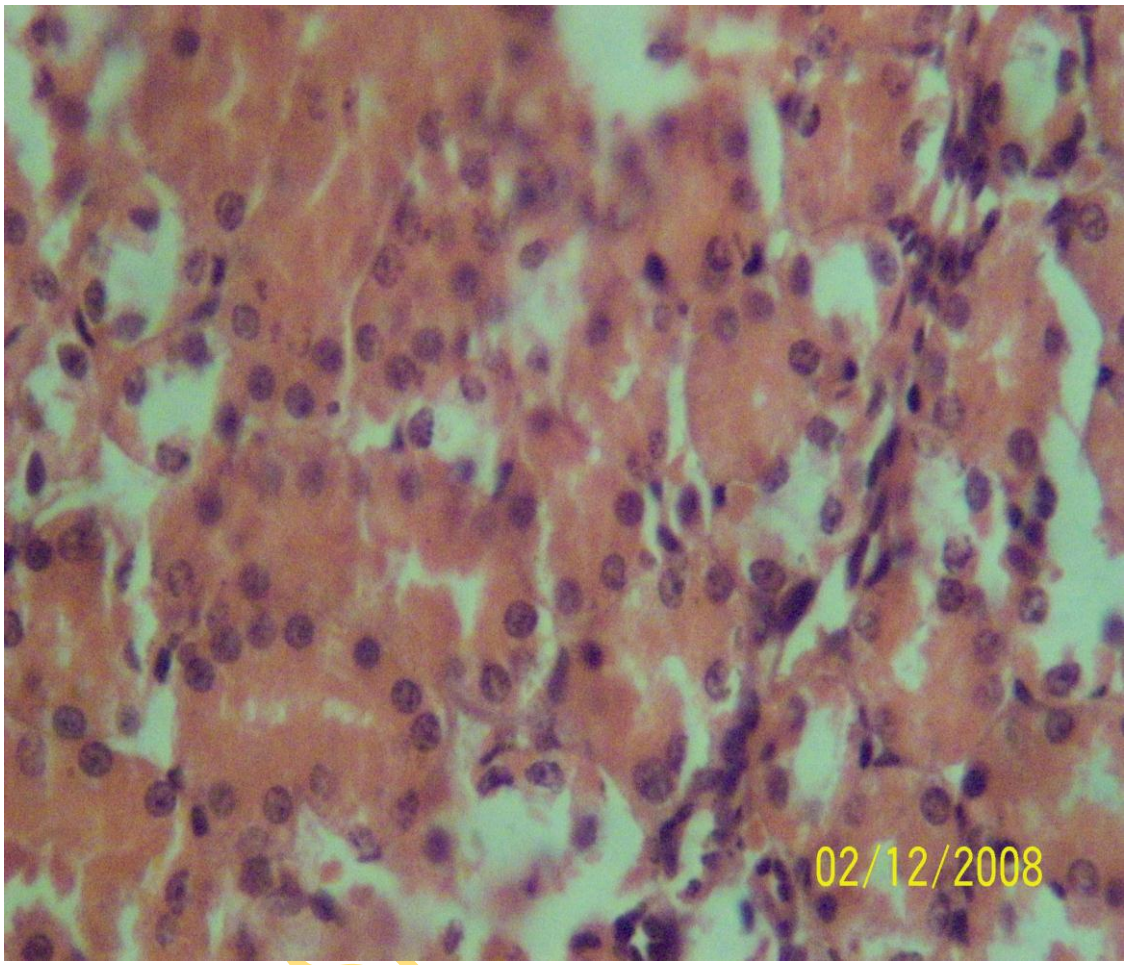


Figure 3E: Section of kidney from rat pretreated with ethanolic extract of *Psidium guajava* (125 mg/kg, p.o.) daily for 7 days before adriamycin injection. Section shows very mild interstitial infiltration by macrophages.

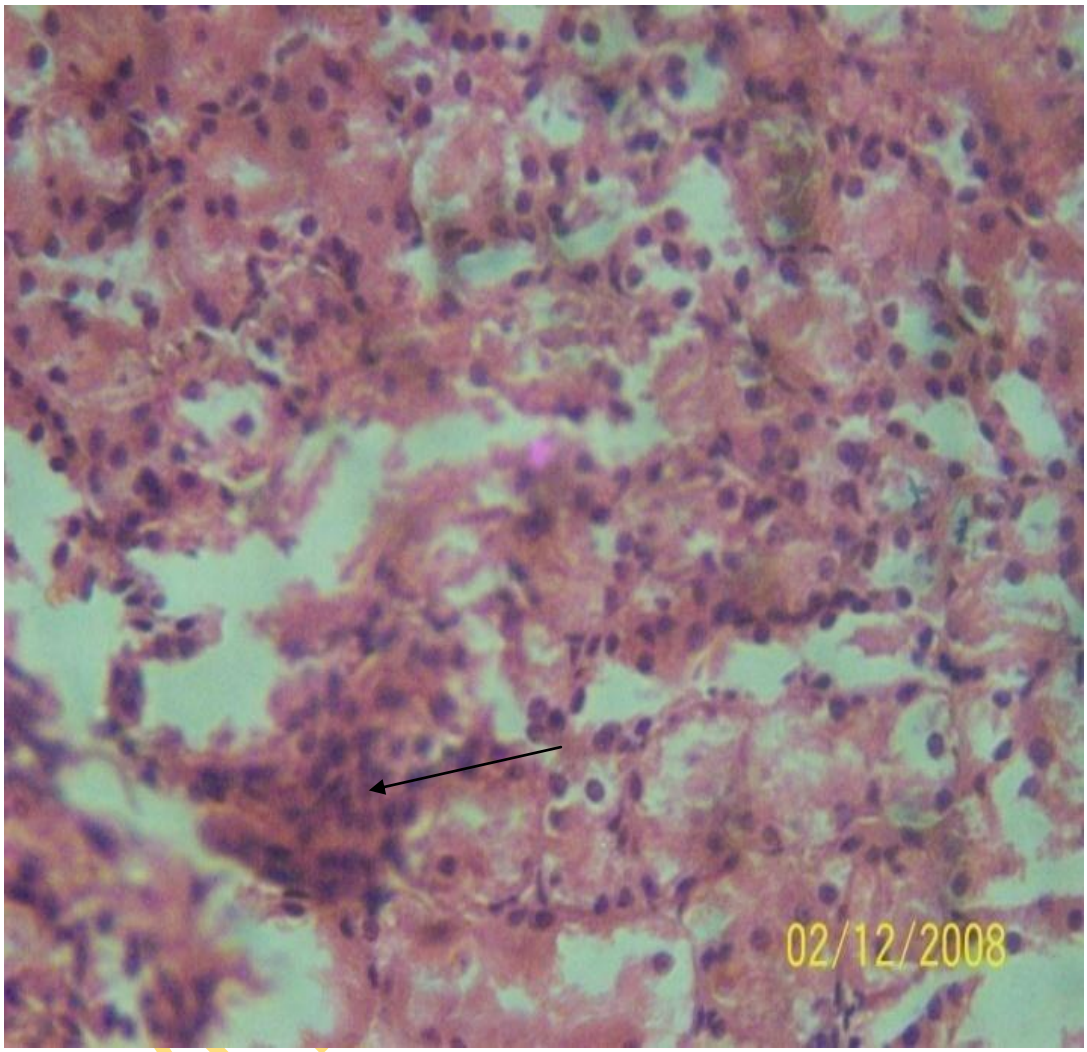


Figure 3F: Section of kidney from rat pretreated with ethanolic extract of *Psidium guajava* (250 mg/kg, p.o.) daily for 7 days plus adriamycin (2.5 mg/kg, i.p.). Section shows very mild hemorrhage (arrow).

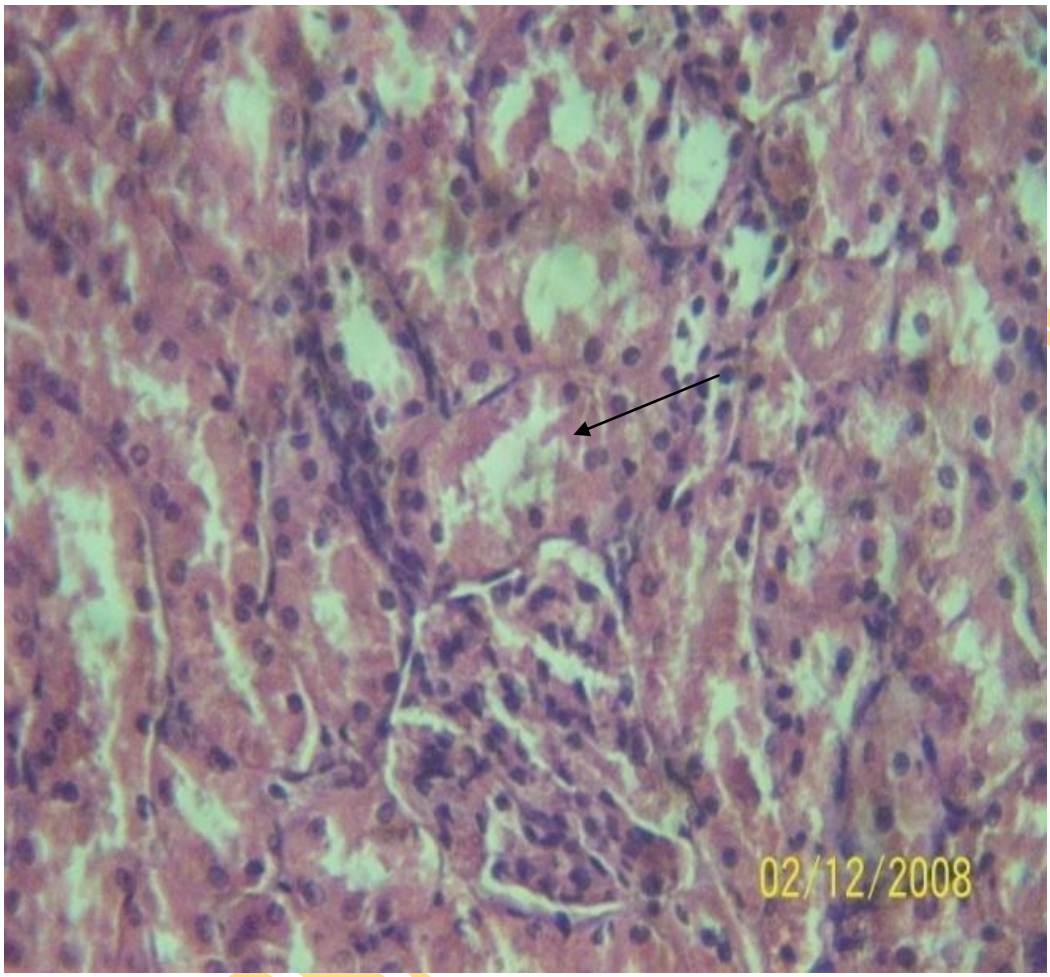


Figure 3G: Section of kidney from rat pretreated with ethanolic extract of *Psidium guajava* (500 mg/kg, *p.o.*) daily for 7 days plus adriamycin (2.5 mg/kg, i.p.). Section shows renal casts and cellular infiltration by macrophages.

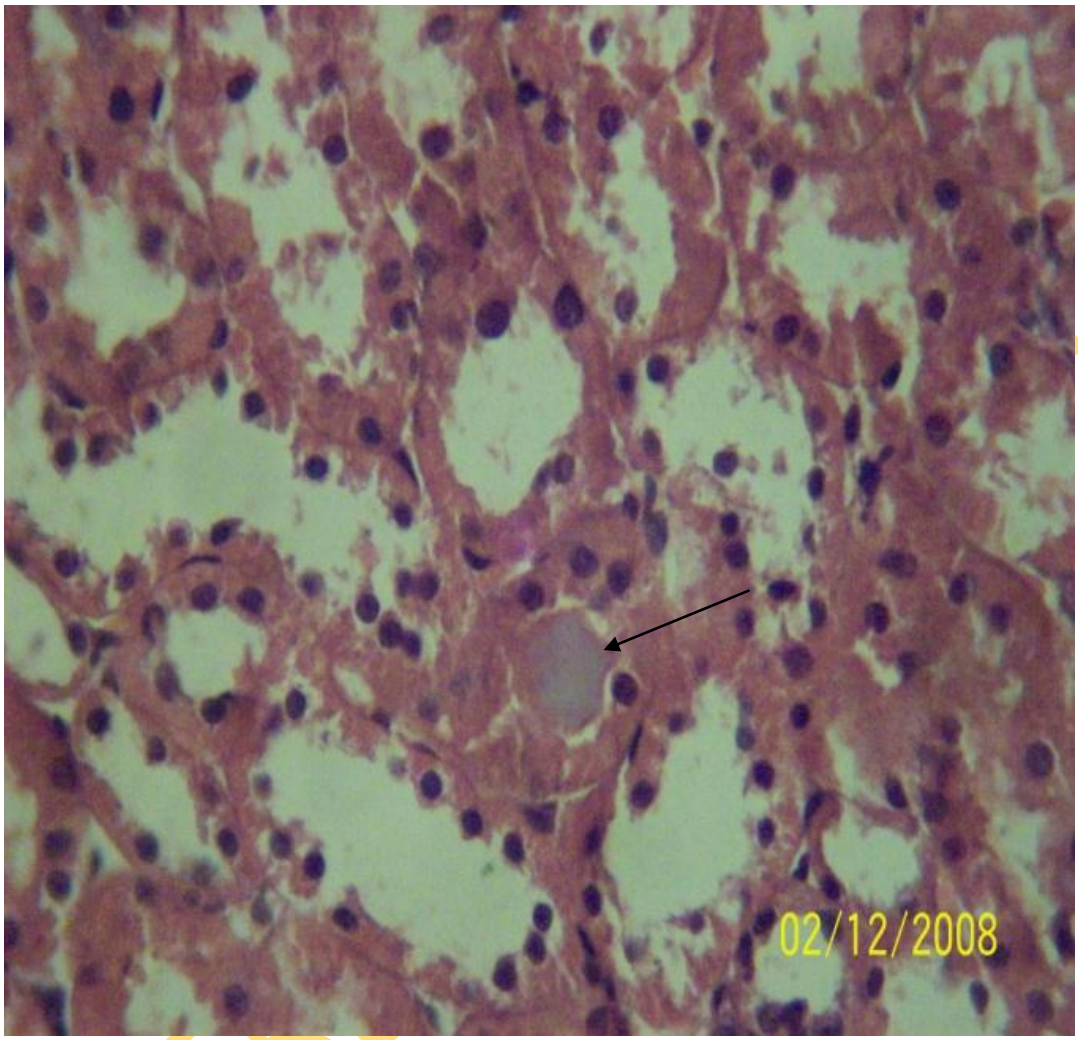
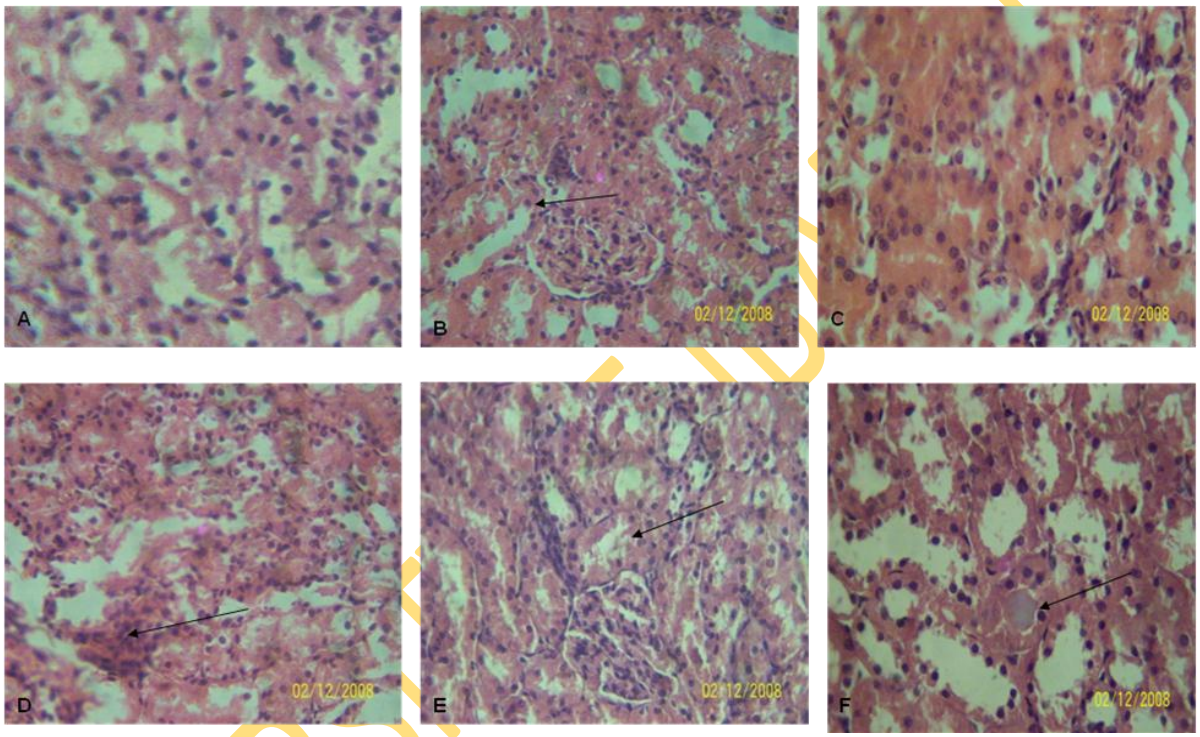


Figure 3H: Section of kidney from rat treated with 500 mg/kg oral dose of the ethanolic extract of *Psidium guajava* daily for 7 days. Section shows tubular necrosis near the renal capsule (black arrow) with mild cellular infiltration by macrophages.



Summary of Photomicrograph of rat kidney section (x400): A. Control (normal saline) rat kidney Section. B. Adriamycin-treated (2.5mg/kg) group showing tubular degeneration. C. Adriamycin (2.5mg/kg) plus guava extract (125mg/kg) treated group showing mild interstitial infiltration by macrophages. D. Adriamycin (2.5mg/kg) plus guava extract (250mg/kg) treated group showing very mild hemorrhage. E. Adriamycin (2.5mg/kg) plus guava extract (500mg/kg) treated group showing renal casts. F. Guava extract (500mg/kg) treated group showing tubular necrosis near the renal capsule.
→ Indicate some of the tubules showing necrosis, mild hemorrhage and renal casts.

Figures 3A and 3B show the changes in blood creatinine, blood urea nitrogen, and nitrate/nitrite concentration following exposure to repeated doses of adriamycin. There was a significant increase in the plasma concentrations of urea and creatinine by 54.8% and 45.0% respectively when compared with the saline (control) group. Plasma nitrate/nitrite level was significantly ($p<0.01$) increased in the adriamycin-treated rats when compared with the control group. Pretreatment with 125 mg/kg, 250 mg/kg and 500 mg/kg of EEPG for 7 days before adriamycin treatment produced a non-significant reduction in BUN and a significant reduction in creatinine concentration respectively. Pretreatment with 125 mg/kg, 250 mg/kg and 500 mg/kg of EEPG caused slight reductions in the plasma levels of nitrate/nitrite concentrations by 18.7%, 14.1% and 16.9% respectively when compared with the group treated with adriamycin alone. Administration of EEPG (500 mg/kg) alone did not cause any significant alteration in BUN, creatinine and nitrate/nitrite levels when compared with control.

Effect of EEPG on the activity of GST activity and GSH level for all experimental groups is shown in Table 3A. Adriamycin significantly ($p<0.05$) decreased GST activity and GSH level by 48.0% and 40.8% respectively when compared with the saline (control) group. Significant preservation of kidney GST activity was observed in the adriamycin-treated rats following pretreatment with 125- and 250- mg/kg doses of EEPG. The highest dose of EEPG (500mg/kg), on the other hand, did not significantly elevate GST activity in the adriamycin-treated rats when compared with the lower doses. Similarly, pretreatment with EEPG produced mild increases in GSH level at the various doses used in this study, but these were not statistically significant when compared with those in the adriamycin only group. The extract treatment alone at the highest dose (500 mg/kg) did not produce any significant change both in GSH level and GST activity when compared with control.

The effects of administration of adriamycin and guava extract on renal activities of catalase, superoxide dismutase (SOD) and hydrogen peroxide (H_2O_2) level are shown in Table 3B. The renal activities of catalase and SOD were reduced in rats that received adriamycin alone (2.5 mg/kg) relative to control. The reduction was only significant in the renal catalase ($p<0.05$). Renal hydrogen peroxide generation also increased significantly ($p<0.05$) by 43.6% in the adriamycin-treated rats when compared with control. Relative to rats challenged with adriamycin alone, pretreatment of rats with varied doses of EEPG prevented the inhibitory effect of adriamycin on renal activities of

catalase and SOD. EEPG at 250 mg/kg and 500 mg/kg doses reduced hydrogen peroxide generation by 28.9% and 26.3% respectively in the adriamycin-treated rats, though not statistically significant ($p>0.05$). Treatment with EEPG (500 mg/kg) had no significant effect on activities of catalase, SOD and hydrogen peroxide generated in the kidney ($p>0.05$).

The levels of renal vitamin C and MDA are presented in table 3C. Adriamycin administration produced mild decrease ($p>0.05$) in vitamin C concentration by 10.8%. Renal level of MDA (an empirical index of lipid peroxidation) was markedly ($p<0.01$) increased in rats given cumulative injection of adriamycin compared to the control. Pretreatment with EEPG at 125 mg/kg, 250 mg/kg and 500 mg/kg for 7 days increased renal levels of vitamin C by 12.8%, 18.1% and 16.8% respectively. However, EEPG pre-treatment for 7 days before adriamycin administration decreased MDA level compared to adriamycin-treated group. The preventive effect was significant ($p<0.05$) at 250 mg/kg and 500 mg/kg doses of the extract. The EEPG (500 mg/kg) alone did not significantly ($p>0.05$) alter both vitamin C and MDA levels when compared with control.

The activities of renal 5'-nucleotidase and glucose-6-phosphatase were significantly reduced following the administration of 6 equal injections of adriamycin (Table 3D). Treatment with EEPG at 125 mg/kg and 250 mg/kg exerted significant ($p<0.01$) increase in the activity of renal 5'-nucleotidase in the adriamycin-treated rats when compared with group that was treated with adriamycin alone. Also, EEPG at 125 mg/kg, 250 mg/kg and 500 mg/kg produced significant increase in glucose-6-phosphatase activity by 34.7%, 36.8% and 30.8% respectively in the adriamycin-treated rats. Treatment with EEPG (500 mg/kg) alone decreased 5' nucleotidase activity and glucose-6-phosphatase activity by 23.6% ($p<0.05$) and 24.6% ($p>0.05$) respectively in adriamycin-treated rats.

Histopathological examination of sections from rat kidney treated with adriamycin show tubular degeneration. Kidney sections from rats pretreated with 125-, 250- and 500- mg/kg doses of EEPG before administration of ADR showed mild interstitial infiltration by macrophages; very mild hemorrhage and renal casts with cellular infiltration by macrophages respectively. The 125- and 250 mg/kg doses of EEPG provided better histological protection against the renal tubular damage induced by adriamycin.

Experiment 3b: Effect of posttreatment with the ethanolic extract of *Psidium guajava* on nephrotoxicity induced by repeated doses of adriamycin

PROCEDURE

Six groups of five rats per group weighing between 200 and 245 g were studied in this experiment; Group I (control) received 0.9% NaCl; Group II was treated with cumulative dose of adriamycin (ADR) in 6 equal injections (2.5 mg/kg b.wt, i.p); Rats in groups III, IV and V received 6 equal injections of adriamycin after which they were then treated with 125, 250 and 500 mg/kg/day of EEPG for 5 days. Group VI was treated with EEPG (500 mg/kg) only. All treatments lasted for 15 days. Rats were sacrificed by cervical dislocation 48 hours after the last injection of adriamycin. Blood samples were collected via cardiac puncture and plasma separated by centrifugation at room temperature. Kidney samples were immediately removed, homogenized and centrifuged at 4^{o}C to separate the postmitochondrial fraction (PMF) as in experiment 1. Renal function was assessed in plasma. Biomarkers of oxidative stress, plasma membrane and tubular damage were evaluated in the PMF. A portion of the kidney was removed before homogenizing and processed for histology.

RESULTS

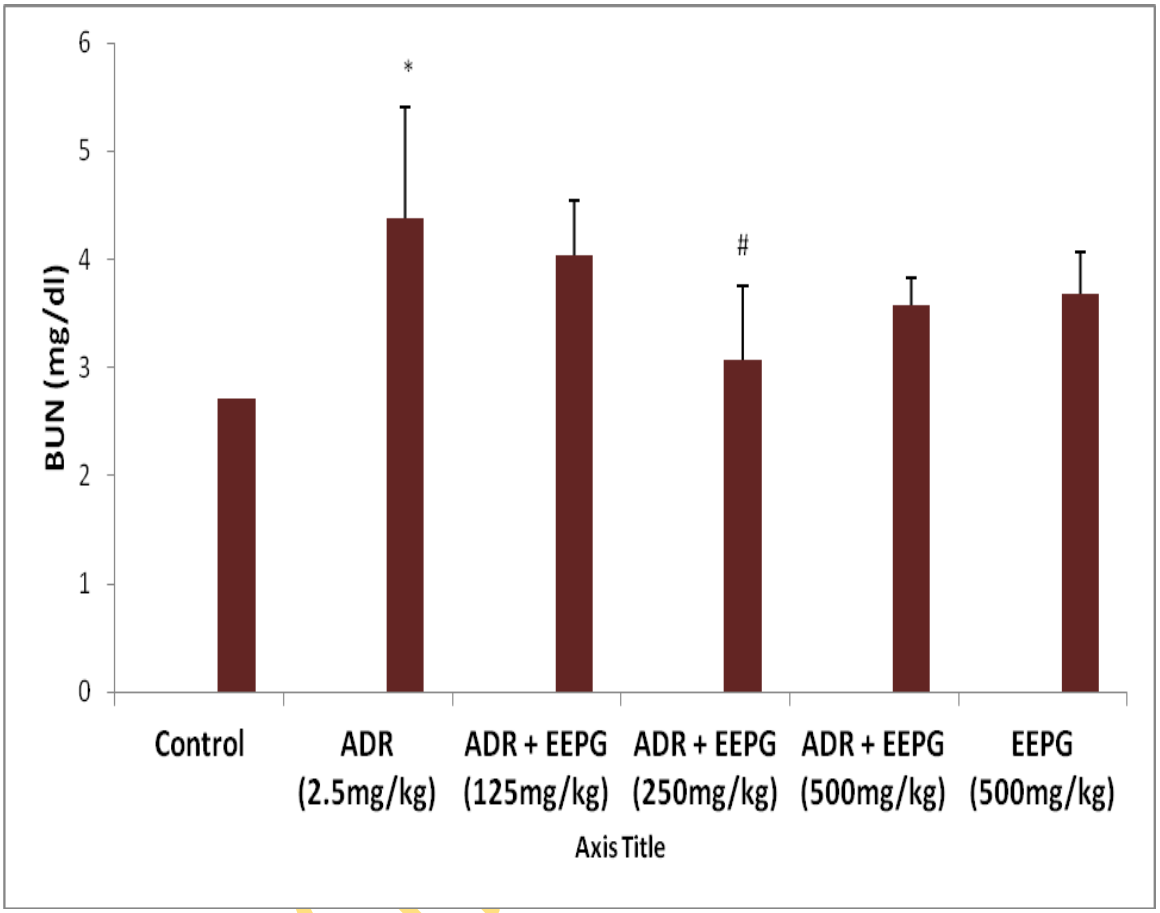


Figure 3I: Effect of posttreatment with ethanolic extract of *Psidium guajava* (EEPG) on changes in blood urea nitrogen (BUN) of normal and adriamycin (ADR)-treated rats.
$^{*}p<0.01$ and $^{\#}p<0.05$ when compared with control and adriamycin respectively.

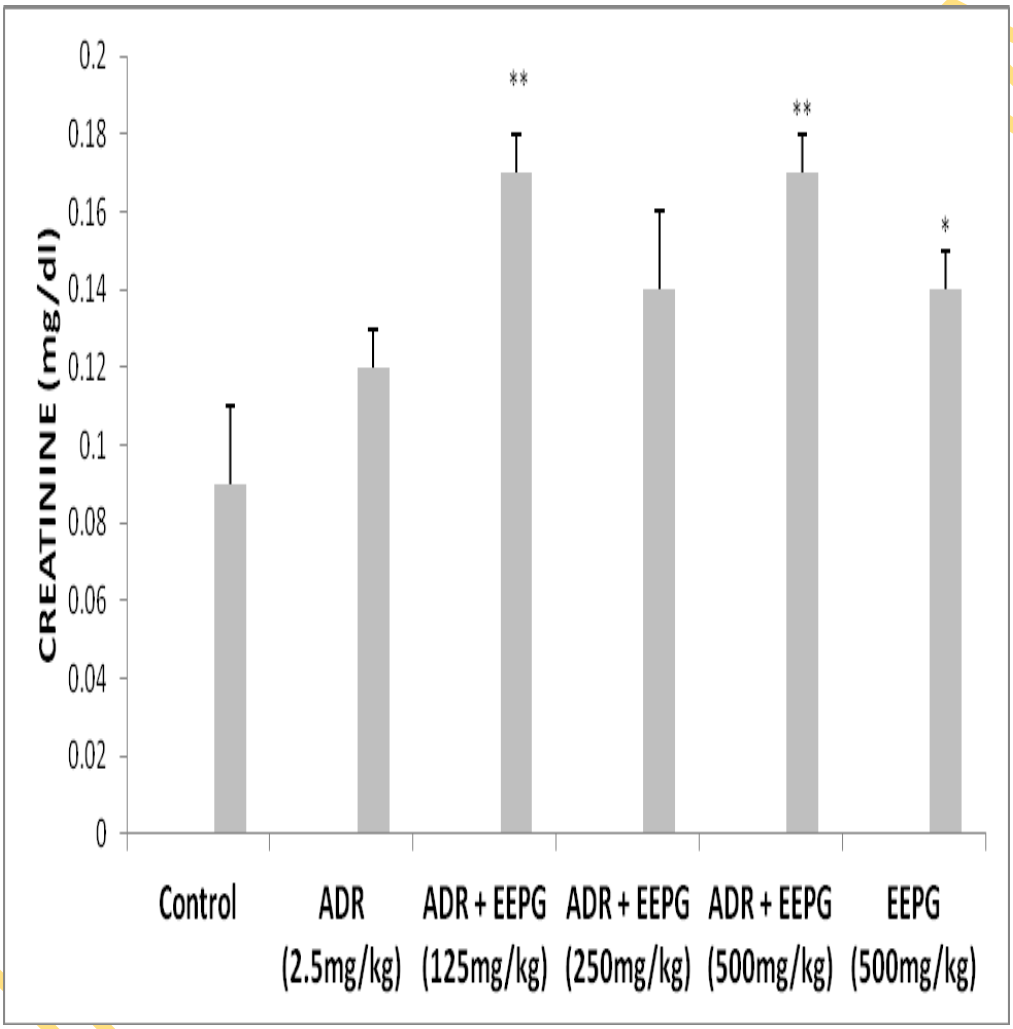


Figure 3J: Effect of posttreatment with ethanolic extract of *Psidium guajava* (EEPG) on changes in plasma creatinine of normal and adriamycin (ADR)-treated rats. $^{*}p<0.01$ and $^{**}p<0.001$ when compared with control and adriamycin respectively.

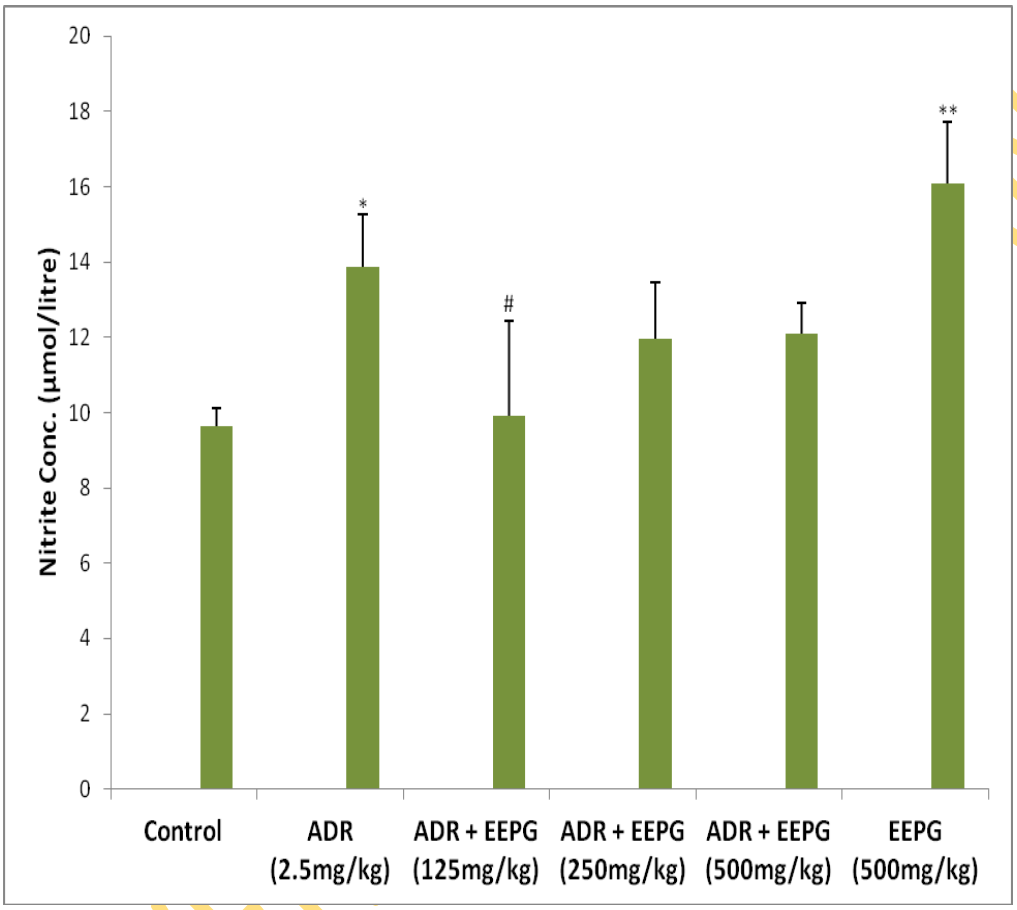


Figure 3K: Effect of posttreatment with ethanolic extract of *Psidium guajava* (EEPG) on nitrite concentration of normal and adriamycin (ADR)-treated rats.
$^{*}p<0.01$ and $^{**}p<0.001$ when compared with control; $^{\#}p<0.01$ when compared with adriamycin.

Table 3E: Effect of posttreatment with ethanolic extract of *Psidium guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in reduced glutathione (GSH) level and glutathione-S-transferase (GST) activity

Treatment group	GST (nmol/mg protein)	GSH (µg/g tissue)
Control (saline)	0.62±0.15	87.0±3.58
ADR (2.5 mg/kg)	0.48±0.07 (23.56)[a]	56.30±2.12[*] (35.29)[a]
EEPG (125 mg/kg) + ADR (2.5 mg/kg)	0.46±0.08 (4.19)[b]	38.64±3.82[**] (31.37)[b]
EEPG (250 mg/kg) + ADR (2.5 mg/kg)	0.51±0.03 (5.73)[c]	39.71±2.25[**] (29.47)[b]
EEPG (500 mg/kg) + ADR (2.5 mg/kg)	0.23±0.04 (51.99)[b]	41.82±2.02[**] (25.72)[b]
EEPG (500 mg/kg)	0.17±0.02[*] (72.12)[d]	47.52±1.67[*] (45.38)[d]

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. [*]Significantly different from control ($p<0.001$); [**]Significantly different from ADR-treated rats ($p<0.001$). Values in parenthesis represent % change; [(a,d)] % change relative to control; [(b,c)] % change relative to ADR.

Table 3F: Effect of posttreatment with ethanolic extract of *Psidium guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in activities of superoxide dismutase (SOD), catalase (CAT) and H_2O_2 concentration.

Treatment Group	Catalase ($\mu moleH_2O_2$ consumed/min)	SOD (Units/mg prot.)	H_2O_2 generated ($\mu moleH_2O_2$ gener/min)
Control (Saline)	0.028±0.02	8.324±1.7	6.715±1.22
ADR (2.5 mg/kg)	0.014±0.004 (50.0)[a]	4.702±1.18[*] (43.51)[a]	9.653±1.84 (30.44)[e]
EEPG (125 mg/kg) + ADR (2.5 mg/kg)	0.018±0.01 (22.22)[c]	3.751±0.50 (20.23)[b]	7.254±0.58 (24.85)[f]
EEPG (250 mg/kg) + ADR (2.5 mg/kg)	0.018±0.003 (22.2)[c]	4.128±0.85 (12.21)[b]	8.998±1.62 (6.79)[f]
EEPG (500 mg/kg) + ADR (2.5 mg/kg)	0.012±0.01 (14.29)[b]	2.956±0.63[#] (37.13)[b]	6.151±0.36[**] (36.28)[f]
EEPG (500 mg/kg)	0.010±0.003 (64.29)[d]	2.084±0.17[*] (74.96)[d]	5.090±0.32 (24.20)[g]

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. [*]Significantly different from control ($p<0.001$); [**]Significantly different from ADR-treated rats ($p<0.01$). Values in parenthesis represent % change; [(a,d,e,g)] % change relative to control; [(b,c,f)] % change relative to ADR.

Table 3G: Effect of posttreatment with ethanolic extract of *P.guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in vitamin C and malondialdehyde level (MDA) levels

Treatment Group	Vitamin C concentration (µg/g tissue)	MDA Level (µmol/mg protein)
Control	34.057±1.73	1.214±0.17
ADR (2.5 mg/kg)	19.841±3.01* (41.74)^{a}	2.08±0.01* (41.63)^{d}
EEPG (125 mg/kg) + ADR (2.5 mg/kg)	20.380±3.42 (2.64)^{b}	0.64±0.06** (69.23)^{e}
EEPG (250 mg/kg) + ADR (2.5 mg/kg)	21.103±1.62 (5.98)^{b}	0.93±0.27** (55.29)^{e}
EEPG (500 mg/kg) + ADR (2.5 mg/kg)	21.012±2.82 (5.57)^{b}	1.76±0.15 (15.38)^{e}
EEPG (500 mg/kg)	24.817±2.60$^{\#}$ (2.713)^{c}	1.24±0.20 (2.09)^{f}

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. $^{*}p<0.001$ and $^{\#}p<0.01$ when compared control; $^{**}p<0.001$ when compared with ADR-treated rats. Values in parenthesis represent % change; $^{(a,c,d,f)}$ % change relative to control; $^{(b,e)}$ % change relative to ADR.

Table 3H: Effect of posttreatment with ethanolic extract of *P.guajava* (EEPG) on normal and adriamycin (ADR) - induced changes in 5'nucleotidase (5′NTD) and glucose-6-phosphatase (G6Pase) activities in rat kidney

Treatment Group	5'Nucleotidase (µmol/g tissue/min)	Glucose-6-phosphatase (µmol/g tissue/min)
Control	30.815±1.11	5.014±0.28
ADR (2.5 mg/kg)	26.649±0.36* (13.52)^{a}	4.223±0.14* (15.78)^{a}
EEPG (125 mg/kg) + ADR (2.5 mg/kg)	25.469±1.24 (4.43)^{b}	4.214±0.23 (0.21)^{b}
EEPG (250 mg/kg) + ADR (2.5 mg/kg)	29.971±0.24$^{\#}$ (11.08)^{c}	4.316±0.09 (2.15)^{c}
EEPG (500 mg/kg) + ADR (2.5 mg/kg)	26.993±1.12$^{\#\#}$ (1.27)^{c}	4.22±0.20 (0.024)^{b}
EEPG (500 mg/kg)	29.607±1.39 (3.92)^{d}	4.685±0.27 (6.56)^{d}

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. Significantly different from control *($p<0.001$); Significantly different from ADR-treated rats ($^{\#}p<0.01$, $^{\#\#}p<0.001$). Values in parenthesis represent % change; $^{(a,d)}$ % change relative to control; $^{(b,c)}$ % change relative to ADR.

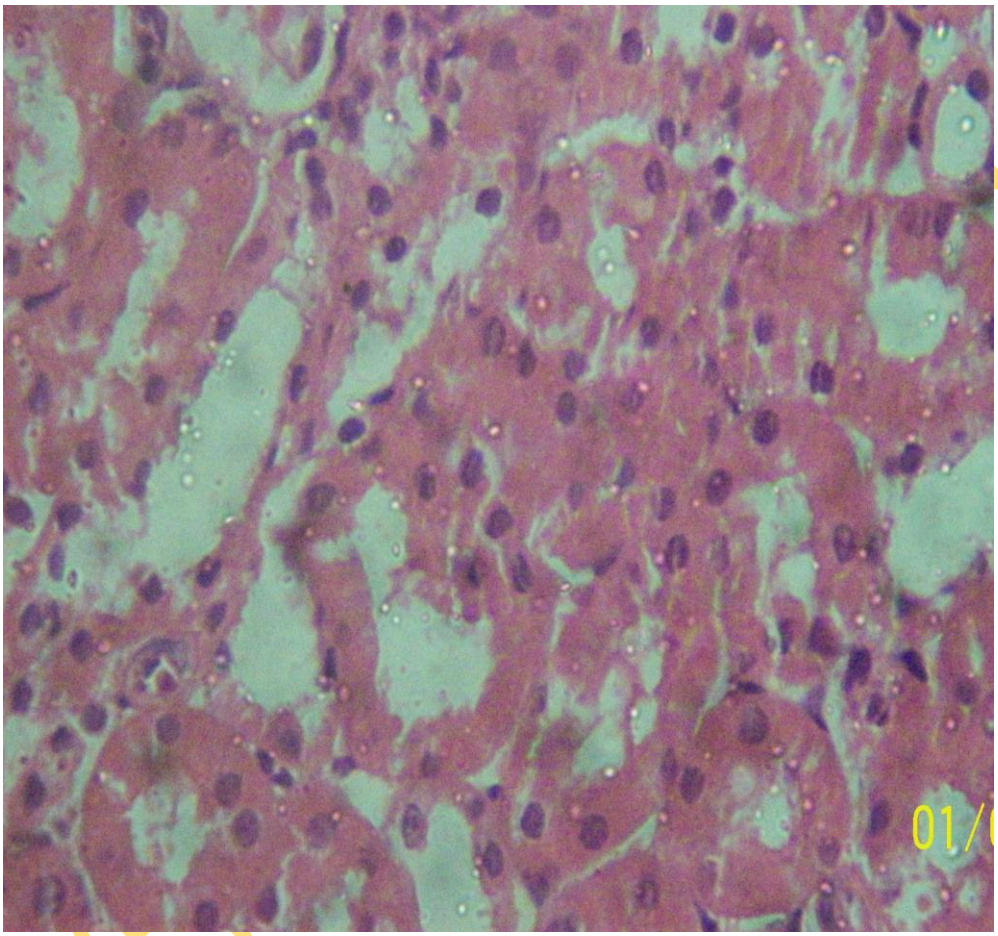


Figure 3L: Kidney section of control rats (normal saline). No visible lesions

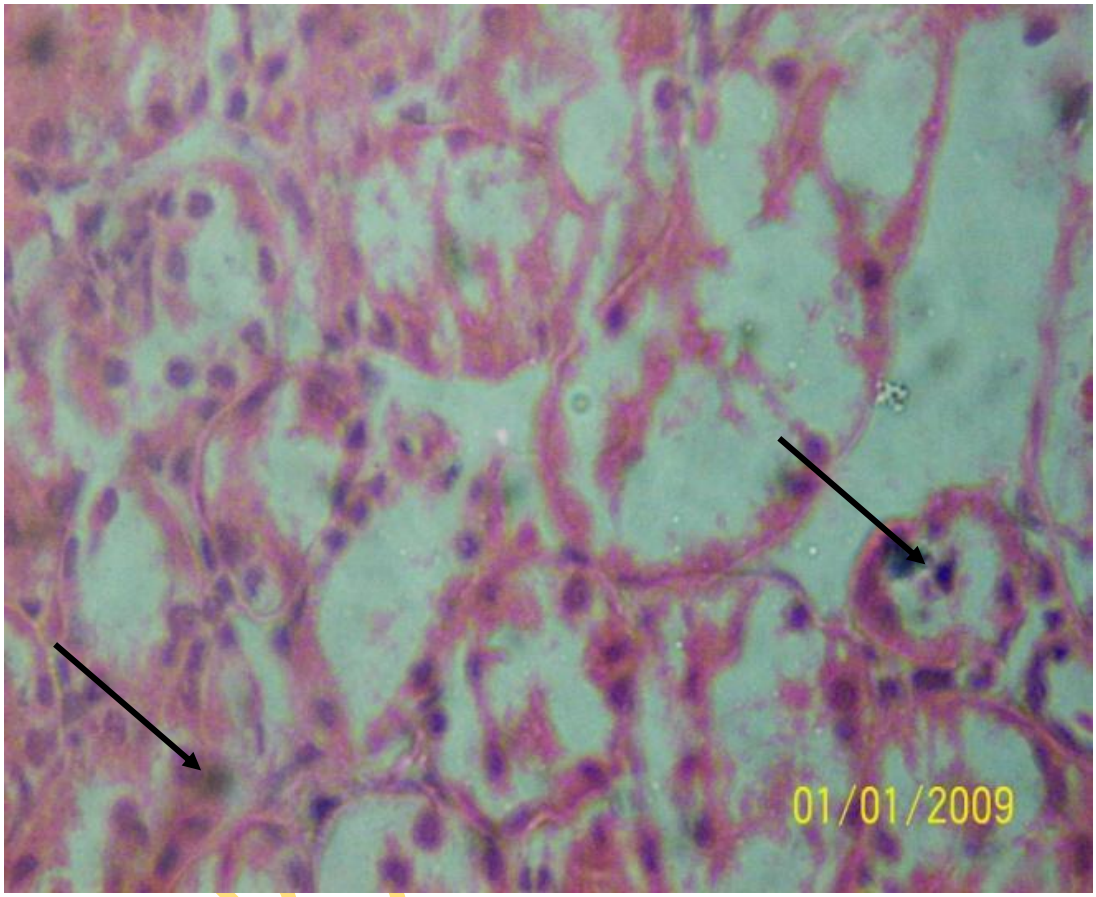


Figure 3M: Kidney section of rats treated with adriamycin (2.5mg/kg) showing renal tubular necrosis and congestion. Mild cellular infiltration by macrophages.

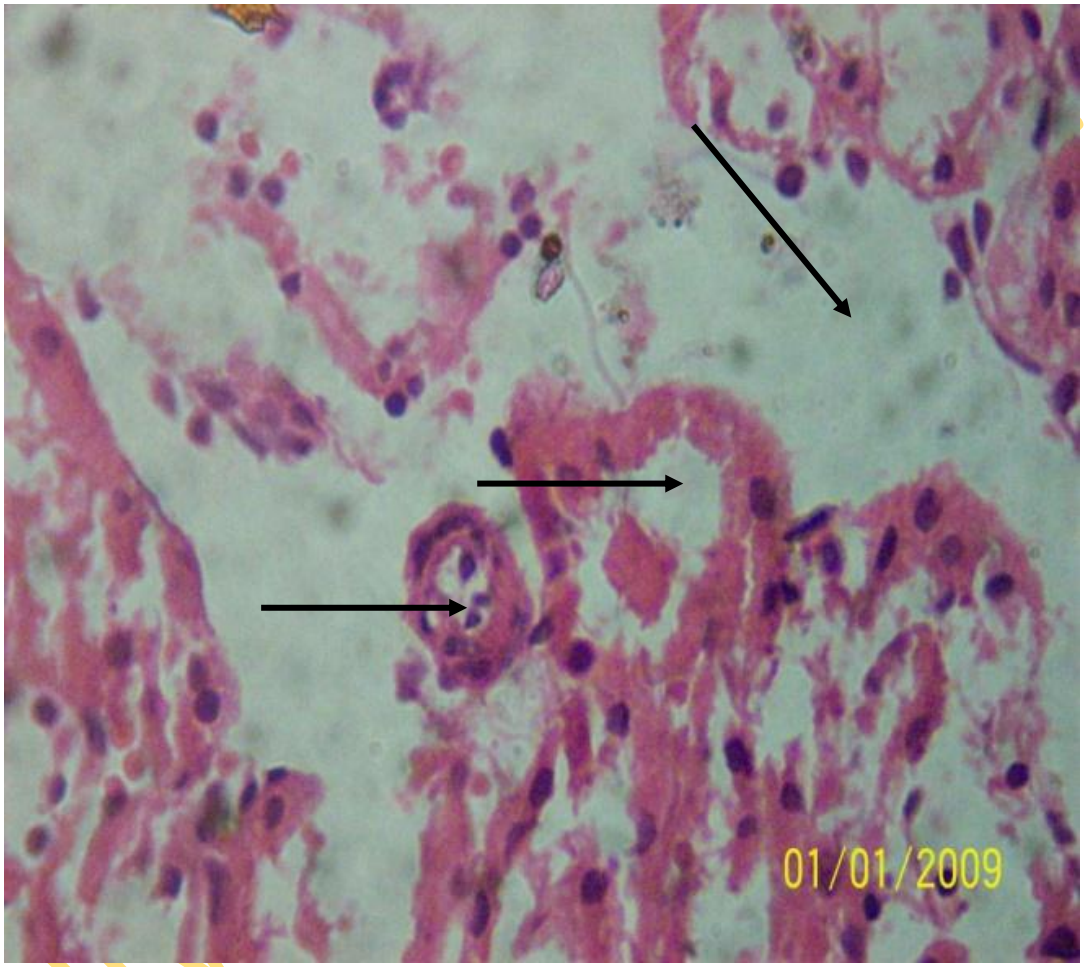


Figure 3N: Kidney section of rats posttreated with ethanolic extract of *Psidium guajava* (125 mg/kg) for 5 days after cumulative dose of adriamycin (2.5 mg/kg). Shows severe tubular necrosis and protein casts in tubular lumen.

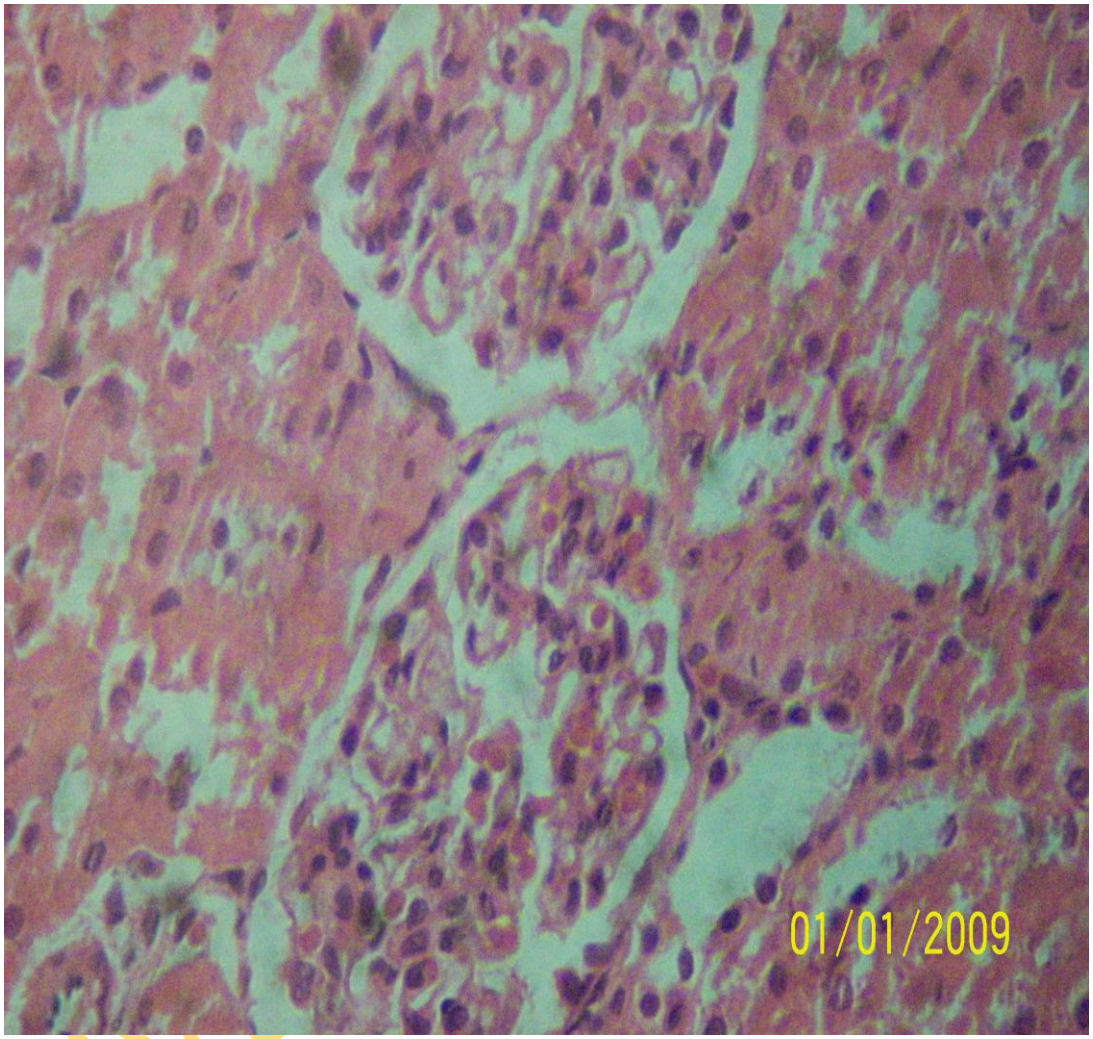


Figure 3O: Kidney section of rats posttreated with ethanolic extract of *Psidium guajava* (250 mg/kg) for 5 days after cumulative dose of adriamycin (2.5 mg/kg). No visible lesion

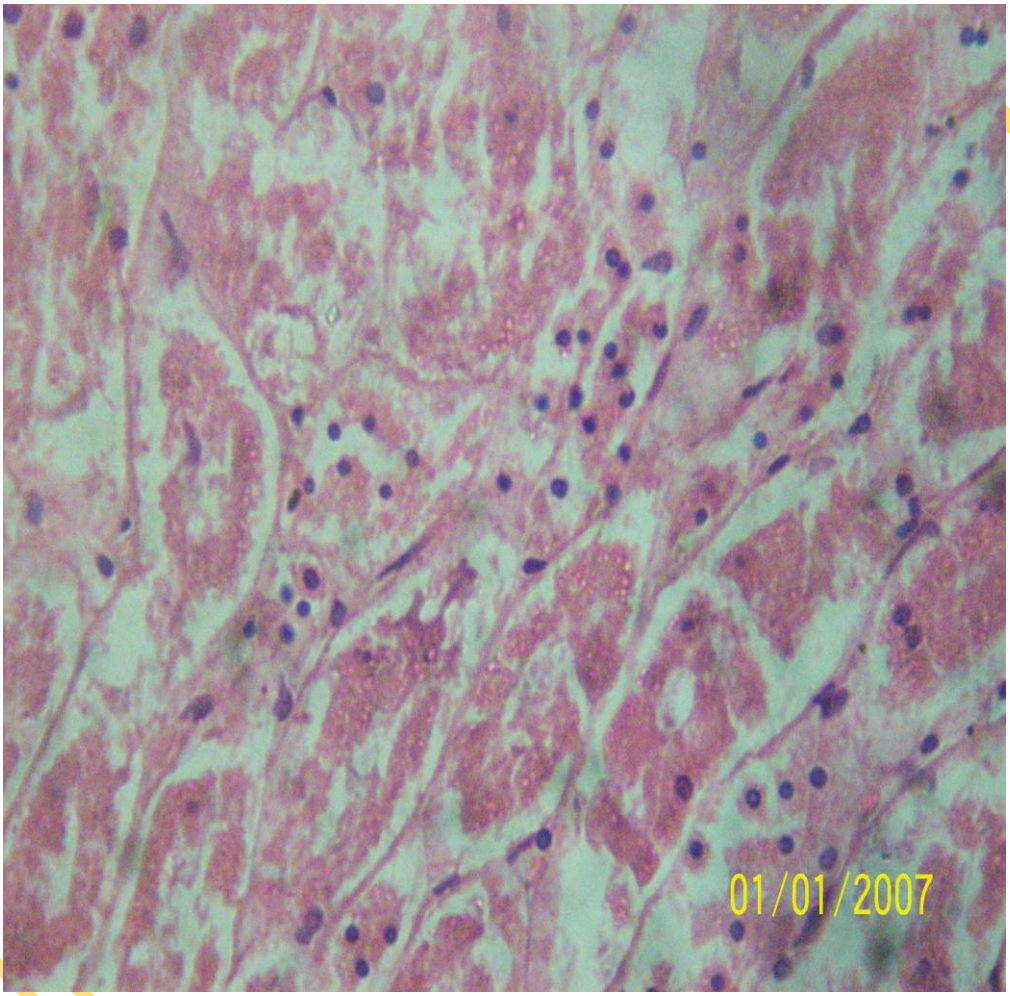


Figure 3P: Kidney section of rats posttreated with ethanolic extract of *Psidium guajava* (500 mg/kg) for 5 days after cumulative dose of adriamycin (2.5 mg/kg) showing mild tubular necrosis.

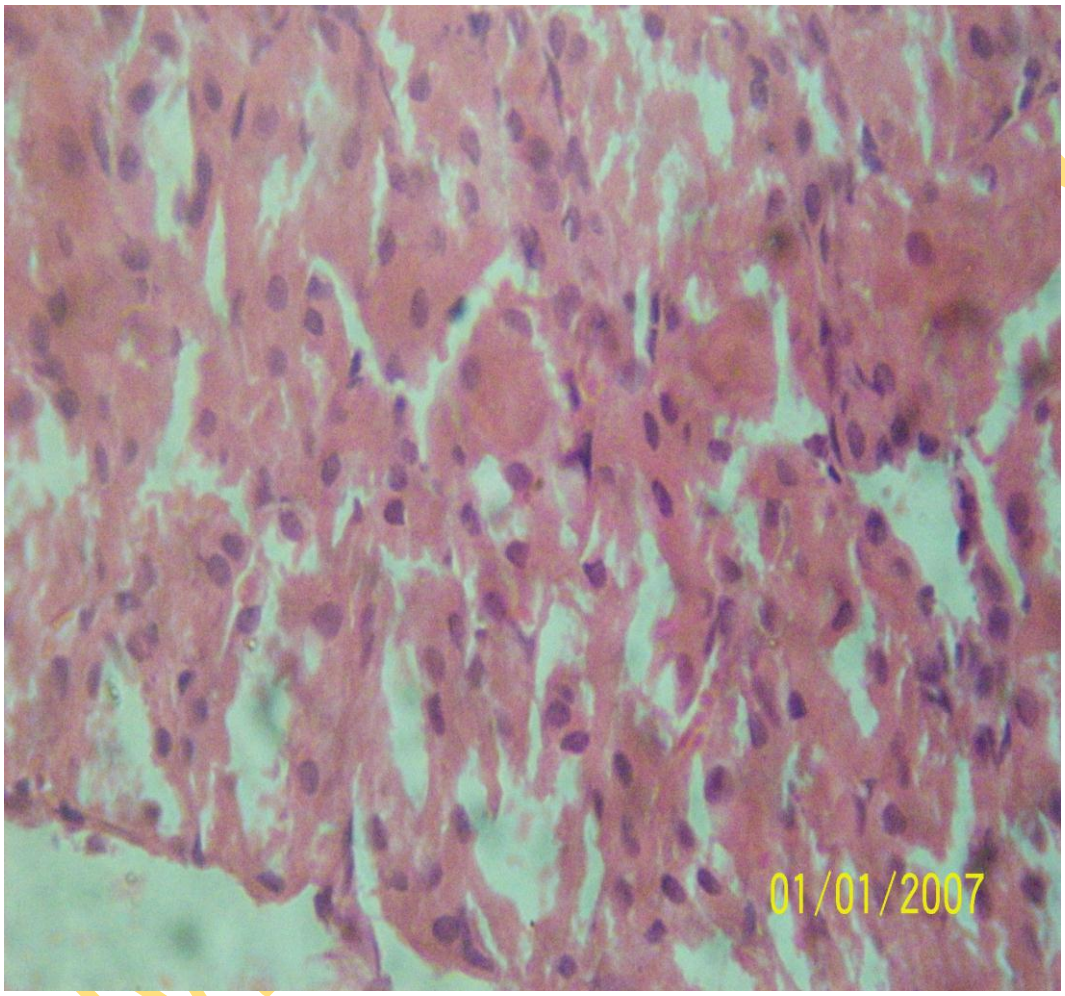
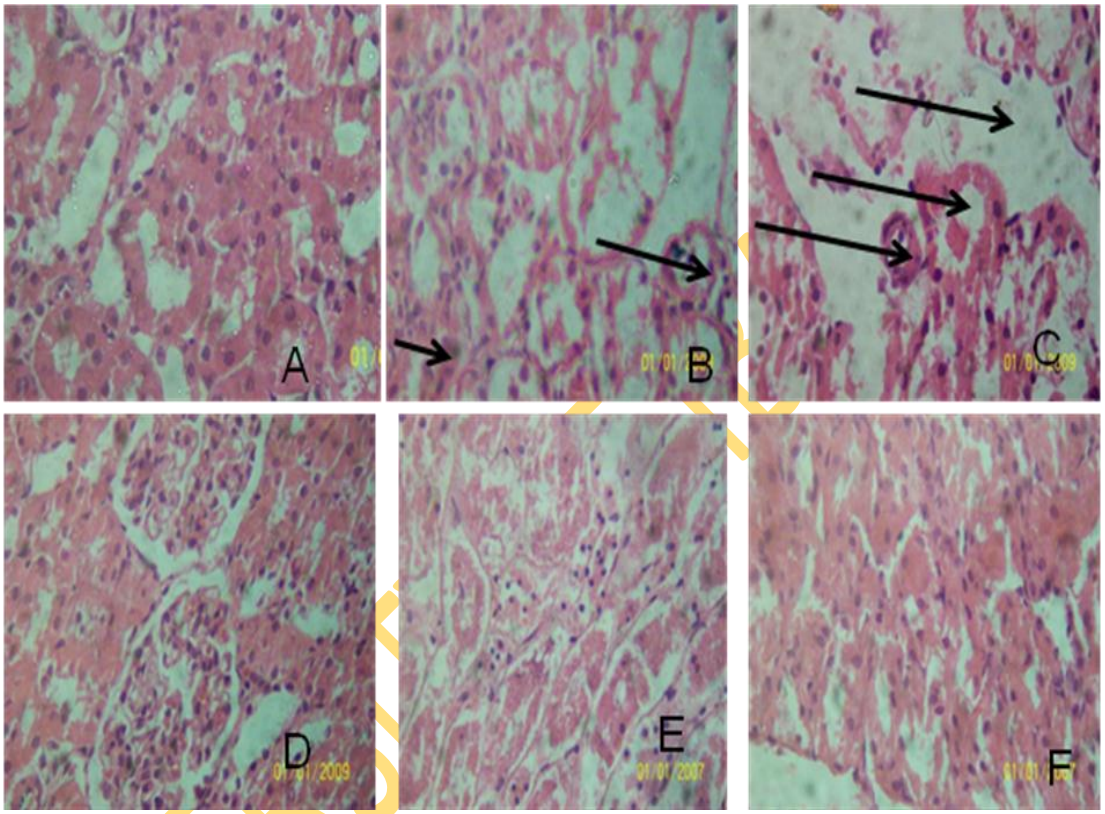


Figure 3Q: Kidney section (x400) of rats treated with ethanolic extract of *Psidium guajava* (500 mg/kg) only for 5 days. There is massive protein leakage, tubular casts, marked cellular infiltration by macrophages.



Summary of Photomicrograph of rat kidney section (x400): A. Control (normal saline), no visible lesions. B. Adriamycin-treated (2.5 mg/kg) group showing renal tubular necrosis. C. Adriamycin (2.5mg/kg) plus ethanolic extract of *Psidium guajava* (EEPG, 125 mg/kg) treated group showing severe tubular necrosis and protein casts in tubular lumen. D. Adriamycin (2.5 mg/kg) plus EEPG (250 mg/kg) treated group showing no visible lesion. E. Adriamycin (2.5 mg/kg) plus EEPG (500 mg/kg) treated group showing mild tubular necrosis. F. EEPG (500 mg/kg) treated group showing massive protein leakage.

Results for renal function tests are depicted in Figures 3I, 3J and 3K. Adriamycin (2.5 mg/kg) administered in 6 equal injections significantly elevated BUN and nitrate/nitrite concentrations by 38.2%, and 30.6% respectively when compared with control (saline-treated) rats ($p<0.01$). Plasma creatinine level was also increased by 22.3% ($p>0.05$) in the adriamycin-treated rats. Posttreatment with 125-, 250-, and 500-mg/kg/day doses of EEPG for 5 days after adriamycin injection (i.p.) reduced BUN by 7.7%, 29.9% and 18.5% and nitrate/nitrite concentration by 28.5%, 13.8% and 12.8% while it increased creatinine concentration by 29.2%, 11.7% and 28.4% respectively when compared with the ADR group. The reduction of these parameters produced by EEPG was statistically significant at 250 mg/kg for BUN, 125-, and 500 mg/kg for plasma creatinine and 125 mg/kg for nitrate/nitrite concentrations. The EEPG (500 mg/kg) when administered alone produced increase in these parameters when compared with control though only statistically significant for plasma creatinine and nitrite/nitrate concentrations.

Treatment with ADR decreased glutathione-S-transferase (GST) activity and also produced significant depletion of reduced glutathione (GSH) level ($p<0.001$) by 23.6% and 35.3% respectively as shown in Table 3E. EEPG administration led to a slight but non-significant increase in GST activity by 4.2%, 5.7% and 51.9% at 125-, 250- and 500- mg/kg doses respectively ($p>0.05$). The GSH level following guava extract administration was decreased by 31.4%, 29.5% and 25.7% at 125-, 250- and 500- mg/kg doses respectively. This decrease produced by EEPG in GSH was significant at all dose levels ($p<0.001$). GST activity and GSH level decreased significantly by 72.1% and 45.4% following only EEPG (500 mg/kg) administration.

Adriamycin renal toxicity was associated with induction of oxidative stress in the rats. Table 3F shows the effect of posttreatment of EEPG on catalase (CAT) and superoxide dismutase (SOD) activities and H_2O_2 generation in ADR-treated rats. CAT and SOD activities decreased by 50.0% and 43.5% respectively following treatment with ADR. This reduction in activity was significant in SOD ($p<0.001$). H_2O_2 generation following adriamycin cumulative injection was increased by 30.4%. EEPG caused non-significant increase in activity of catalase by 22.2% at 125- and 250mg/kg and 14.3% at 500 mg/kg. Following treatment with EEPG at 125- and 250- mg/kg, activity of SOD and H_2O_2 generation was decreased by 20.2%, 12.2% and 24.9%, 6.8% respectively. At 500 mg/kg of the extract, activity of SOD and H_2O_2 generation was significantly reduced by 37.1% and 36.4% respectively in the ADR-treated rats.

ADR-induced decrease in antioxidant defense system resulted in significant increase in renal lipid peroxidation (LPO) as there was increase in MDA level by 41.6% when compared with control as indicated in Table 3G. EEPG significantly reduced renal LPO by 69.2% and 55.3% at 125-, and 250- mg/kg respectively ($p<0.001$) and non-significantly by 15.4% at 500 mg/kg when compared with ADR-treated rats. EEPG when administered alone at 500 mg/kg, however, did not significantly ($p>0.05$) affect LPO level as only slight increase of 2.1% was recorded.

The vitamin C concentration in the kidney reduced significantly by 41.7% following adriamycin injection (Table 3G). Posttreatment with EEPG resulted in slight and non-significant increase in vitamin C concentration at all dose levels. EEPG (500 mg/kg), on the other hand, significantly reduced vitamin C level ($p<0.01$) when administered alone.

The effect of EEPG on ADR-induced decreases in renal glucose-6-phosphatase (G6Pase) and 5′-nucleotidase (5′-NTD) are shown in Table 3H. ADR significantly decreased G6Pase and 5′-NTD activities by 13.5% and 15.8% respectively when compared with control. EEPG at all dose levels did not produce any significant change in glucose-6-phosphatase activity when compared with the group that received cumulative dose of adriamycin. EEPG was most effective in reducing the effect of ADR on 5′-NTD activity at 250- and 500- mg/kg doses. EEPG (500 mg/kg) alone also decreased the activities of these enzymes, though not significantly.

Histopathological examination of sections from rat kidney treated with adriamycin show renal tubular necrosis and congestion. Kidney sections from rats posttreated with 125-, 250- and 500- mg/kg doses of EEPG for 5 days after adriamycin administration showed severe tubular necrosis and protein casts in tubular lumen; no visible lesions and mild tubular necrosis respectively. The 500 mg/kg dose of EEPG when administered alone produced massive protein leakage, tubular casts and marked cellular infiltration by macrophages.

CONCLUSION

The above studies showed that ethanolic extract of *Psidium guajava* may not be effective as a therapeutic agent since it exerted no beneficial effects in the posttreatment schedule.

EXPERIMENT 4: EVALUATION OF THE EFFECT OF ETHANOLIC EXTRACT OF *PSIDIUM GUAJAVA* ON CYP3A4 - MEDIATED DEALKYLATION OF 7-BENZOYLOXY-4-TRIFLUOROMETHYL COUMARIN.

INTRODUCTION

Cytochrome P450 (CYP) enzymes belong to heme-containing monooxygenases and are recognized to be responsible for drug metabolism, carcinogenesis and degradation of xenobiotics. Among the family of CYP enzymes, CYP3A4 is the most abundant enzyme in human liver microsomes and intestinal epithelium; ~30% of the total CYP was suggested to be CYP3A4 (Shimada *et al*., 1994), and >50% of clinically used drugs are oxidized by it (Guengerish, 1997). Doxorubicin and other drugs like cyclophosphamide, etoposide, and vincristine have been found to be metabolized by CYP3A4 (Baumhakel *et al*., 2001).

Metabolism of adriamycin by CYP3A4 yields adriamycinol (active) and aglycone (inactive) which are metabolites that result in free radical formation and toxicity. This would also result in reduction in the bioavailability of this drug (Benet and Cummins, 2001) and its effectiveness. Therefore, there is a need to circumvent this toxicity due to accumulation of toxic metabolites from adriamycin metabolism by CYP3A4. Since expression of CYP3A4 can be regulated via a number of different mechanisms ranging from transcriptional regulation to enzyme stability and inhibition, this study aimed at investigating the effect of EEPG on the activity of CYP3A4 as this will also shed more light on the mechanisms of action of EEPG.

PROCEDURE

Wistar albino rats weighing between 170 – 250 g were used for this study. Rats were divided into ten groups of five animals. Group 1 (control) received normal saline. Group II received repeated doses of adriamycin (ADR) in 6 equal injections (2.5 mg/kg b.wt, i.p). Adriamycin was concurrently administered with 125, 250 and 500 mg/kg of EEPG to rats in groups III, IV and V respectively. Ketoconazole (400 mg/kg) was concurrently administered with ADR to rats in group VI while those in groups VII, VIII and IX received 125, 250 and 500 mg/kg of EEPG only respectively. Group X received 400 mg/kg of ketoconazole only. Rats were sacrificed by cervical dislocation 24 hours after the last treatment. Kidneys were immediately removed, rinsed in ice-cold 1.15% KCl, blotted and weighed. The kidneys were then minced with scissors in 4 volumes of ice-cold potassium phosphate buffer (0.1M, pH 7.4) and homogenized in a potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 g for 20 minutes at 4^{O}C and the supernatant was recentrifuged at 100,000 *g* for 1 hour to get the microsomes which were resuspended in 0.25 M sucrose solution. Aliquots of this suspension were stored at -20^{O}C and thawed before use.

RESULTS

Table 4A: Percentage inhibition of CYP3A4-mediated dealkylation of 7-benzoyloxy-4 trifluoromethyl coumarin by ethanolic extract of *Psidium guajava* in adriamycin-treated rats

Group	Absorbance	% inhibition
Control	9.2±0.38	-
ADR (2.5mg/kg)	9.0±0.13	3.1
EEPG (125 mg/kg)	8.4±0.14	8.7
EEPG (250 mg/kg)	8.2±0.13	13.5
EEPG (500 mg/kg)	7.8±0.17	15.6
KET (400 mg/kg)	7.9±0.13	14.5
ADR + EEPG (125 mg/kg)	8.9±0.27	2.9
ADR + EEPG (250 mg/kg)	7.9±0.3**	14.5
ADR + EEPG (500 mg/kg)	8.2±0.27*	13.5
ADR + KET (400 mg/kg)	7.9±0.17**	14.5

Values expressed as mean ± standard error of mean (SEM) for five rats in each group.
*p<0.01 and **p<0.001 when compared with adriamycin.
KET: Ketoconazole; ethanolic extract of *Psidium guajava* (EEPG).

Results for CYP3A4 inhibition by ethanolic extract of *Psidium guajava* is depicted in Table 4A. In Table 4A, administration of the extract alone at 125-, 250- and 500- mg/kg inhibited CYP3A4 activity by 8.7%, 13.5% and 15.6% respectively. Ketoconazole (400 mg/kg) also inhibited CYP3A4 activity by 14.5%. All these effects, however, were not significantly ($p>0.05$) different from those of the control.

The activity of CYP3A4 in rats in which EEPG was concurrently administered with adriamycin (15 mg/kg) was significantly ($p<0.01$) inhibited by 14.5% and 13.5% at 250 mg/kg and 500 mg/kg respectively. The inhibitory effect of EEPG (250 mg/kg) was comparable with that of ketoconazole (400 mg/kg) which also significantly ($p<0.001$) inhibited the enzyme by 14.5% when concurrently administered with adriamycin. The lowest dose of the guava extract i.e 125 mg/kg, when concurrently administered with ADR did not significantly ($p>0.05$) alter the effect of CYP3A4 when compared with rats that received ADR only.

CONCLUSION

EEPG inhibited the activity of CYP3A4. This result suggests that there may be presence of an active component in EEPG contributing to this inhibitory effect.

EXPERIMENT 5: GENOTOXICITY INDUCED BY ADRIAMYCIN AND THE EFFECT OF ETHANOLIC EXTRACT OF *PSIDIUM GUAJAVA*

INTRODUCTION

Testing chemicals for the ability to induce numerical or structural chromosomal damage is easily accomplished by using the micronucleus assay. A "micronucleus" is literally a small nucleus. The nucleus is the organelle in the cell that contains the genetic material (DNA) that directs normal cellular function and cellular reproduction. In cells of eukaryotic organisms, the nucleus contains DNA packaged into chromosomes. Chromosome shape, size, and number are constant for species. During cell division, the genetic material replicates and then divides equally between the two daughter cells that are produced. If the process is disrupted, or the chromosomes are broken or damaged by chemicals or radiation, then the distribution of genetic material between the two daughter nuclei during cell division may be affected and pieces or entire chromosomes may fail to be included in either of the two daughter nuclei. When this occurs, the genetic material that is not incorporated into a new nucleus may form its own "micronucleus" which is clearly visible with a microscope. Thus, in the micronucleus test, animals are treated with a chemical and then the frequency of micronucleated cells is determined at some specified time after treatment. If a treated group of animals shows significantly higher frequencies of micronucleated cells than do the untreated control animals, then the chemical is considered to be capable of inducing structural and/or numerical chromosomal damage. Erythrocytes (red blood cells) are the cells that are scored in the bone marrow or the blood for presence of micronuclei.

Adriamycin is an anthracycline antibiotic used as an antitumor agent against human malignancies but which also has a wide variety of toxic side-effects, including cardiotoxicity, cytotoxicity and the induction of chromosomal aberrations. The protective effect of the ethanolic extract of *Psidium guajava* against genotoxicity induced by adriamycin was evaluated in this present study.

PROCEDURE

Thirty male Wistar rats (aged 10 - 12 weeks) with body weight ranging between 80 – 120 g were used for this study. Rats were divided into six groups of five animals per group. Group 1 (control) received normal saline; Group II received single intraperitoneal injection of adriamycin (ADR, 20 mg/kg b.wt). Groups III, IV and V received 125, 250 and 500 mg/kg/day of EEPG *p.o.* respectively for 7 days before

adriamycin injection. Group VI was treated with 500 mg/kg/day of EEPG only. Rats were sacrificed 24 hours after ADR injection. Bone marrow was flushed from both femurs of each rat using fetal calf serum and spread onto slides. Slides were coded and then air-dried, fixed with methanol and stained with maygrunward stain. Bone marrow cells were then examined microscopically and scored per animal for frequency of micronucleated polychromatic cells in each of five animals per dose group.

RESULTS

Table 5A: Genotoxicity induced by adriamycin and the effect of ethanolic extract of *Psidium guajava*

Treatment Group	No of micronucleated cells/1000 polychromatic erythrocyte cells
Control	1.15±0.05
ADR (20 mg/kg)	19.25±0.48*
ADR +EEPG (125 mg/kg)	11.80±0.92#
ADR+EEPG (250 mg/kg)	8.80±0.49#
ADR+EEPG (500 mg/kg)	3.40±0.40#
EEPG only (500 mg/kg)	1.17±0.17

Values expressed as mean ± standard error of mean (SEM). *Significantly different from control ($p<0.001$); #Significantly different from ADR-treated rats ($p<0.001$).

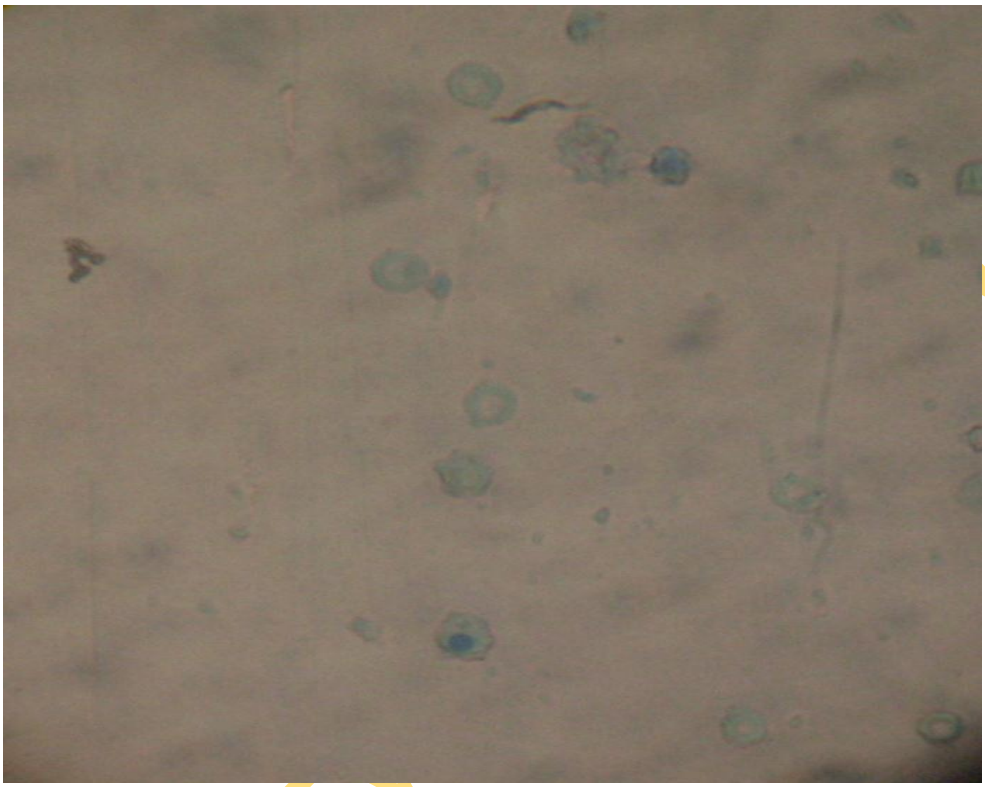


Figure 5A: Maygrunward stained slide showing the occurrence of micronucleated cells in the erythrocyte

The data presented in table 5A showed that animals treated with adriamycin (20 mg/kg) exhibited a significantly ($^{*}p<0.001$) high frequency (19.25 ± 0.48) of micronucleated polychromatic erythrocytes in bone marrow cells as compared with control (1.15 ± 0.05). Dose-related decrease in the number of micronucleated cells was observed in rats pretreated with ethanolic extract of *Psidium guajava* (EEPG) for 7 days. Treatment of rats with EEPG (500 mg/kg) alone did not induce any significant variation in the incidence of micronucleated polychromatic erythrocytes (1.17 ± 0.17) when compared with control (1.15 ± 0.05).

CONCLUSION

The significant increase in micronuclei formation in adriamycin-treated rats observed in this study corroborates previously reported DNA damage associated with adriamycin toxicity. Guava (EEPG) treatment decreased the frequency of micronucleated cells in adriamycin-treated rats. These results demonstrate the efficacy of EEPG as a protective agent against adriamycin-induced genotoxicity.

EXPERIMENT 6: ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF THE ETHANOLIC EXTRACT OF *PSIDIUM GUAJAVA IN VITRO*

INTRODUCTION

Plants produce a higher number of naturally occurring secondary metabolites, many of them with unique pharmacologic activities. These metabolites include the flavonoids, phenols and phenolic glycosides, saponins, cyanogenic glycosides, unsaturated lactones and glucosinolates (Podolak *et al*., 2007; Kappel *et al*., 2008). Phytochemicals, especially plant phenolics constitute a major group of compounds that act as primary antioxidants (Hatano *et al*., 1989). They can react with active oxygen radicals, such as hydroxyl radicals (Hussain *et al*., 1987), superoxide anion radicals (Afanaslev *et al*., 1989) and lipid peroxy radicals (Torel *et al*., 1986), and inhibit lipid oxidation at an early stage. In the past, herbs often represented the original sources of most drugs and herbal remedies, but nowadays, alternative medicines are used widely in all over the world (Cooper, 2004 and Cooper, 2005). Herbal-derived remedies need a powerful and deep assessment of their pharmacological qualities (Fabio and Luigi, 2007).

With increasing recognition of herbal medicine as an alternative form of health care, screening of medicinal plants for biologically active compounds has become an important source of antibiotic prototypes and cancer-related drugs (Koduru *et al*., 2006). Hence, for selecting crude plant extract with potential useful properties, *in vitro* screening methods have been used for further in-depth chemical elucidation and pharmacological investigations (Mathekaga and Meyer, 1998). In this study, the antioxidant and free radical scavenging activities of EEPG was evaluated *in vitro*.

PROCEDURE

The antioxidant activity of EEPG was investigated using the trolox equivalent antioxidant capacity (TEAC), reducing power (RP) and AAPH-induced lipid peroxidation. The radical scavenging activity (RSA) of EEPG against chemically generated hydroxyl ($OH^{\cdot}$) radical in the deoxyribose assay, hydrogen peroxide, 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* was also evaluated.

RESULTS

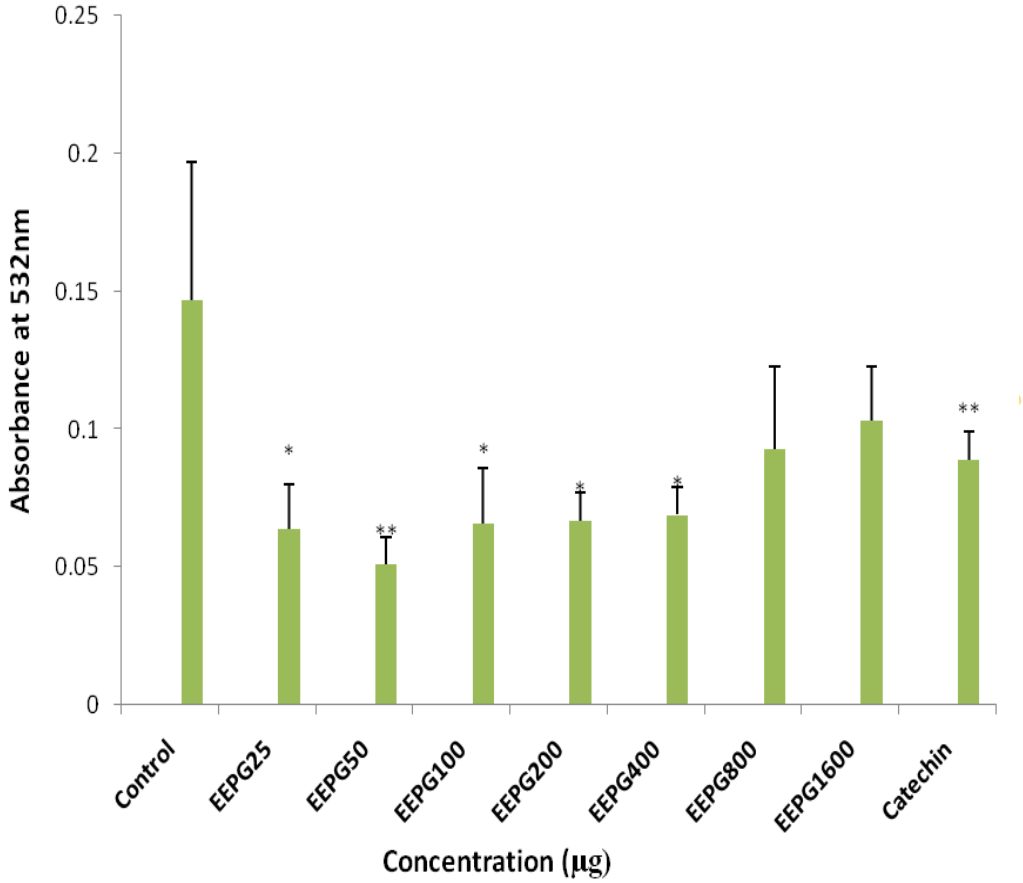


Figure 6A: Effect of ethanolic extract of *Psidium guajava* on AAPH-induced lipid peroxidation.
$^{*}p<0.05$ and $^{**}p<0.01$ when compared with control.

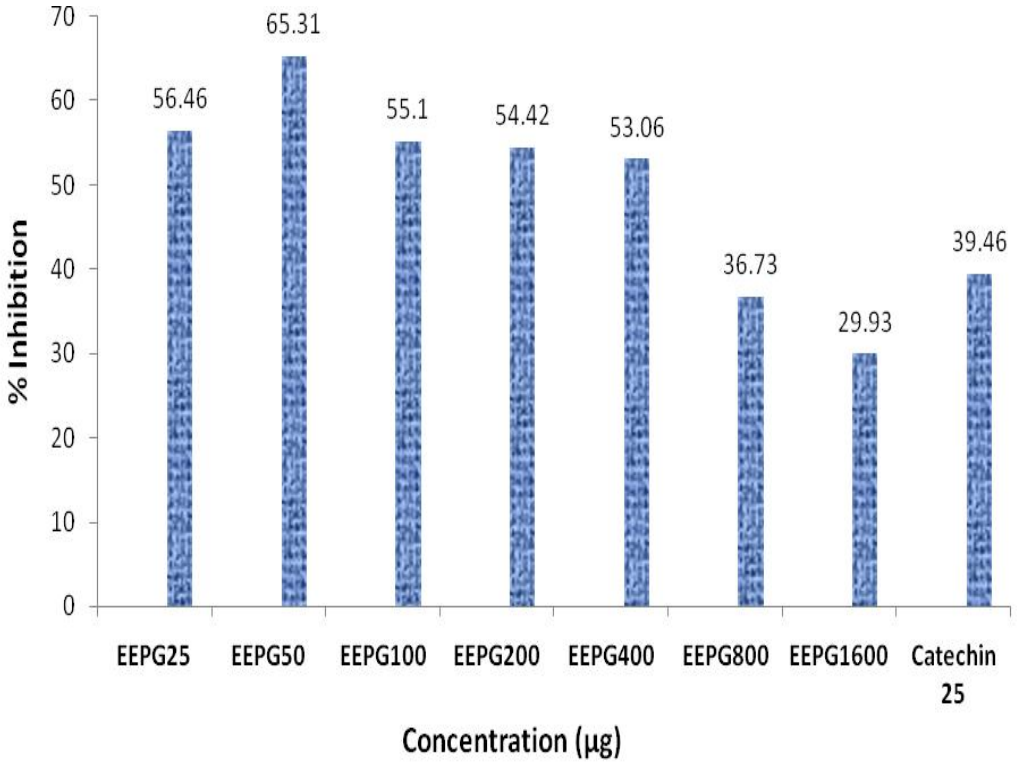


Figure 6B: % Inhibition of AAPH-induced lipid peroxidation by ethanolic extract of *Psidium guajava* (EEPG) and catechin

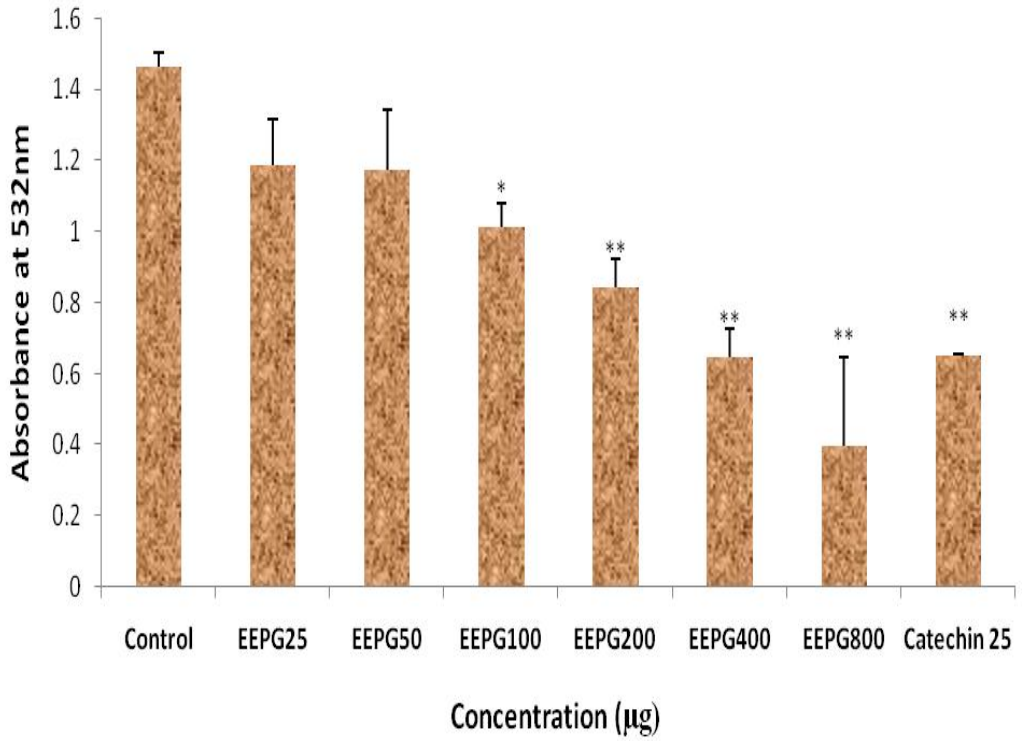


Figure 6C: Inhibition of deoxyribose oxidation by ethanolic extract of *Psidium guajava*.
$^{*}p<0.01$ and $^{**}p<0.001$ when compared with control

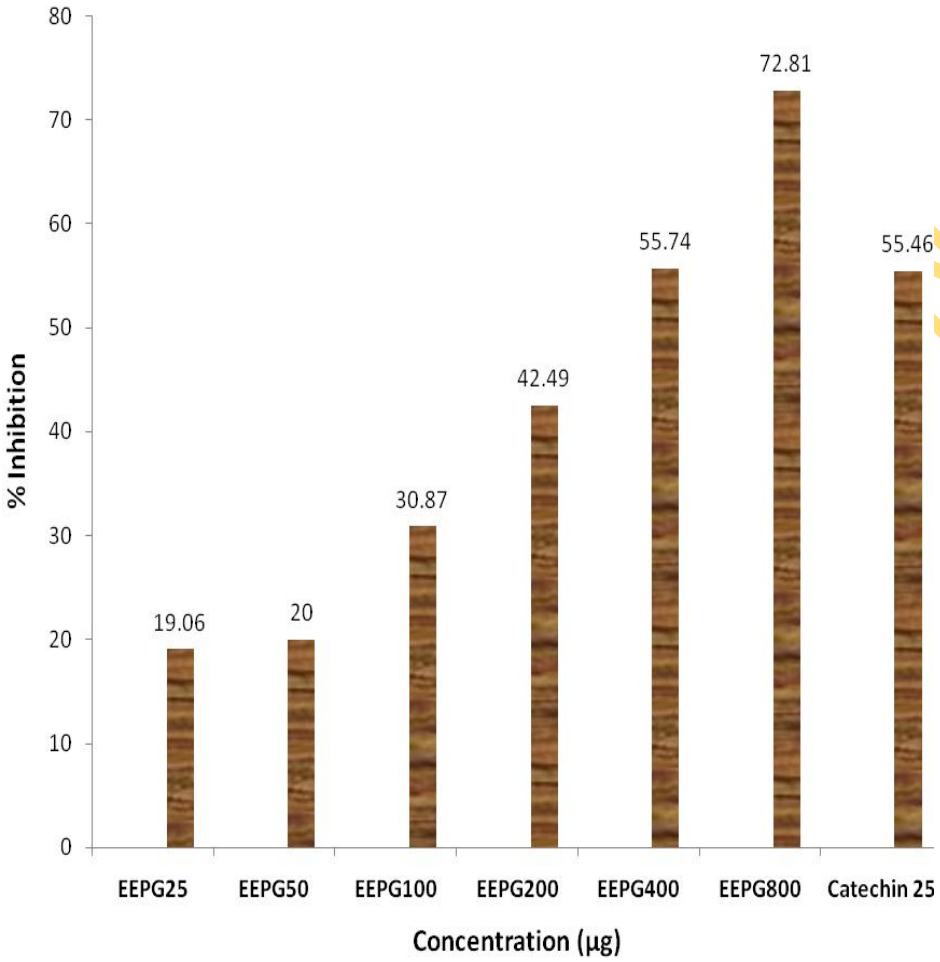


Figure 6D: Percentage inhibition of deoxyribose oxidation by ethanolic extract of *Psidium guajava*

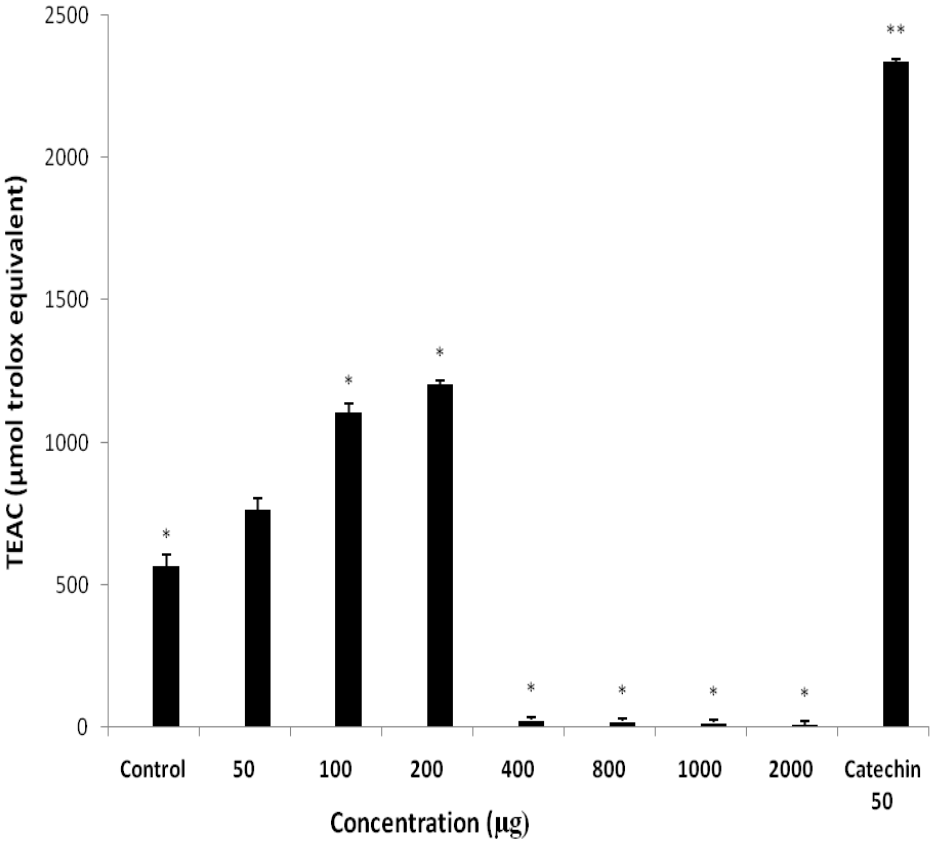


Figure 6E: Trolox antioxidant capacity (TEAC) of ethanolic extracts of *Psidium guajava* (EEPG)

*p<0.001 and **p<0.0001 when compared with control

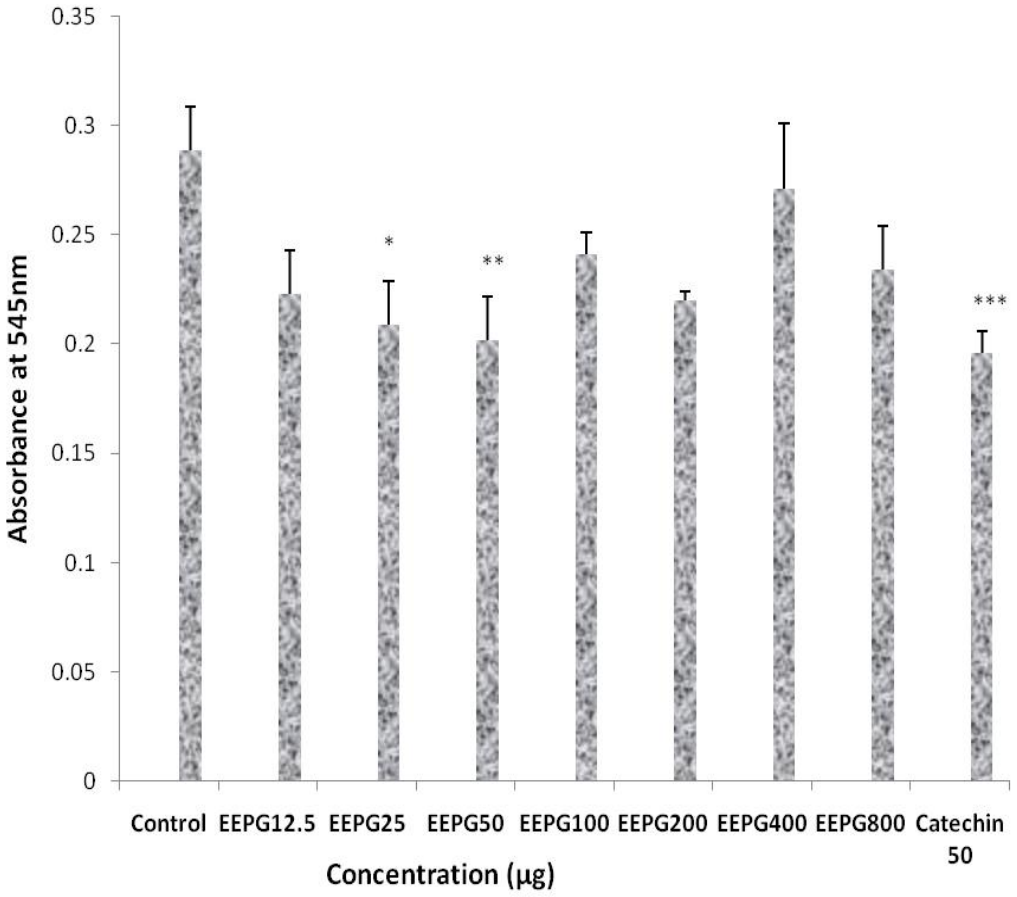


Figure 6F: Nitric oxide ($NO^{\cdot-}$) radical scavenging activity of ethanolic extract of *Psidium guajava*
$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ when compared with control

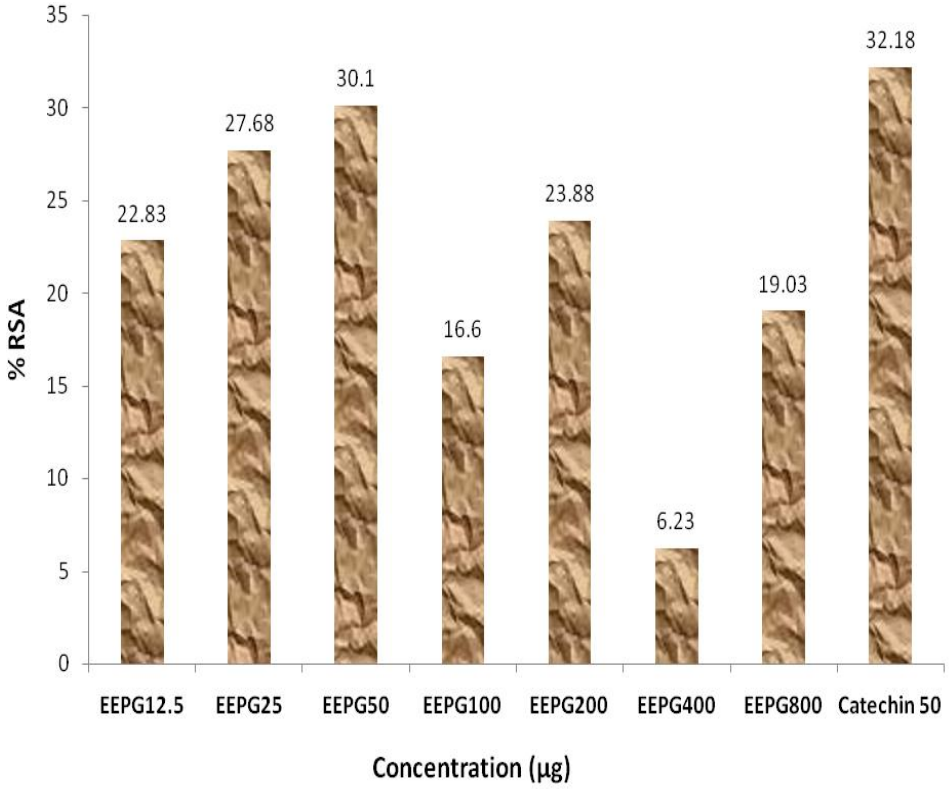


Figure 6G: Nitric oxide ($NO^{\cdot}$) radical scavenging activity (RSA) of ethanolic extract of *Psidium guajava* (EEPG).

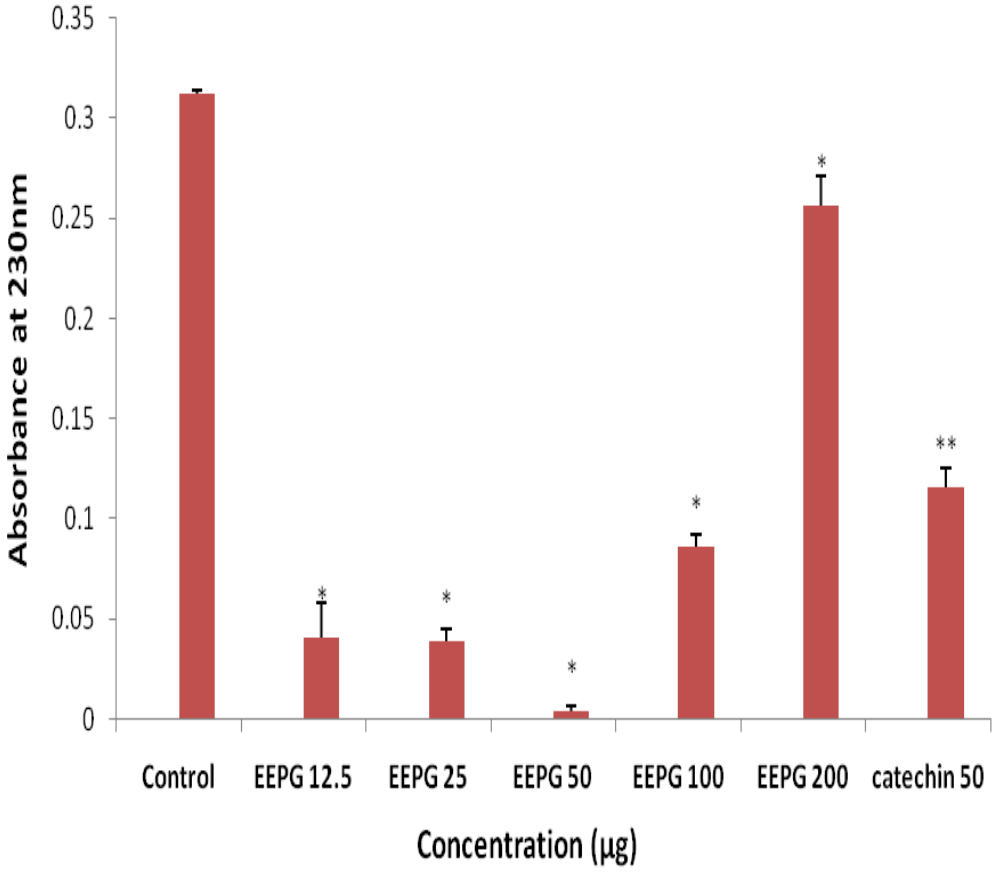


Figure 6H: Determination of hydrogen peroxide scavenging ability of ethanolic extract of *Psidium guajava*
$^{*}p<0.001$ and $^{**}p<0.0001$ when compared with control

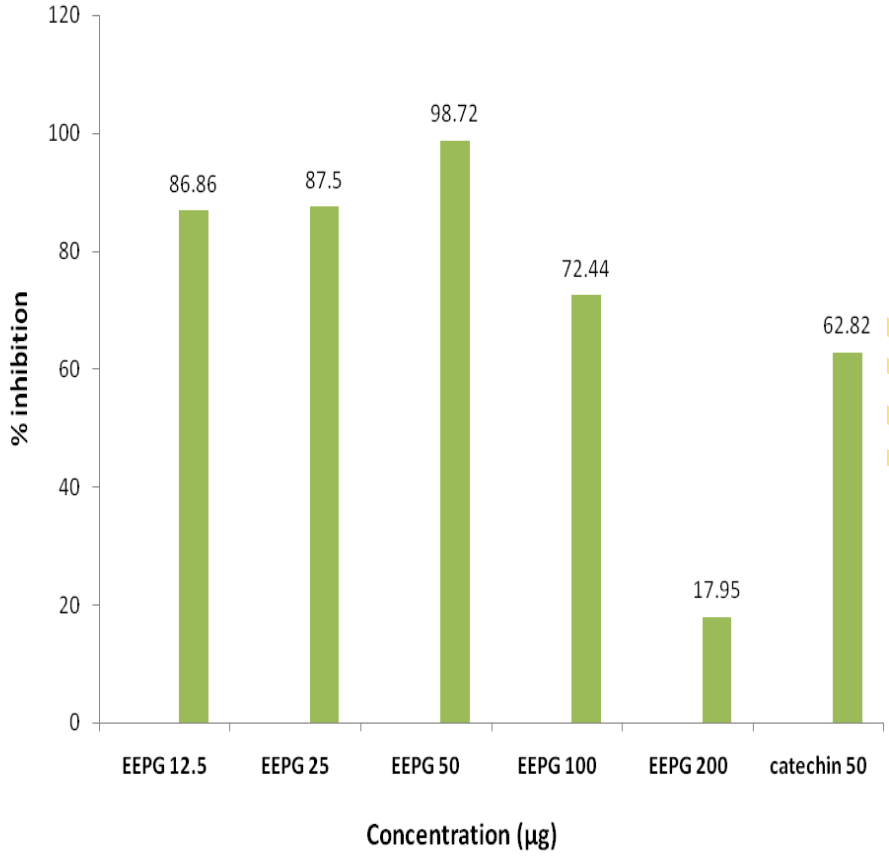


Figure 6I: % Inhibition of hydrogen peroxide generation by ethanolic extract of *Psidium guajava* (EEPG) and catechin

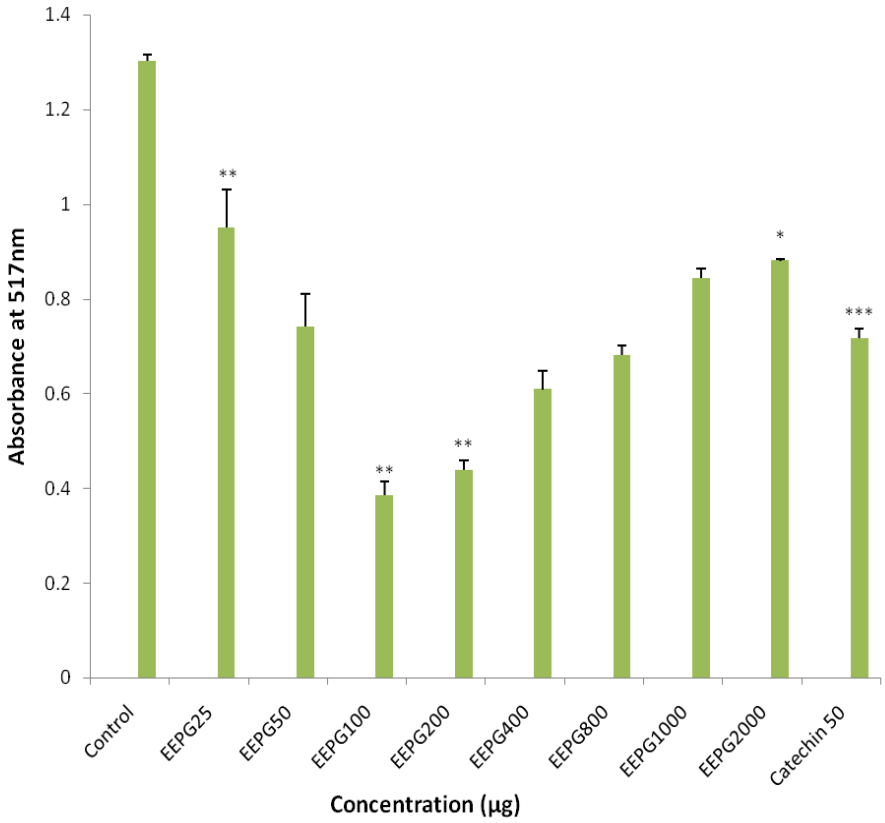


Figure 6J: DPPH· radical scavenging activity (RSA) of ethanolic extract of *Psidium guajava* (EEPG)
*p<0.05, **p<0.001 and ***p<0.0001 when compared with control

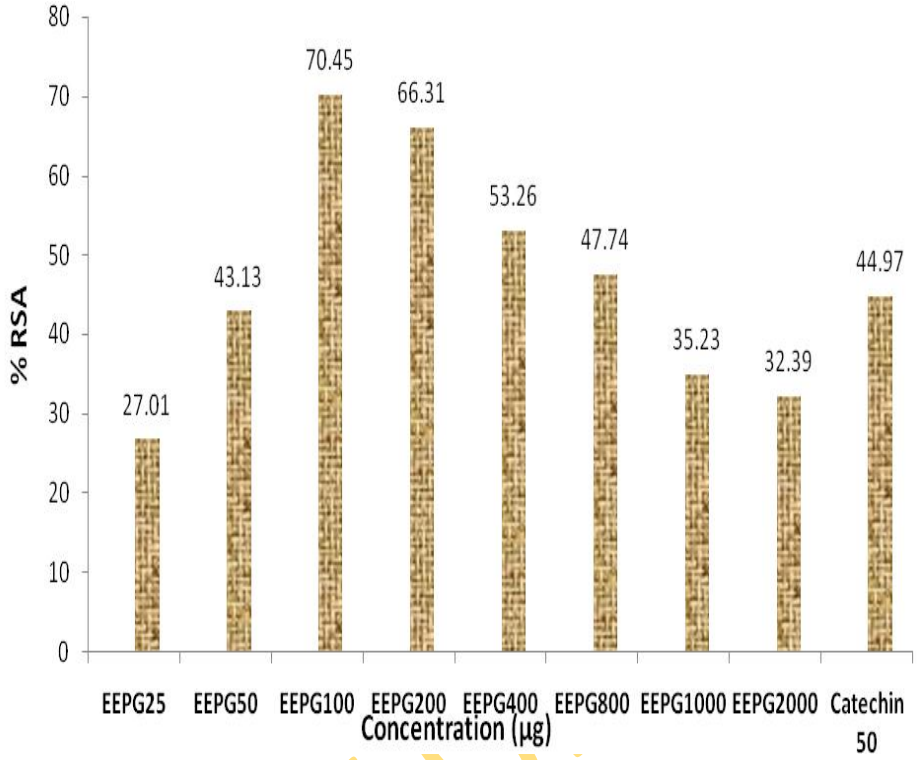


Figure 6K: DPPH radical scavenging activity (RSA) of ethanolic extract of *Psidium guajava* (EEPG)

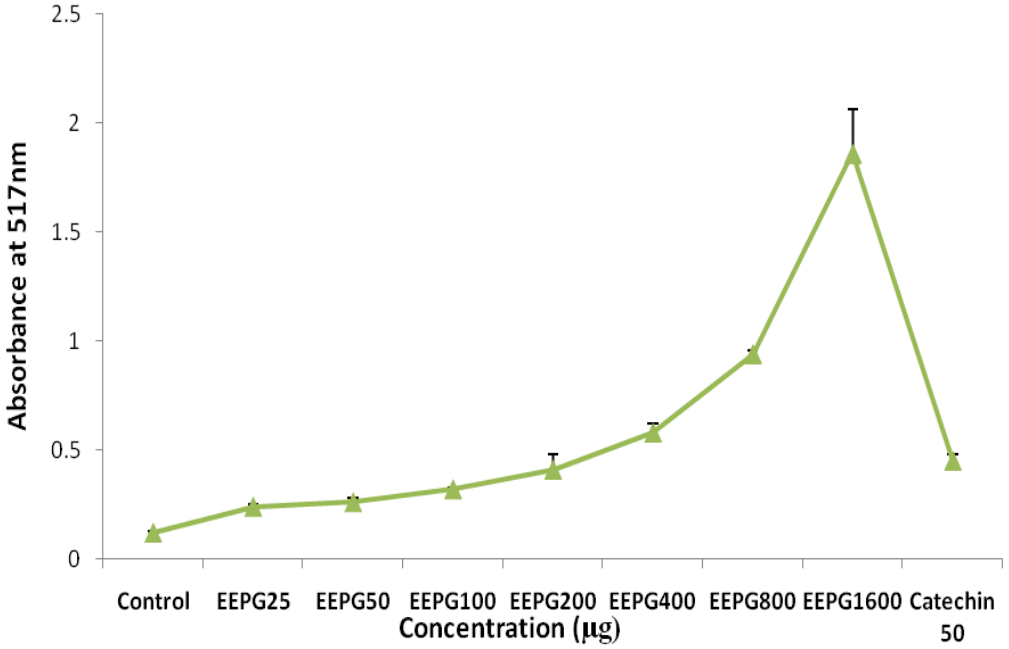


Figure 6L: Reducing power of ethanolic extract of *Psidium guajava*

The antioxidant and free radical scavenging activities of EEPG was evaluated. The effect of EEPG on AAPH- induced lipid peroxidation (LPO) is shown in Figures 6A and 6B. EEPG inhibited LPO induced by AAPH as indicated by the decrease in absorbance when compared with control (i.e. reaction mixture without EEPG) (Figure 6A). LPO was significantly inhibited by 56.5%, 65.3%, 55.1%, 54.4%, 53.1%, at 25 – 400 µg and non-significantly inhibited by 36.7% and 29.9% ($p>0.05$) at 800 – 1600 µg respectively (Figure 6B). This shows that the extract showed significant capacity to suppress AAPH-induced lipid peroxidation at 25 – 50 µg after which there was a decrease in percentage inhibition of AAPH-induced lipid peroxidation from 100 – 1600 µg. The reference drug, catechin at 50 µg, produced 39.46% inhibition of LPO induced by AAPH respectively with respect to control.

Figures 6C and 6D show hydroxyl radical scavenging potential of EEPG via inhibiting deoxyribose oxidation. EEPG produced significant inhibition of deoxyribose degradation at 100 – 800 µg concentrations by 30.9% ($p<0.01$), 42.5%, 55.7%, 72.8% ($p<0.001$) respectively with respect to control (Figure 6D). Catechin also shows higher potency than EEPG, producing 55.46% inhibition at 25 µg ($p<0.001$). Furthermore, the antioxidant activity of EEPG ranged from 763.08 to 1206.2 µmol trolox equivalent in the TEAC assay at 50 – 200 µg ($p<0.001$) and 24.62 to 14.77 µmol trolox equivalent at 800 – 2000 µg ($p<0.001$). Catechin exhibited a TEAC value of 2338.5 µmol trolox equivalent ($p<0.001$) (Figure 6E).

Result of the radical scavenging activities (RSA) of the extract against $NO^{\cdot}$, H_2O_2, and $DPPH^{\cdot}$ radicals are presented in figures 6F, 6H and 6J respectively. EEPG exhibited maximum RSA of 30.1% on $NO^{\cdot}$ radical ($p<0.01$), 98.72% on H_2O_2 ($p<0.001$) at 50 µg and 70.45% on $DPPH^{\cdot}$ radical ($p<0.001$) at 100 µg. EEPG also demonstrated marked reducing power, increasing absorbance values from 0.24±0.01 to 1.86±0.20 at 25 – 1600 µg (Figure 6L). The reducing power of EEPG at 200 µg (0.41±0.07) almost compares with that of catechin at 50 µg (0.45±0.028). The total phenolic and proanthocyanidin contents of the plant were 0.11 and 5.0 mg gallic acid equivalent/g of extract respectively. The total flavonoid content of *P.guajava* was 20 mg/g of extract expressed as catechin equivalent.

CONCLUSION

The ethanolic extract of *Psidium guajava* (EEPG) exhibited significant free radical scavenging and antioxidant activities *in vitro.*

EXPERIMENT 7: PHYTOCHEMICAL ANALYSIS AND ISOLATION OF BIOACTIVES FROM ETHANOLIC EXTRACT OF *PSIDIUM GUAJAVA*

Free radicals are produced in normal and/or pathological cell metabolism. Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, the uncontrolled production of oxygen derived free radicals has been reported to be involved in the onset of many diseases such as cancer, rheumatoid arthritis, cirrhosis and arteriosclerosis as well as in degenerative processes associated with aging (Halliwell and Gutteridge, 2003). Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and tissue damage (Halliwell and Gutteridge, 2003). In recent years, there has been increasing evidence that reactive oxygen species (ROS) are associated with pathological conditions such as atherosclerosis (Patel *et al*., 2000) and carcinogenesis (Emerit, 1994), as well as with aging (Hipkiss, 2006). Thus, a lot of attention has focused on dietary antioxidants which may have a potential for therapeutic use and prevention of these diseases.

Investigations into the health maintaining properties of plants have resulted in the identification of a wide array of bioactive compounds in plants that include flavonoids, phenolics, limonoids, carotenoids, coumarins, phytosterols, etc. Based on recent research, several compounds from fruits and vegetables were found to possess anticarcinogenic and antioxidant activities (Okonogi *et al*., 2007). Antioxidant activity by scavenging of reactive oxygen species is important in preventing potential damage to cellular components such as DNA, proteins, and lipids. Guava (*Psidium guajava* L., Myrtaceae) leaves have a long history of medicinal uses. The leaves have been used in folk medicine of Thailand as an antidiarrheal (Lutterodt, 1989) and antidysenteric; externally, they have been used as a deodorant of mouth odour (Brand-Williams *et al*., 1995).

Few of these plants are biologically and chemically investigated in order to determine their effectiveness and active constituents. The aim of this work was to conduct some phytochemical analysis of guava extract in order to isolate bioactive compounds and also identify their chemical structure.

PROCEDURE

Infra red (IR) spectra were recorded with a PERKIN ELMER FT/IR-80857 spectrophotometer. NMR spectra were recorded on a Varian NMR 200 spectrometer (200 MHz for 1H). Chemical shifts are shown as δ values. Adsorption column chromatography (CC) was performed with Kieselgel 60 (ASTM 70-230 mesh). Gel permeation was achieved using Sephadex LH-20. 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 detected by the use of iodine, ferric chloride (5%) and DPPH (0.2%) spray reagents.

RESULTS

Table 7A: Fractionation of GEEF (guava extract ethanolic fraction) on Vacuum Liquid Chromatography and detection of antioxidant and phenolic compounds

Fractions	Codes	Reaction with DPPH	Reaction with $FeCl_3$
6-8	GEFA	No activity	High activity
10-13	GEFB	No activity	High activity

Table 7B: Fractionation of GEFB on open column Chromatography and identification of antioxidant and phenolic compounds

Fractions	Codes	Reaction with DPPH	Reaction with $FeCl_3$
117-119	GEFBP	High activity	High activity

Table 7C: Fractionation of GEFBP on Sephadex LH-20 and identification of antioxidant and phenolic compounds

Fractions	Codes	Reaction with DPPH	Reaction with $FeCl_3$
8-9	GEFBP′	High activity	High activity

The active purified compound present in GEFBP′ was obtained as a pale yellow powder and was analysed for its chemical structure by IR and H-NMR analyses. Results revealed the active compound as compound **1** as follows:

IR absorption band (cm^{-1})	Corresponding functional groups
4327	Hydroxyl group
2852	C-H stretching vibration
1569 and 1458	Double bond
721 and 668	Unsaturated cyclo compound.

The proton NMR and IR of this compound are shown in Figures 7A and 7B respectively. H-NMR of the same compound shows clustering nature of the hydrogen signals between δ 1.0 and 2.0.). The small offshot signal at δ 5.2 is the characteristic of unsaturated system. Signals at 2.9 or 2.8 is a typical behaviour of hydroxyl moiety. From these results, compound **1** was considered triterpene and its proposed chemical structure is shown in Figure 7C.

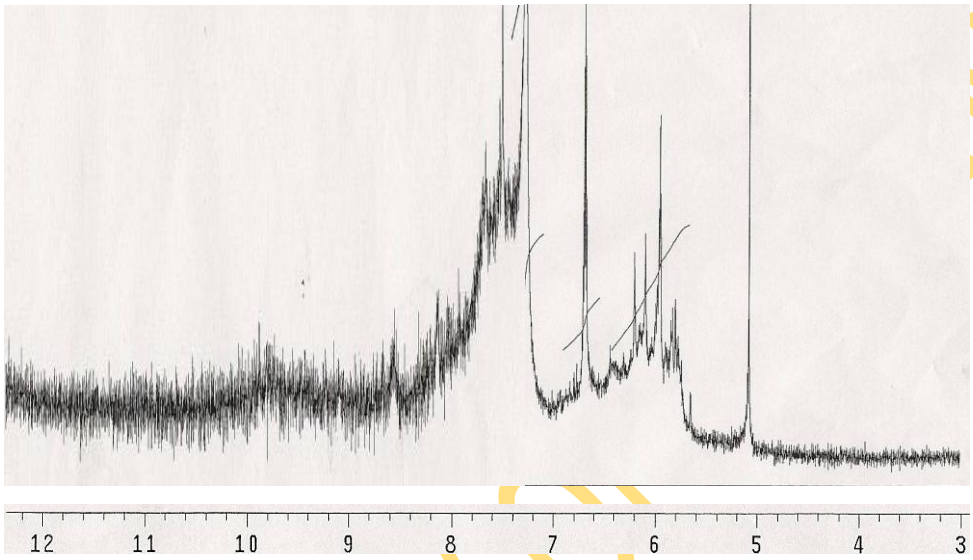


Figure 7A: H-NMR spectrum of compound 1

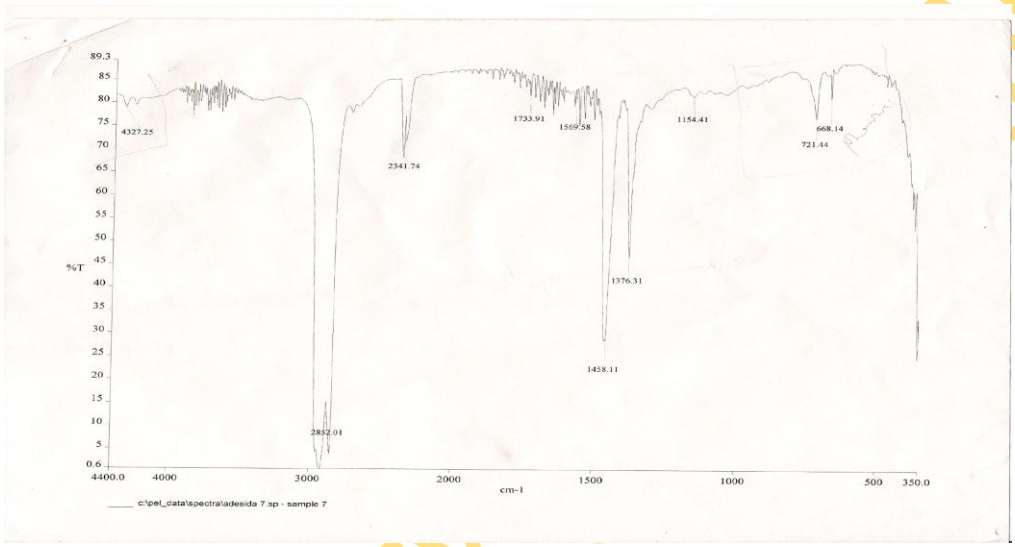


Figure 7B: IR spectrum of compound 1

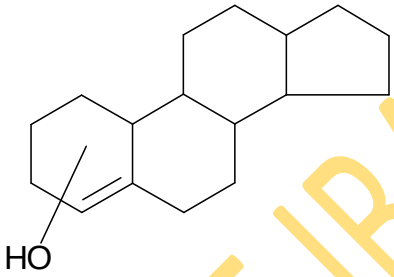


Figure 7C: Proposed chemical structure of the isolated compound named triterpene

CONCLUSION

The active compound isolated from EEPG is alkenol and demonstrated antioxidant properties. The NMR and IR reveal that it contains hydroxyl group, unsaturated group and some series of alicyclic group. The antioxidant activity could be related to the presence of the hydroxyl group.

CHAPTER FIVE

5.1 DISCUSSION

Adriamycin (doxorubicin hydrochloride) is a drug that is widely used in the treatment of variety of human malignancies, including breast cancer, small cell carcinoma of the lung and acute leukemia (Priestman, 2008). Despite the remarkable efficiency with which adriamycin kills cancer cells, its efficacy continues to be challenged by significant toxicities including nephrotoxicity (Kalaiseivi *et al*., 2005) and bone marrow toxicity (Singal *et al*., 2000). ROS produced during metabolism of adriamycin are purported to play an important role in the pathogenesis of experimental adriamycin nephropathy in rats (Liu *et al.*, 2007). The participation of the adriamycin semiquinone radical in oxido-reduction cycling seems to be important for the induction of the cellular damage (Trush and Kensler, 1991). The need of search for effective agents or compounds to protect against adriamycin toxicity to allow for its safer use in clinical practice is imperative.

5.1.1 Adriamycin Nephrotoxicity and Protective effect of EEPG

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) and *Psidium guajava* has been found to contain as part of its chemical composition alkaloids, terpenols and flavonoids which are compounds that have received considerable attention in recent years due to their diverse chemopreventive effects (Boudet, 2007). In view of these facts, the present study was designed to test the hypothesis whether a nutritional strategy like guava extract administration could prevent adriamycin-induced nephrotoxicity, genotoxicity and oxidative stress.

In this study, nephrotoxicity was induced at an intraperitoneal single dose of 20 mg and repeated doses of 2.5mg of adriamycin per kg body weight. Since studies have shown that repeated administration of adriamycin led to substantial glomerular and tubular lesion, we therefore used higher than the single dose in order to analyse toxicity that accumulate and also to examine the effect of concurrent administration of EEPG as this will also shed more light on the mechanism(s) of protection of EEPG against toxicity induced by adriamycin.

Single dose of adriamycin at 20 mg/kg and repeated doses at 2.5mg/kg resulted in the development of acute renal failure in the rats. This was evident from the renal function test as plasma levels of urea and creatinine significantly increased ($p<0.05$) when compared with those of the untreated (control) rats. This result is in agreement with Yilmaz

et al. (2006) who observed acute cardio–renal failure in rats after 72 hrs of a single dose of adriamycin administration. They observed that the administration of ADR to rats caused an increase in plasma creatinine and urea levels, indicating a decrease in glomerular filtration. Adriamycin-induced renal damage was found to be associated with increased renal vascular resistance and histological damage to tubular cells (Saad *et al.*, 2001). Injac and Strukelj (2008) and Lahouel *et al* (2010) attributed this increase in plasma creatinine and urea levels to renal insufficiency which occurred in association with the sudden fall in glomerular filtration rate. This is because majority of adriamycin administered enters specifically the proximal tubular epithelial cells and binds to anionic phospholipids in the target cells inducing abnormalities in the function and metabolism of multiple intracellular membranes and organelles. This develops injury in the proximal tubular epithelial cells of kidney causing acute renal failure.

Treatment with the ethanolic fractions of *P. guajava* (EEPG) at various doses (i.e 125 mg/kg, 250 mg/kg and 500 mg/kg) before adriamycin administration at 20mg/kg decreased the elevated plasma concentrations of urea and creatinine (indicators of impaired glomerular function (Poggio and Hall, 2006). The 250 mg/kg dose of the extract seemed to be the most effective in protecting the kidney against the adriamycin-induced renal injury. The prevention of increase in plasma creatinine which was significant following concurrent administration of the extract with adriamycin (2.5mg/kg) suggests that the extract may have prevented renal impairment induced by adriamycin. The extract alone administered at 500 mg/kg for 13 days increased the plasma concentrations of urea and creatinine suggesting that, the EEPG in large doses could interfere with glomerular function. The extract alone at the highest dose of 500 mg/kg when administered concurrently with adriamycin had no significant effect on the levels of urea and creatinine suggesting that the extract showed no sign of toxicity. This could be as a result of a short-term exposure to the extract (6 exposures) when compared with the long-term treatment with the extract at this same dose for 13 consecutive days.

Adriamycin treatment at single and repeated doses induced significant decrease in renal glutathione-S-transferase (GST) activity with a corresponding depletion of reduced glutathione (GSH). GSH plays an important role in the detoxification of xenobiotic compounds and in the antioxidation of reactive oxygen species and free radicals. Ayla *et al* (2011) demonstrated reduction in GSH level after adriamycin administration. This corroborates with the finding in this study that adriamycin treatment caused depletion of GSH. The decline in GSH cellular level has been considered to be indicative of oxidative stress (Farombi *et al*., 2008). The above result confirms that, adriamycin nephrotoxicity is

associated with the cellular depletion of GSH. The observed glutathione depletion could be as a result of extensive oxidation of GSH by adriamycin as observed with other redox-active agents causing oxidative stress (Magda *et al*., 2005). Since GSH is a hydroxyl radical scavenger Yadav and Mishra (2013), it therefore suggests that hydroxyl radicals may play a role in adriamycin nephrotoxicity (Santos *et al*., 2008). Treatment with EEPG prevented the adriamycin-induced depletion of cellular GSH and decreased GST activity. The significant protection offered at 125 and 250 mg/kg doses of the extract demonstrates that, EEPG improved the endogenous antioxidant reserve and this could improve kidney structure and function. Thus, increase in both enzymatic and non-enzymatic antioxidants may be playing significant role in the mechanism of the protective effect of guava. Flavonoids have been found in *Psidium guajava* and are antioxidants known to be excellent scavengers of free radicals (Aboseif, 2012). This may also explain the protection afforded by EEPG against the renal dysfunction and observed in this study.

The slight decrease in kidney GSH level, though not significant, and significant decrease in GST activity observed with the extract alone when administered consecutively for 13 days at its highest dose may be associated with increased utilization of GSH as a hydroxyl radical scavenger. It may also imply that the extract is exhibiting prooxidative effect at this dose. Indeed, some antioxidants have been reported to possess prooxidant effects at higher doses. The flavonoids: quercetin, myricetin, kaempferol (Sahu and Gray, 1996), and curcumin (Tanwar *et al.*, 2010) were found to mediate induction of reactive oxygen species at high concentration. Some studies have also suggested a similar prooxidant effect for CoenzymeQ10 *in vitro* (Linnane *et al*., 2007).

Furthermore, this study revealed that adriamycin nephrotoxicity is associated with decrease in the activities of superoxide dismutase and catalase. Montilla *et al* (1997) has also reported a decrease in the activity of catalase with adriamycin administration. Superoxide dismutase and catalase are major primary antioxidant defense components that primarily catalyze the dismutation of superoxide radical (O_2^-) to H_2O_2 and decomposition of H_2O_2 to H_2O respectively (Singal *et al*., 2000). Studies have shown that, redox cycling of adriamycin results in formation of superoxide radicals (Bachur *et al*.,1979) and that, superoxide dismutase has the ability to inhibit this reaction (Kostrzewa-Nowak *et al*., 2005). In this study, the decrease in the activity of catalase induced by adriamycin when compared with control group would probably result in accumulation of H_2O_2 which reacts with metal ions to promote additional radical generation, with the release of the particularly reactive hydroxyl radicals (Goyal *et al*., 2012). 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). This result lends credence to the report by Abo-Salem (2012) that ROS have been suggested to play an important role in the mechanism of nephrotoxicity following adriamycin administration Also the involvement of reactive oxygen species in the impairment of glomerular filtration rate (GFR) has been reported (Martinez-Salgado *et al*., 2007). The non-significant decrease in the activity of superoxide dismutase following repeated administration of adriamycin when compared with control is also supported by the fact that, at longer post-treatment durations, there was no change in the superoxide dismutase activity (Siveski-IIiskovi *et al.,* 1994). The significant increase in activities of catalase and superoxide dismutase produced at 250mg/kg of extract in single dose of adriamycin could strengthen antioxidant defenses and prevent the kidney from the oxidative stress due to adriamycin. The increase in activity of catalase may prevent exaggerated production of reactive oxygen species and oxidative damage. This is in agreement with the fact that, elevation in glomerular antioxidant enzymes protects renal function against the injury induced by reactive oxygen species (Yoshika *et al*., 1990). It is also reasonable to suggest that EEPG by increasing SOD activity, might prevent $O_2^{\cdot -}$ accumulation and reduce the production of the toxic reaction product, peroxynitrite, which can arise from reaction with $NO^{\cdot}$ to protect against adriamycin-induced nephrotoxicity.

Rats treated with the largest dose of EEPG (500 mg/kg) for 13 days exhibited a significant reduction in superoxide dismutase and catalase activities. This seems to further explain that, the extract in large doses may predispose to oxidative stress and contribute to renal impairment.

The activities of glutathione peroxidase and glutathione reductase were decreased in the adriamycin-treated rats. The decreases were statistically significant ($p<0.05$). The decreases in the activities of these enzymes may induce oxidative stress resulting in kidney damage. This is in agreement with other findings that, adriamycin inhibits the activity of glutathione peroxidase (Dbrowska *et al*., 2008). The possibility of oxidative inactivation of antioxidant enzyme also can not be ruled out (Timao and Pawan, 2000). The EEPG on the other hand prevented the decreases in the activities of both enzymes induced by adriamycin. A possible mechanism for this protection by the extract is modulation of endogenous antioxidant enzymes which could help in the detoxification of radicals as adriamycin has been shown to promote production of free radicals (Liu *et al*., 2007). A way of detoxifying these toxic oxygen species is via reduction by GSH

which is converted to oxidized glutathione (GSSG) in the process and in turn reduced by glutathione reductase in the presence of NADPH (Den Boer *et al*., 1990).

In the present study, adriamycin administration at 20mg/kg and 2.5mg/kg led to accumulation of malondialdehyde (MDA) which is one of the end products of lipid peroxidation (LPO). This is similar to observation made by Yagmurca *et al* (2004) that 20 mg/kg single dose of adriamycin resulted in renal LPO in rats, thus validating the finding in this study. The reduction in the activities of GST, catalase, SOD, glutathione peroxidase, and glutathione reductase as well as depletion of renal GSH may explain the increased lipid peroxidation observed in the adriamycin-treated rats in this study. The results in this study are very similar to the results obtained by Deman *et al*. (2001). They demonstrated that ADR treatment significantly reduces antioxidant capacity in kidney, which accounts for the increased susceptibility to oxidative stress of the cellular structures. Patel *et al.* (2010) reported that oxidative stress leads to lipid peroxidation, which is the result of an interaction between free radicals of diverse origin and unsaturated fatty acids typically in membrane lipids. Malondialdehyde, may diminish enzyme activity by oxidizing the active site or by forming protein cross-links (Choi and Tappel, 1969). In the presence of semiquinone in the tetracyclic aglycone molecules of adriamycin, the drug is reported to increase the oxygen radical activity (Kalyanaraman *et al*., 1980) as well as peroxidation of polyunsaturated fatty acids within the membrane phase (Kim *et al*., 2005). Lipid peroxidation can liberate metabolites causing DNA damage (Vaca and Harms-Ringdahi, 1989) and irreversible modifications of membrane structure and function (Odom *et al*., 1992). Since hydroxyl radical, highly reactive and damaging, has been found to induce peroxidation of cell membrane lipids (Hemnani, and Parihar, 1998), adriamycin toxicity may be associated with generation of hydroxyl radical. The result from this study however, showed that EEPG is capable of ameliorating adriamycin - induced oxidative renal damage. Administration of EEPG at 125 and 250 mg/kg suppressed the increased lipid peroxidation observed in the animals treated with single dose of adriamycin, which was statistically significant. Also 250mg and 500mg/kg of EEPG significantly prevented increase in lipid peroxidation caused by repeated doses of adriamycin. This action could be ascribed to the phenolic phytochemicals present in the guava leaves as they have been documented to possess antioxidant action and are able to inhibit peroxidation reaction in living systems (Van acker *et al*., 1995; Qian and Nihorimbere 2004). Additionally, the decrease in lipid peroxidation produced by *Psidium guajava* can be correlated with the induction of antioxidant enzymes. Treatment with the highest dose of EEPG (500 mg/kg) significantly brought the thiobarbituric acid reactive

substances near the adriamycin level. This shows that, the extract at this dose may not be offering any protection against adriamycin-induced lipid peroxidation.

The present study also revealed that, adriamycin administration resulted in a significant reduction in vitamin C concentration. Vitamin C (ascorbic acid) acts as a potent water-soluble antioxidant in biological fluids (Frei *et al*., 1990) by scavenging physiologically relevant reactive oxygen species (ROS) and reactive nitrogen species (RNS). It is also thought to be involved in recycling the α-tocopheryl radical back to α-tocopherol (Halliwell and Gutteridge, 1989). Evidence also showed that ascorbic acid regenerates other smaller molecular antioxidants such as GSH, urate, and β-carotene, from their respective radical species (Halliwell, 1996). From the study, administration of adriamycin could have resulted in the generation of reactive oxygen species which overwhelms the antioxidant defense system of the renal tissue. This will ultimately lead to reduction in ascorbic acid concentration. EEPG administration was found to increase significantly the vitamin C level in the groups treated with 20mg/kg and 2.5mg/kg of adriamycin. One of the possible mechanisms via which ascorbic acid acts as an antioxidant could be its ability to donate a reducing equivalent which helps to quench the damaging effects of ROS. Thus, this increase in the level of vitamin C may be playing a significant role in the mechanism of the protective effect of EEPG.

Adriamycin nephrotoxicity is associated with decrease in the activities of renal 5'-nucleotidase (5'-NTD) and glucose-6-phosphatase (GPase). These are suitable marker enzymes for plasma membranes. The decrease in activities of these microsomal marker enzymes reveals microsomal damage from ADR-induced oxidative stress leading to disruption in the membrane function and its eventual collapse. This may contribute to the proximal tubular damage during adriamycin treatment. Intervention of the guava extract preserved the activities of 5'-nucleotidase and glucose-6-phosphatase by raising their activities in the kidney. This data demonstrate that, EEPG protected against adriamycin-induced microsomal damage by suppressing oxidative stress and increasing 5'-NTD and GPase activities. However, treatment with EEPG alone at its highest dose (500 mg/kg) for 13 days consecutively produced a significant reduction in the activities of both enzymes. Thus, administration of guava extract at a minimal dose could be a promising approach in the treatment of nephrosis/microsomal damage caused by adriamycin.

Histological sections from rats kidney treated with single dose of adriamycin (20 mg/kg) revealed the presence of severe peritubular and periglomerular infiltration which also confirms the renal tubular damage. Kidney sections from rats treated with 125 and

250 mg/kg doses of EEPG before administration of single dose of adriamycin show mild tubular necrosis to mild congestion of renal blood vessels respectively. The 250 mg/kg dose of the extract appears to show better histological protection against adriamycin-induced renal damage.

Adverse histopathological change in form of eosinophilic material in the lumen was observed following repeated administration of adriamycin to the experimental rats. The ethanolic extract of guava, at all doses, was able to protect against histopathological change induced by adriamycin.

In an attempt to further identify safe dose, duration and the most appropriate time of intervention concerning the protective effect of EEPG on adriamycin-induced nephrotoxicity, the present study was undertaken to evaluate EEPG pretreatment and posttreatment schedules in rats treated with repeated doses of adriamycin (2.5mg/kg).

Administration of repeated doses of ADR (2.5 mg/kg) produced marked acute renal failure as indicated by the significant elevation in blood urea nitrogen (BUN) and plasma creatinine when compared with the untreated rats (saline group). Renal production of nitric oxide $(NO)^-$ was also monitored in the plasma by measuring NO_2^-/ NO_3^- concentrations (Moncada *et al*., 1991). There was significant increase in the NO (nitric oxide) levels of the renal tissue which have been claimed to be important in the pathogenesis of adriamycin nephropathy (Mohamed and Amr, 2007). Elevation of plasma NO_2^-/ NO_3^- observed following repeated administration of adriamycin suggests an increase in NO production in ADR-treated rats. And since the primary mechanism for clearance of nitrate is renal (Guillermo *et al.*, 1995), accumulation of nitrate might be indicating renal impairment. Renal morphologic examination which revealed severe tubular necrosis further confirmed renal tubular damage. Pretreatment with EEPG at all doses for 7 days attenuated the adriamycin-mediated increase in BUN and plasma creatinine. A greater effect was observed at 500 mg/kg of the extract. Posttreatment with EEPG at the doses of 125 mg/kg and 500 mg/kg did not produce any significant decrease in urea level in the adriamycin-treated rats. This suggests that there was no protection offered at these doses by the extract against adriamycin-induced increase in urea concentration. However, 250 mg/kg dose of the extract significantly decrease the plasma concentration of urea. Following posttreatment with EEPG at 125 mg/kg, 250 mg/kg and 500 mg/kg, the extract further increased plasma creatinine concentration in the adriamycin-treated rats.

Decrease in the high levels of NO in kidney of adriamycin-treated rats by pretreatment with guava extract could be through participation in the regulation of NO

production. This seems to suggest that EEPG is capable of decreasing excessive $NO^{\cdot}$ generation that characterizes adriamycin-induced nephrotoxicity However, in the posttreatment schedule, the reduction in the nitrate/nitrite concentration in the adriamycin-treated rats with 125 mg/kg ($p<0.01$), 250 mg/kg and 500 mg/kg ($p>0.05$) of ethanolic extract of *P.guajava* may result from the NO consumption by its reaction with superoxide anion to generate peroxynitrite (Radi *et al*., 2004). Peroxynitrite has been suggested to be an important causative agent in the pathogenesis of renal damage and dysfunction (Ferraro *et al*., 2003).

The results of the present study also show that renal SOD, CAT, GST activities as well as GSH and vitamin C levels (non-enzymic antioxidants) decreased in the ADR-treated animals with increased lipid peroxidation when compared with control as was also observed in the previous study. These observations support the hypothesis that the mechanism of nephrotoxicity in ADR-treated animals is related to depletion of antioxidant defense system. This decrease in the intracellular antioxidant defense could generate a state of oxidative stress that sensitizes cells to damage by adriamycin as observed by other toxins (Arteel, 2003). Under pathophysiological conditions, deterioration of antioxidant defense system or an upsurge in the production of free radicals results in cell damage (Thamiselvan, 2005). The loss of catalase activity following repeated doses of adriamycin as earlier mentioned may be responsible for the build up of H_2O_2 generated in the kidney. This increased H_2O_2 may thus exhaust intracellular GSH when the GSH system attempts to detoxify it (Po-Len *et al.*, 2005). The excess intracellular H_2O_2 may then work with superoxide (O^{2-}) to form hydroxyl radicals which will cause greater damage (Thannickal and Fanburg, 2000).

Pretreatment with EEPG for 7 days led to increase in the activities of SOD, catalase, GST and protected against depletion of GSH and vitamin C levels in the adriamycin-treated rats. The most abundant reactive oxygen species (ROS) generated in living cells are superoxide anion and its derivatives, particularly highly reactive and damaging hydroxyl radical, which induces peroxidation of cell membrane lipid (Hemnani and Parihar, 1998). In this respect, any increase in SOD activity of the organ appears to be beneficial in the event of increased free radical generation. However, it has been reported that a rise in SOD activity, without a concomitant rise in the activity of catalase might be detrimental (Harman, 1991). It is due to the fact that SOD generates hydrogen peroxide as a metabolite, which is cytotoxic and has to be scavenged by catalase. Thus a simultaneous increase in catalase activity observed in this study is essential for an overall beneficial effect of increase in SOD activity. The peroxidative

alteration in the kidney was inhibited by pretreatment with the extract, as the MDA level was significantly reduced especially at the 250 mg/kg and 500 mg/kg of the extract and the GSH content was dramatically increased in a dose-dependent manner by guava extract. This inhibition of lipid peroxidation might contribute to the beneficial effect of ethanolic extract of guava in reducing ADR-induced renal toxicity.

Posttreatment of EEPG with adriamycin led to a further decrease in SOD activity and this could result in increase in the intracellular production of superoxide. This enhancement of superoxide production by the combined treatment may have led to a greater production of intracellular reactive oxygen species to induce greater toxicity. Pacher *et al*., 2007 stated that NO^- is the only biological molecule that reacts faster with superoxide and is produced in high concentrations to outcompete endogenous levels of superoxide dismutase. This could be another possible reason for the observed decrease in SOD activity.

Extract at 125 mg/kg and 250 mg/kg caused a slight increase in the activity of catalase which could have led to some reductions in the H_2O_2 level observed. This slight increase may serve as an adaptive mechanism to protect cells in response to the ensuing oxidative injury. However, this was not sustained as the 500 mg/kg dose of the extract caused a reduction in catalase activity higher than the adriamycin-treated group.

Further reduction of GSH level by posttreatment with EEPG when compared with the adriamycin-treated group may be suggesting that the extract has pro-oxidative potential. Nakamura *et al* (2000) in his study on protocatechuic acid reported that chemopreventive agents that act in the initiation stage do not necessarily exert beneficial effects in the post-initiation phase and moreover, radical scavengers are known to have pro-oxidative potential because of their conversion to more reactive or stable radicals after they react directly with reactive oxygen species, which may contribute to the induction of secondary oxidative damage on the target organs.

The loss of GST activity produced by EEPG in the posttreatment schedule especially at 500 mg/kg may be partly due to lack of the decreased availability of its substrate, GSH. This inhibition in enzyme activity may also be due to the toxicity exhibited by the extract alone at 500 mg/kg. Overall, the disturbance of reactive oxygen specie detoxification systems such as not only GSH but also GST, CAT and SOD may be partially involved in enhanced oxidative stress induced by the extract.

Decrease in renal activities of 5'-nucleotidase and glucose-6-phosphatase was also observed after adriamycin treatment. Administration of ethanolic extract of *Psidium guajava* for 7 days prior to cumulative injection of adriamycin preserved the

activities of these enzymes. Posttreatment with ethanolic extract of *Psidium guajava* at 125 mg/kg, 250 mg/kg and 500 mg/kg did not show any significant prevention against this reduction in activities of these enzymes.

The histological sections of the kidney posttreated with ethanolic extract of *Psidium guajava* revealed severe tubular necrosis which corroborated the results of the biochemical assays.

It is pertinent to conclude here that, although EEPG is capable of preserving the activities of antioxidant enzymes and renal function, continuous daily administration throughout the period of treatment, however, may predispose to a decline in antioxidant defense and oxidative stress in the adriamycin-treated rats. Also, the results so far suggest that ethanolic extract of *Psidium guajava* may only act as a chemopreventive agent without any beneficial effects in the posttreatment phase and may exert a pro-oxidative action.

5.1.2 Effect of ethanolic extract of *Psidium guajava* on CYP3A4 activity

CYP3A4 is a phase 1 enzyme that participates in the metabolism of adriamycin. It is widely known that concomitant oral administration of several foods and herbs affects drug metabolism in humans by inhibiting CYP3A4 activity. Foodstuffs, beverages and herbs can either inhibit the transport and metabolism of various drugs to increase their bioavailability or induce drug transport and changes in metabolism to reduce drug bioavailability. These effects are mediated mainly by cytochromes P450 (CYP) and the drug transporters p-glycoprotein (P-GP) and organic anion transporting polypeptide (OATP) in the intestine (Chen and Raymond, 2006). CYP inhibition measurement is almost always performed by analysing inhibition of substrate metabolism. When there is induction of CYP3A4, its availability will increase and serum levels of its substrate (drug) will be reduced, (as a result of increase in the metabolism of the drug which would result in decrease in drug bioavailability). This can result in reduction in drug effectiveness and possibly induce therapeutic failure. There are two proposed mechanisms by which adriamycin has been found to act in the cancer cell. The first is intercalation into DNA and disruption of topoisomerase-II-mediated DNA repair and the second one is generation of free radicals and their damage to cellular membranes, DNA and proteins (Gewirtz 1999). Our focus in this study has been on the area of generation of free radicals as one of the mechanisms by which adriamycin exerts its toxicity. Adriamycin during its metabolism, is oxidized to semiquinone radical, which is converted back to adriamycin in a process that releases reactive oxygen species and adriamycinol, its active metabolite that

interferes with iron which is capable of producing hydroxyl ion which is a more potent reactive oxygen specie. Reactive oxygen species can lead to lipid peroxidation and membrane damage, DNA damage, oxidative stress, and triggers apoptotic pathways of cell death (Doroshow, 1986). Finding a way to maintain the efficacy and reduce toxicity has been one of the major areas of focus of anthracycline research. Our goal is to assess if ethanolic extract of *Psidium guajava* could increase the bioavailability of adriamycin by inhibiting the metabolic activity of CYP3A4 and thus preventing the production of the active metabolites responsible for cellular damage and toxicity. This would provide a therapeutic benefit as more adriamycin will be available in the plasma to exert its antitumor action with reduced toxicity.

At low dose of 125 mg/kg of guava extract, there was no inhibitory effect when compared with the ADR-treated group. When the doses of the extract were increased to 250 mg/kg and 500 mg/kg, the activity of CYP3A4 was inhibited. This inhibition of the activity of CYP3A4 by EEPG will result in an increase in the bioavailability of adriamycin because there is a decrease in its metabolism. This will also lead to increase in its therapeutic effectiveness and most importantly, prevention of generation of its metabolites that could result in toxicity. Extracts of other plants such as *Sutherlandia* and grapefruit juice have been found also to inhibit CYP3A4 activity (Greco *et al.*, 1995). P-glycoprotein and CYP3A4 act in concert to reduce absorption of xenobiotics along the gastrointestinal tract and increase drug elimination from the liver. The repeated extrusion and subsequent reabsorption of drug along the gastrointestinal tract increases drug metabolism by repeated exposure to CYP3A4 and as a result reduces drug bioavailability (Benet and Cummins, 2001). These results suggest that EEPG induced increase in bioavailability of adriamycin via inhibition of CYP3A4 activity. The hydroxyl group in the ethanolic fraction of guava extract as shown in the IR could be an important functional group responsible for the inhibitory effect on CYP3A4. Ho and Saville (2001) have reported that hydroxyl substitution in flavonoids of grape fruit juice is an important functional group for inhibitory effect on CYP3A4. . This *in vivo* inhibition of CYP3A4 activity exhibited by guava extract suggests that guava leaves may contain active component(s) responsible for this action. Further studies are still needed to establish this.

Therefore, use of guava extract may provide a therapeutic benefit in adriamycin administration

5.1.3 Effect of ethanolic extract of *Psidium guajava* on genotoxicity induced by adriamycin

Adriamycin is an anthracycline antibiotic used as an antitumor agent against human malignancies but which also has a wide variety of toxic side-effects, including genotoxicity. The reduction of oxidative DNA damage by antioxidants has been evaluated as a chemotherapeutic approach for reducing damage caused by chemotherapy agents such as adriamycin (Quiles *et al.*, 2002). According to Gewirtz (1999), doxorubicin is believed to be involved in induction of DNA damage through the inhibition of topoisomerase II and free radical generation by redox reactions.

Treatment with single dose of adriamycin showed a significant increase in the incidence of structural chromosomal aberrations in bone marrow cells of rats as indicated by the increase in number of micronucleus cells formed when compared with control. This significant increase in micronuclei formation by adriamycin observed in this study correlates with previously reported DNA damage associated with adriamycin toxicity (Rodrigues *et al.,* 1989). These results are in agreement with other reports concerning adriamycin toxicity. Anderson *et al*. (1997) demonstrated that adriamycin has genotoxic and clastogenic effects. Villani *et al*. (1998) reported that adriamycin induced genetic damage in human lymphocytes. Antunes and Takahashi (1998) and Duffaud *et al*., (1998), reported that adriamycin is genotoxic, it intercalates into DNA molecule generating free radicals and giving positive results with micronucleus and chromosome aberration tests. The formation of iron-adriamycin complex, which generates free radicals and induce lipid peroxidation has also been considered a participating mechanism (Myers *et al.,* 1982).

Although, antitumor action of adriamycin may be mediated by a wide number of mechanisms, free radical production is among the main causes of its toxicity. This fact could be used in a trial to reduce the toxic effects of adriamycin without interfering with its antitumor properties. The most immediate approach has been the combination of the drug delivery together with an antioxidant in order to reduce oxidative stress (Singal *et al.,* 2000). Pretreatment with ethanolic extract of *Psidium guajava* for 7 days prior to ADR treatment reduced the clastogenic effect of adriamycin as shown by the significant decrease in the frequency of micronuclei formation in a dose-dependent manner. It has been reported that quercetin, the main flavonoid present in *Psidium guajava* leaf has many pharmacological activities. Flavonoids existing in the extract could play an important

role in reducing the clastogenic effect of adriamycin. These phenolic compounds are widely distributed in the plant kingdom and have several pharmacological properties such as spasmolytic (Capasso *et al*., 1991) and antidiarrhoeal (Tona *et al*., 1999) activities. Flavonoids have been reported to have free radical scavenging properties (Bharani *et al*., 1995). van Acker *et al* (1995) reported that flavonoids are antioxidants found usually in plants, fruits and vegetable and are known to be excellent scavengers of free radicals. These results demonstrate the efficacy of EEPG and may act as a protective agent against adriamycin-induced genotoxicity. EEPG when administered at 500 mg/kg did not cause any significant change in activity of bone marrow cells when compared with control suggesting that substances present in the extract are not clastogenic nor do they promote DNA damage.

5.1.4 Free Radical Scavenging and Antioxidant Activities of EEPG

Interest in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants has been increased considerably. Restrictions on the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and BHT are being imposed due to their carcinogenicity (Mahdavi and Salunkhe, 1995); therefore, a need for identifying alternative natural and safe sources of antioxidants, especially of plant origin, has increased in recent years (Zainol *et al*., 2003).

Many herbal products have traditional uses that are now being investigated to create an evidence base that will facilitate their inclusion in general medical practice. With increasing recognition of herbal medicine as an alternative form of health care, screening of medicinal plants for biologically active compounds has become an important source of antibiotic prototypes and cancer-related drugs (Koduru *et al*., 2006). Hence, for selecting crude plant extract with potential useful properties, *in vitro* screening methods have been used for further in-depth chemical elucidation and pharmacological investigations (Mathekaga and Meyer, 1998). It was discovered that the therapeutic benefit of medicinal plants usually contributes to their antioxidant properties (Rice-Evans, 2004 and Dixon *et al*., 2005). Phenolic compounds possess diverse biological activities such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities. These activities might be related to their antioxidant activity (Chung *et al*., 1998). Other studies showed that there were significant correlations between phenolic compounds and antioxidant properties of medicinal plants under investigations (Gao *et al*., 2008 and Kim *et al*., 2008).

The leaves of guava have been found to be rich in flavonoids, and also found to show antioxidant and free radical scavenging capacity (Chen and Yen, 2006). Results from previous experiments in this study have demonstrated the ability of the ethanolic extract of guava (EEPG) to protect against adriamycin-induced nephrotoxicity and acute renal failure. The protective action by this extract was suggested to be largely dependent on the observed antioxidant activity. 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*.

2-2-azobis-2′ amidinopropane-hydrochloride (AAPH) is a free radical generator. It is a peroxyl radical initiator which can attack the polyunsaturated lipids initiating lipid peroxidation. Data from AAPH establishes the antioxidant and free radical scavenging properties of EEPG. EEPG showed significant capacity to suppress AAPH-induced lipid peroxidation at concentration range between 25 – 400 µg when compared with control. This result demonstrates the ability of EEPG to scavenge peroxyl radical generated by AAPH. The inhibition of AAPH-induced lipid peroxidation provides further evidence for the antioxidant and protective effects demonstrated by the extract *in vivo*. Also, EEPG produced 31 – 73% inhibition of deoxyribose oxidation at concentration range of 100 – 800 µg. The reference drug, catechin, produced 55% inhibition at 25 µg, demonstrating the greater potency of EEPG as an antioxidant. The deoxyribose method is a simple assay to determine the rate constants of hydroxyl radicals production (Ilavarasan *et al.*, 2005). It is to determine the hydroxyl radical ($OH^{\cdot}$) scavenging potential of the extract. 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 malondialdehyde-like compound (MDA) that forms a chromogen with thiobarbituric acid. The decrease in deoxyribose degradation produced by EEPG 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 EEPG as concentration increases in this study further explains its effectiveness *in vivo* in attenuating adriamycin-induced renal oxidative damage.

Furthermore, the antioxidant activity of EEPG was also demonstrated in the TEAC system and catechin showed greater potency than EEPG in this system. The TEAC system involved the potential scavenging of an artificially generated $ABTS^{+}$ radical (Campos and Lissi, 1997). This method has produced useful information regarding the antioxidant activities of phytochemicals (Rice-Evans *et al.*, 1995 and Salah

et al., 1995**)**. EEPG increased the TEAC values between concentration range of 50-200 µg when compared with control after which there was a decrease in TEAC values from 400-2000 µg showing that the extract exerted significant scavenging effect against the artificially generated $ABTS^{+}$ at lower concentrations (50 - 200 µg).

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, 2003). 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). EEPG in this study exerted scavenging effect on $DPPH^{\cdot}$ radical at all concentrations used (25 – 2000 µg). This is depicted by the decrease in absorbance values of reaction mixtures containing EEPG at these concentrations when compared with control (without EEPG) (result shown in Figure 6J). Thus, antioxidant capacity is all about ability to reduce the absorbance of DPPH. It is known that a freshly prepared $DPPH^{\cdot}$ 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 $DPPH^{\cdot}$ (by providing hydrogen atoms or by electron donation, conceivably via a free radical attack on the $DPPH^{\cdot}$ 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 EEPG with DPPH solution on TLC, which gave distinct yellow colour.

EEPG in this study also exhibited marked scavenging effects on $NO^{\cdot}$ radical at moderately low concentrations (Figure 6F). The scavenging effect on this radical decreased with increasing concentration of the extract. 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 adriamycin-treated rats. Overproduction of NO and its metabolites, in particular the deleterious molecule peroxynitrite ($ONOO^{-}$) has been suggested to contribute to numerous pathological conditions associated primarily with inflammatory disorders (Clancy and Abramson, 1995). EEPG also showed significant ability to scavenge H_2O_2 generated in the reaction medium and this was between 12.5 – 100 µg of the extract. Hydrogen peroxide can cross membranes and may oxidize a

number of compounds. The scavenging activity showed that the extract has H_2O_2 detoxification capacity.

The reducing power increased with increasing concentration of the extract (result depicted in figure 6L), 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 reducing ability of a compound generally depends on the presence of reductones, which exhibits antioxidative potential by breaking the free radical chain, donating a hydrogen atom (Gordon, 1990). 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, EEPG 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 EEPG at 1600 µg (1.86±0.2) compares with that of the reference standard, catechin (0.45±0.03), at 50 µg indicates that the reducing power of EEPG was much more than that of catechin. The absorbance values ranged between 0.24±0.01 to 1.86±0.20 at EEPG concentration of 25 – 1600 µg showing that the reducing ability of EEPG was dose dependent and significantly higher than the control (0.12±0.01). Since it is more potent than catechin, polyphenolics in EEPG 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.

In conclusion, EEPG 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 EEPG in nephrotoxicity and oxidative damage induced by adriamycin. This points to the potential benefits and future use of guava extract in oxidative and free radical pathologies.

5.1.5 Purification of GEFBP8 and GEFBP9 from GEFB on sephadex LH-20

A lot of attention has focused on dietary antioxidants which may have a potential for therapeutic use and prevention of diseases. Investigations into the health maintaining properties of plants have resulted in the identification of a wide array of bioactive compounds in plants that include flavonoids, phenolics, limonoids, carotenoids, coumarins, phytosterols, etc. Based on recent research, several compounds from fruits and vegetables were found to possess anticarcinogenic and antioxidant activities (Okonogi *et al.*, 2007). Few of these plants are biologically and chemically investigated

in order to determine their effectiveness and active constituents. The aim of this work was to identify and possibly isolate some bioactive compounds from guava leaves and also identify their chemical structure.

Results from this experiment indicated the presence of phenolic compounds with antioxidant activity in the ethanol fraction of *Psidium guajava*. Fractions 117, 118 and 119 (GEFBP) obtained from GEFB on open column chromatography (Table 7B) were detected to be phenolics exhibiting antioxidant activity by using the DPPH spray system. Further fractionation of GEFBP yielded 13 fractions (GEFBP1 to GEFBP13) among which are GEFBP8 and GEFBP9 (Table 7C). The purification of GEFBP8 and GEFBP9 was achieved through the use of column chromatography loaded with Sephadex LH-20. The column was eluted with 1 solvent system of acetone: H_2O (90:10). 13 fractions were collected. TLC analysis of these fractions showed that GEFBP8 and GEFBP9 are relatively pure and gave a positive test to DPPH and $FeCl_3$. They were bulked together based on having similar TLC pattern and coded GEFBP′. The sample was submitted for spectroscopic analysis.

GEFBP′: The IR (infrared) shows the following signals:
4327cm^{-1} corresponding to hydroxyl group
2852cm^{-1} for C-H stretching vibration
1569cm^{-1} and 1458cm^{-1} corresponding to double bond

The finger tip region at 721cm^{-1} and 668 cm^{-1} characterize the presence of unsaturated cyclo compound. However, the H-NMR of the same compound shows the characteristic signal of triterpene. This is substantiated by the clustering nature of the hydrogen signals between δ 1.0 and 2.0. Also, chemical analysis of guava extract has revealed the presence of triterpenes (Adeniyi *et al*., 1996; Begum *et al*., 2002). The small offshot signal at δ 5.2 is the characteristic of unsaturated compound. Signals at 2.9 or 2.8 are a typical behaviour of hydroxyl moiety. Tentatively, the IR as well as NMR indicated the presence of hydroxyl group, unsaturated compound and some series of alicyclic compound. The hydroxyl group could serve as proton donor to free radicals and might have been responsible for the antioxidant behaviour exercised by this fraction. The tentative structure of the compound isolated is already shown in the result.

5.2 CONCLUSION

Results of the present study show that single and cumulative doses of adriamycin induced oxidative stress, thus supporting the role of oxidative stress in its toxicity.

EEPG used in this study when administered alone consecutively showed signs of toxicity while its cumulative treatment did not present any sign of toxicity. It appears that the effects of guava extract administered at varied doses were found to be dependent on the duration of exposure.

Also, the results so far suggested that ethanolic extract of *Psidium guajava* may only act as a chemopreventive agent with no beneficial effects in the posttreatment phase as it may exert pro-oxidative action.

In vivo inhibition of CYP3A4 activity exhibited by guava extract suggests that guava leaves contain active component(s) responsible for this action. Further studies are still needed to establish this. The hydroxyl compound in the ethanolic fraction of guava extract as shown in the IR could be an important functional group for the inhibitory effect on CYP3A4.

Taken together, the present study reveals that administration of adriamycin to rats induced oxidative stress. Ethanolic extract of *Psidium guajava* protected rats against adriamycin-induced nephrotoxicity and genotoxicity. Free radical scavenging property (*in vitro*) and antioxidant activity of ethanolic extract of *Psidium guajava* (*in vivo*) may be responsible for this protective effect.

5.3 CONTRIBUTION TO KNOWLEDGE

1. Data from this study provide information for the first time that renal injury and genotoxicity associated with adriamycin chemotherapy may be ameliorated with a dietary supplement with guava extract. This reveals additional potential therapeutic or chemoprophylactic benefit of naturally occurring constituents present in guava.
2. This study contribute 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 adriamycin- induced nephrotoxicity.
3. This study provides information for the first time on the possible pro-oxidant potential of guava extract when consumed at high dose levels.
4. This study identified and isolated a triterpene-like compound with the presence of functional group like hydroxyl group detected by NMR and IR.
5. Data from this study reveal for the first time that ethanolic extract of *P. guajava* is capable of inhibiting CYP3A4 *in vivo*.
6. This study provides useful information for future clinical trials involving evaluation of chemoprophylactic or therapeutic potentials of guava constituents as well as the safe and effective doses in 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:

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

where S_o 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 $K_{(o)}$ at 0 min determined by extrapolation.

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

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

iii. Superoxide Dismutase (SOD) activity

$$\text{Increase in absorbance per minute} = \frac{A_3 - A_o}{2.5}$$

where A_o=absorbance after seconds

A_3=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}

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

$$= \quad \mu\text{mole/min/mg protein.}$$

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. Calculation of Glucose-6-Phosphatase activity

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

$$= \text{xnM}$$

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

$$= \text{n x 6.6nM}$$

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

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

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

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

vii. Calculation of 5′- Nucleotidase activity

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

= xnM

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

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

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

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

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

$= \frac{\text{ODT}}{\text{ODS}} \times \frac{2640000}{30\text{mg/ml protein}}$ } enzyme unit

Concentration of standard = 0.04M
= 40,0000µM

IN VITRO ASSAYS

i. Inhibition of Deoxyribose degradation (Deoxyribose assay)

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

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

A_{test} = absorbance of sample with extract

A_{blank} = absorbance of blank

ii. 1,1-Dipheny-2-picrylhydrazyl ($DPPH^{\cdot}$) 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 $DPPH^{\cdot}$ solution without extract.

iii. Inhibition of 2,2'-Azobis (2-amidinopropane) hydrochloride (AAPH) - induced Lipid peroxidation

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

where A_{uinh} = 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

$$\% \text{ RSA} = \left[1 - \frac{\Delta\text{Abs of sample}}{\Delta\text{Abs of control}}\right] \times 100$$

Table 6A: Effect of ethanolic extract of *Psidium guajava* (EEPG) on AAPH-induced lipid peroxidation.

Concentration (µg)	Absorbance	% Inhibition
Control	0.147±0.05	-
EEPG		
25	0.064±0.016*	56.46
50	0.051±0.01**	65.31
100	0.066±0.02*	55.10
200	0.067±0.01*	54.42
400	0.069±0.01*	53.06
800	0.093±0.03	36.73
1600	0.103±0.02	29.93
Catechin		
25	0.019±0.001*	39.46

Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates.

*p<0.05 and **p<0.01 when compared with control.

Table 6B: Inhibition of deoxyribose oxidation by ethanolic extract of *Psidium guajava* (EEPG)

Concentration (µg)	Absorbance	% Inhibition
Control	1.464±0.04	-
EEPG		
25	1.185±0.13	19.06
50	1.172±0.17	20.0
100	1.012±0.07*	30.87
200	0.842±0.08**	42.49
400	0.648±0.08**	55.74
800	0.398±0.25	72.81
Catechin		
25	0.652±0.004**	55.46

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 6C: Nitric oxide ($NO^{\cdot-}$) radical scavenging activity (RSA) of ethanolic extract of *Psidium guajava* (EEPG)

Concentration (µg)	Absorbance	% RSA
Control	0.289±0.02	-
EEPG		
12.5	0.223±0.02	22.83
25	0.209±0.02*	27.68
50	0.202±0.02**	30.10
100	0.241±0.01	16.60
200	0.220±0.004	23.88
400	0.271±0.03	6.23
800	0.234±0.02	19.03
Catechin		
50	0.196±0.01***	32.18

Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates.

*p<0.05, **p<0.01 and ***p<0.001 when compared with control.

Table 6D: Hydrogen peroxide scavenging activity of ethanolic extract of *Psidium guajava* (EEPG)

Concentration (µg)	Absorbance	% RSA
Control	0.312±0.002	-
EEPG		
12.5	0.041±0.017*	86.86
25	0.039±0.006*	87.5
50	0.004±0.003*	98.72
100	0.086±0.006*	72.44
200	0.256±0.015*	17.95
Catechin		
50	0.116±0.009**	62.82

Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates.

*p<0.001 and **p<0.0001 when compared with control.

Table 6E: DPPH· radical scavenging activity (RSA) of ethanolic extract of *Psidium guajava* (EEPG).

Concentration (µg)	Absorbance	% RSA
Control	1.303±0.013	-
EEPG		
25	0.951±0.08**	27.01
50	0.741±0.07	43.13
100	0.385±0.03**	70.45
200	0.439±0.02**	66.31
400	0.609±0.04	53.26
800	0.681±0.02	47.74
1000	0.844±0.02	35.23
2000	0.881±0.003*	32.39
Catechin		
50	0.717±0.02**	44.97

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 6F: Standard curve for total flavonoid content of ethanolic extract of *Psidium guajava* (EEPG).

Concentration (µg)	Absorbance
EEPG 1000	0.011
Catechin	
0	0.00033
20	0.055
40	0.099
60	0.153
80	0.198
100	0.271

Absorbance values expressed as mean of three replicates.

Table 6G: Standard curve for total phenolic content of ethanolic extract of *Psidium guajava* (EEPG).

Concentration (µg)	Absorbance
EEPG 1000	0.291
Gallic acid	
0	0.026
100	1.321
200	2.190
300	2.281
400	2.528
500	2.304

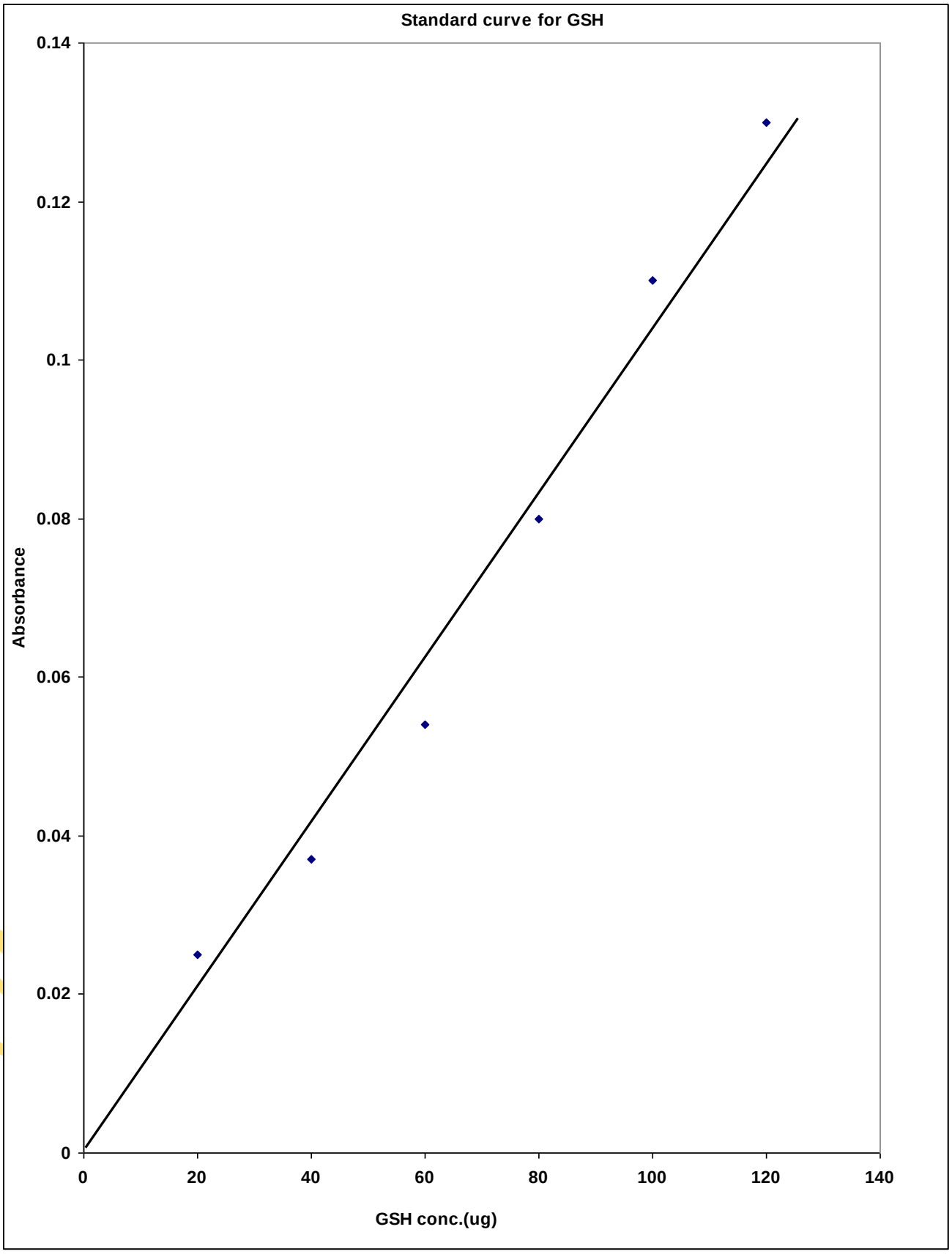
Absorbance values expressed as mean of three replicates.

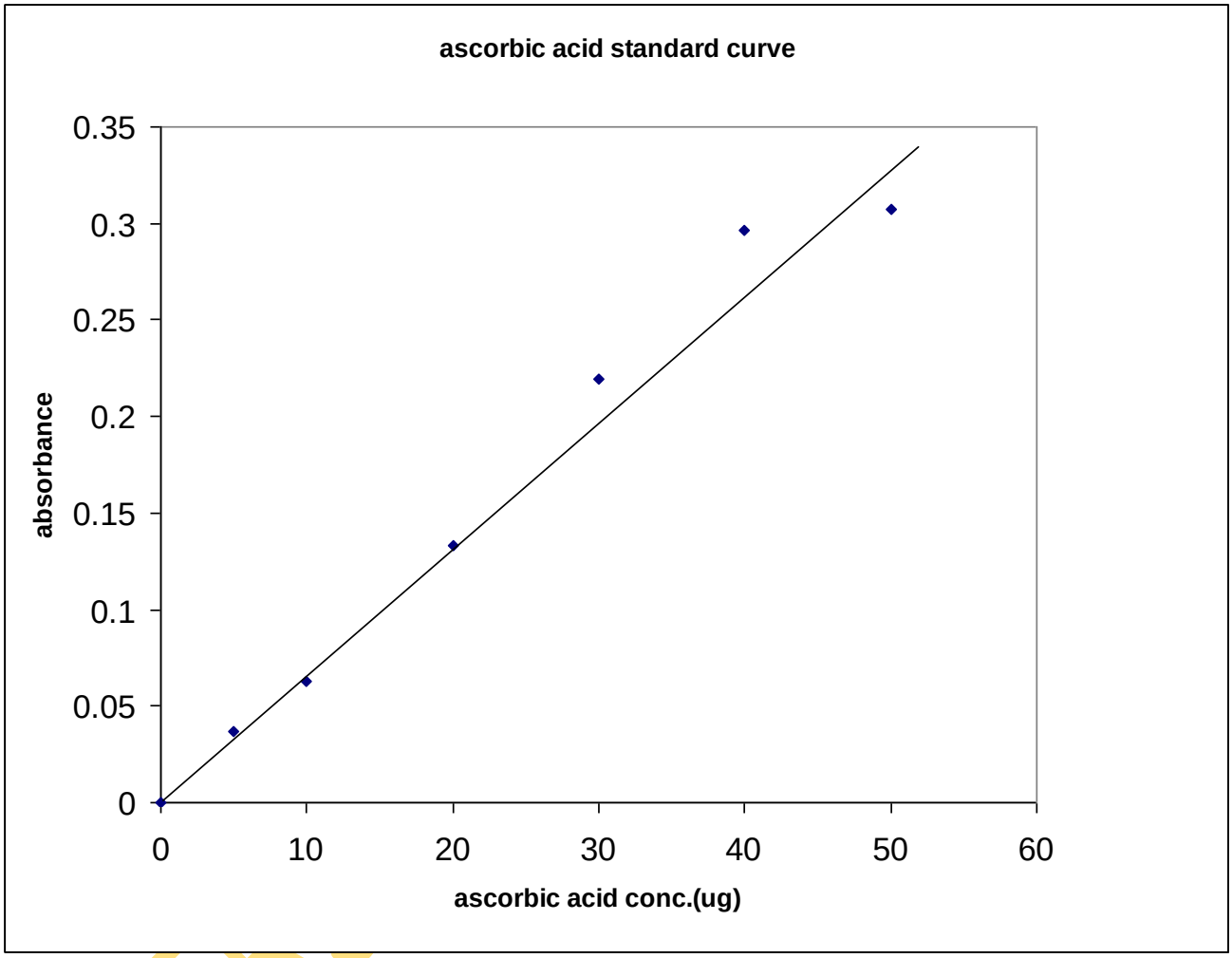
Table 6H: Standard curve for total proanthocyanidin content of ethanolic extract of *Psidium guajava* (EEPG).

Concentration (µg)	Absorbance
EEPG 1000	0.380
Gallic acid	
0	0.371
100	0.373
200	0.375
400	0.395
500	0.400

Absorbance values expressed as mean of three replicates.

FIGURES


Standard curve for GSH
Absorbance
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
20
40
60
80
100
120
140
GSH conc.(ug)


ascorbic acid standard curve
absorbance
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
ascorbic acid conc.(ug)