DOSE-AND TIME DEPENDENT EFFECTS OF OIL-PALM (*ELAEIS GUINEENSIS*) LEAF EXTRACT IN STREPTOZOTOCIN-INDUCED OXIDATIVE STRESS AND RENAL DAMAGE IN DIABETIC RATS

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ABSTRACT

Diabetic nephropathy is a major microvascular complication of diabetes and is the leading cause of end-stage renal disease. Treatment with antioxidants produced marginal benefit in preventing diabetic renal complications. Human studies showed that high dose of vitamin E failed to impart beneficial effect. The outcomes of these studies thus lay emphasis on the significance of developing better novel antioxidant treatments for reducing diabetic nephropathy. Oil palm (*Elaeis guineensis*) leaves are underutilized in tropical countries including Malaysia, and the ethanol extract of oil palm leaves (OPLE) is rich in flavonoids and catechins. The present study aimed to investigate the effectiveness of OPLE in attenuating hyperglycaemia-mediated oxidative stress and renal dysfunction in streptozotocin-induced diabetic rats. Sprague-Dawley rats received OPLE at a dose of 200, 500 or 1000 mg/kg/day for 4 or 12 weeks after diabetes induction with streptozotocin (60 mg/kg, *i.p.*). At the end of 4 or 12 weeks, blood glucose level, body and kidney weight ratio, urine flow rate (UFR), glomerular filtration rate (GFR), fractional sodium (FNa^+) and absolute potassium (K^+) excretions, urinary 8-hydroxy-2-deoxy guanosine (8-OHdG) and proteinuria were assessed. Glutathione (GSH), lipid peroxides (LPO), western blotting assay and immunohistochemistry staining for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase p22-phox and p67-phox subunits, and morphology were analysed in renal tissue while transforming growth factor- beta1 (TGF-β1) was measured in plasma. Significant increase in blood glucose, decreased body weight and increased kidney weight to body weight ratio were observed in diabetic rats. Proteinuria, UFR, GFR, FNa^+ and K^+ excretions were increased. Concomitant

with these alterations, an increase in LPO and a decrease in GSH in renal tissues were observed while urinary 8-OHdG and plasma TGF-β1 were elevated. Histological evaluation demonstrated glomerulosclerosis and tubulointerstitial fibrosis. Consistent with these reports, NADPH oxidase p22phox and p67phox subunits were enhanced as evaluated by western blotting and immunohistochemistry in rats with 4 and 12 weeks diabetes, respectively.

OPLE at all tested doses (200, 500 and 1000) mg/kg/day prevented these changes and preserved renal architecture in the 4-week study. In contrast, only 500 mg/kg/day of OPLE in the 12-week study exhibited a significant improvement in the renal functional changes and morphology, concurrently with improvement in the oxidative stress biomarkers, plasma TGF-β1, and the NADPH oxidase p22phox and p67phox subunits. OPLE 200 mg/kg/day in the 12-week study exhibited improvements only with respect to renal blood flow, protein and K^+ excretions, and NADPH oxidase p22phox and p67phox subunits. Paradoxically, 1000 mg/kg/day of OPLE in the 12-week study exhibited no significant improvement in the above parameters; renal injury was aggravated due to pro-oxidant action. Proteinuria, UFR, GFR, FNa^+ and K^+ excretions were increased. Concomitant with these alterations, renal LPO was increased and renal GSH was decreased whilst urinary 8-OHdG and plasma TGF-β1 were elevated. Histological evaluation demonstrated glomerulosclerosis and tubulointerstitial fibrosis. Consistent with these results, immunohistochemistry and western blotting assay demonstrated an increased in the NADPH oxidase p22phox and p67phox subunits. These results indicate the pro-oxidant action of OPLE at high dose (1000 mg/kg/day) when administered for longer duration (12 weeks).

ABSTRAK

Nefropati diabetes merupakan komplikasi mikrovaskular utama dalam penyakit diabetes melitus dan adalah punca utama penyakit ginjal tahap akhir. Rawatan dengan antioksidan menghasilkan manafaat marginal dalam pencegahan komplikasi ginjal disebabkan diabetes. Kajian terhadap manusia menunjukkan bahawa dos tinggi vitamin E gagal memberikan kesan yang baik. Hasil daripada kajian ini seterusnya memberi penekanan mengenai kepentingan menemukan rawatan antioksidan novel yang lebih baik untuk mengurangkan nefropati diabetes. Daun kelapa sawit (*Elaeis guineensis*) kurang digunakan di negara-negara tropika termasuk Malaysia, dan ekstrak etanol daun kelapa sawit (OPLE) didapati kaya dengan flavonoid dan catechin. Tujuan kajian ini adalah untuk mengkaji keberkesanan OPLE dalam mengurangkan stres oksidatif yang dicetuskan oleh hiperglisemia dan seterusnya disfungsi ginjal dalam tikus diabetes yang diaruhkan oleh streptozotocin.

OPLE pada dos 200, 500 atau 1000 mg/kg/hari diberikan kepada tikus Sprague-Dawley selama 4 atau 12 minggu selepas induksi diabetes dengan streptozotocin (60 mg/kg, ip). Pada penghujung 4 atau 12 minggu, paras glukosa darah, nisbah berat ginjal kepada berat badan, kadar aliran urin (UFR), kadar penapisan glomerular (GFR), ekskresi natrium pecahan (FNA^+) dan kalium mutlak (K^+), 8-hydroxy -2-deoxy guanosine (8-OHdG) dalam urin dan proteinuria ditentukan. Paras glutathione (GSH), peroksida lipid (LPO), subunit oksidase fosfat dinukleotide nikotinamide adenina (NADPH) p22-phox dan p67-phox yang dinilai melalui “western blotting” dan immunohistokimia diukur dalam tisu ginjal

manakala “transforming growth factor β1” (TGF-β1) diukur dalam plasma. Morfologi ginjal dianalisis bagi menentukan tahap kecedaraan organ tersebut.

Terdapat peningkatan ketara paras glukosa darah pada tikus diabetes bersempena dengan pengurangan berat badan dan peningkatan nisbah berat ginjal kepada berat badan. Proteinuria, UFR, GFR, perkumuhan FNa^+ dan K^+ didapati meningkat dalam tikus diabetes. Selaras dengan perubahan-perubahan ini, LPO diperhatikan meningkat dan GSH berkurangan manakala 8-OHdG dalam urin dan TGF-β1 dalam plasma meningkat. Penilaian histologi menunjukkan glomerulosklerosis dan tubulointerstitial fibrosis. Bersamaan dengan perubahan-perubahan ini, analisis “western blotting” dan immunohistokimia menunjukkan bahawa subunit oksidase NADPH p22phox dan p67phox meningkat pada tikus yang mengalami diabetes selama 4 atau 12 minggu.

OPLE pada kesemua dos yang diuji (200, 500 dan 1000) mg/kg/hari menghalang perubahan buruk di atas dan didapati memelihara ginjal dalam kajian 4 minggu. Sebaliknya, hanya 500 mg/kg/hari OPLE dalam kajian 12-minggu mempamerkan penambahbaikkan fungsi serta morfologi ginjal, serentak dengan penambahbaikkan dalam penanda biologi stres oksidatif, TGF-β1 dalam plasma, dan subunit oksidase NADPH p22phox dan p67phox. OPLE pada 200 mg/kg/hari dalam kajian 12-minggu diperhatikankan hanya menambahbaikkan aliran darah ginjal, perkumuhan protein dan K^+, dan subunit oksidase NADPH p22phox dan p67phox. Paradoksnya, 1000 mg/kg/hari OPLE dalam kajian 12-minggu tidak menunjukkan penambahbaikan parameter di atas, sebaliknya terdapat kecederaan yang semakin teruk pada ginjal disebabkan oleh tindakan prooksidan.

Proteinuria, UFR, GFR, perkumuhan FNa^+ dan K^+ didapati meningkat. Selaras dengan perubahan buruk ini, peningkatan LPO dan penurunan GSH dalam ginjal berserta dengan peningkatan 8-OHdG dalam urin dan TGF-β1 dalam plasma diperhatikan. Penilaian histologi menunjukkan glomerulosklerosis dan tubulointerstitial fibrosis. Selaras dengan keputusan ini, subunit oxidase NADPH p22phox dan p67phox diperhatikan meningkat berdasarkan "western blotting" dan immunohistokimia. Keputusan ini jelas menunjukkan tindakan pro-oksidan OPLE pada dos yang tinggi (1000 mg/kg/hari) apabila pemberiannya dilakukan untuk tempoh yang lebih lama (12 minggu).

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LIST OF ABBREVIATIONS

ABP	Arterial Blood Pressure
AChE	Acetylcholinesterase
AGE	Advanced Glycated Endproducts
$AlCl_3$	Aluminium Trichloride
ANOVA	Analysis of Variance
AP	Alkaline Phosphatase
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
BHT	Butylated Hydroxytoluene
Blk	Blank
BSA	Bovine Serum Albumin
β-actin	Beta Actin
B_0	Binding Wells
CHAPS	3-[(3-cholamidopropyl) dimethyl ammonio]-1-propanesulfonate
Cu^{2+}	Copper Ion
Cu^{+}	Cuprous Ion
^0C	Degree Centigrade
DAB	Diaminobenzidine
DDI	Deionized
DM	Diabetes Mellitus
DMSO	Dimethyl Sulphoxide
DN	Diabetic Nephropathy
DNA	Deoxyribonucleic Acid

DPPH	Di-Phenyl Picryl Hrdrazine
DPX	Dextropropoxyphene
DTNB	Dithio-bis-2-Nitrobenzoic Acid
ECM	Extracellular Matrix
ECL	Enhanced Luminol-Based Chemiluminescent
EDTA	Ethylene Diamine Tetra Acetic Acid
EGCG	Epigallocatechin Gallate
ELISA	Enzyme Linked Immuno Suppressant Assay
ESRD	End-Stage Renal Disease
$FeCl_3$	Ferric Chloride
FRAP	Ferric Reducing Antioxidant Power Assay
G	Gram
GAE	Gallic Acid Equivalent
GBM	Glomerular Basement Membrane
GFR	Glomerular Filtration Rate
GSH	Glutathione
GSSG	Glutathione Disulfide
HbA1c	Glycosylated hemoglobin
HCL	Hydrochloric Acid
hr	Hour
H & E	Haematoxylin and Eosin
HNE	Hydroxy Nonenal
HPST	Hydroperoxide Values of the Sample Tubes
HRP	Horseradish Peroxidase
H_2O_2	Hydrogen Peroxide
IDDM	Insulin Dependent Diabetes Mellitus

IDF	International Diabetes Federation
IgG	Immunoglobulin G
i.p	Intra-Peritoneal
i.u.	International Unit
i.v	Intra-Vascular
KCL	Potassium Chloride
kDa	Kilodalton
kg	Kilogram
LPO	Lipid Peroxides
MABP	Mean Arterial Blood Pressure
MD	Macula Densa
MDA	Malondialdehyde
MES	Morpholino Ethanesulphonic Acid
MPA	Metaphosphoric Acid
mg	Milligram
min	Minute
mL	Milliliter
mmol/L	Millimole Per Litre
µmol	Micromole
NaCl	Sodium Chloride
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NIDDM	Non-Insulin Dependent Diabetes Mellitus
no	Number
NP	NonylPhenoxypolyethoxylethanol

n.s.	Non- Significant
NSB	Non-Specific Binding
OPLE	Oil Palm Leaf Ethanol Extract
OHdG	Hydroxy-2-Deoxy Guanosine
PAS	Periodic Acid Schiff
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly-ADP-Ribose Polymerase
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline Tween-20
PKC	Protein Kinase C
p.o	Per-Oral
PP	Polypropylene
Pt	Proximal Tubules
PUFAs	Polyunsaturated Fatty Acids
PVDF	Polyvinylidene Difluoride
QE	Quercetin Equivalent
RAS	Renin-Angiotensin System
RAAS	Renin-Angiotensin Aldosterone System
RBF	Renal Blood Flow
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic Acid
s.c	Sub-Cutaneous
SD	Sprague Dawley
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error Mean

STZ	Streptozotocin
SV	Volume of Original Sample
TA	Total Activity
TBE	Tris-Borate-Ethylenediamine
TBS	Tris-Buffered Saline
TEAM	Triethanolamine
TEM	Transmission Electron Microscope
TFC	Total Flavonoid Content
TGF-α	Transforming Growth Factor Alpha
TGF-β1	Transforming Growth Factor Beta 1
TGF-β2	Transforming Growth Factor Beta 2
TGF-β3	Transforming Growth Factor Beta 3
TNB	Thio-Nitrobenzoic Acid
TPC	Total Phenolic Content
UAER	Urine Albumin Excretion Rate
UFR	Urine Flow Rate
VE	Volume of Extract
WHO	World Health Organisation
H_2SO_4	Sulphuric Acid
$ZnSO_4.7H_2O$	Zinc Sulphate Heptahydrate
13-HpODE	13-Hydroperoxy Octadecadienoic Acid
IC_{50}	Half Maximal Inhibitory Concentration
%	Percentage

A. LIST OF COMMUNICATIONS IN CONFERENCES

- **Varatharajan Rajavel,** Munavar S Abdul Sattar and Nor Azizan Abdullah. The antioxidative effect of *Elaies guineensis* (oil palm) ethanolic leaf extract against development of diabetic nephropathy.
  From Nature to Medicine through Sustainable Education, R&D and Practice, International conference on natural products 2010 Penang, **Malaysia,** 10th-12th December **2010**, Pp: 67. (Poster presentation) **Appendix 1.1**

- **Varatharajan Rajavel,** Munavar S Abdul Sattar, Mohamood Ameen Abdulla and Nor Azizan Abdullah. Effects of oil palm (*Elaeis guineensis*) leaf extract on hyperglycaemia-induced oxidative stress in kidney of diabetic rats.
  Translational research for sustainable wellness: From perception to reality, 26th Scientific Meeting of Malaysian Society of Pharmacology & Physiology, Penang, **Malaysia** 18th-20th May **2012**, Pp: 65. (Oral presentation) **Appendix 1.2**

- **VaratharajanRajavel,** Munavar S Abdul Sattar, Mohamood Ameen Abdulla, Ivy Chung and Nor Azizan Abdullah. Oil palm *(Elaeis guineensis)* leaf extract produced anti-oxidant effect and renal protection in STZ induced diabetic rat: A duration-dependent outcome.
  45th Annual Conference of Indian Pharmacological Society and International Conference on Navigating Pharmacology towards Safe and Effective Therapy, Nagpur, India. **India,** 4th-7th January **2013**, Pp: 149. (Poster presentation) **Appendix 1.3**

B. LIST OF MANUSCRIPTS PUBLISHED

Varatharajan Rajavel, Munavvar Zubaid Abdul Sattar, Mahmood Ameen Abdulla, Normadiah M. Kassim and Nor Azizan Abdullah. Chronic Administration of Oil Palm (*Elaeis guineensis*) Leaves Extract Attenuates Hyperglycaemic-Induced Oxidative Stress and Improves Renal Histopathology and Function in Experimental Diabetes.

Evidence-Based Complementary and Alternative Medicine. 2012, doi:10.1155/2012/195367. **(Impact Factor: 4.774 (2012), 2.175 (current)).** **Appendix 2.1**

Varatharajan Rajavel, Munavvar Zubaid Abdul Sattar, Ivy Chung, Mahmood Ameen Abdulla, Normadiah M. Kassim and Nor Azizan Abdullah. Antioxidant and Pro-oxidant Effects of Oil Palm (*Elaeis guineensis*) Leaves Extract in Experimental Diabetic Nephropathy: A Duration-Dependent Outcome.

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Varatharajan Rajavel, Munavar S Abdul Sattar, Mohamood Ameen Abdulla, Ivy Chung and Nor Azizan Abdullah. Oil palm (*Elaeis guineensis*) leaf extract produced anti-oxidant effect and renal protection in STZ induced diabetic rat: A duration-dependent outcome.

Indian Journal of Pharmacology. Volume 44, December 2012 Supplement. **(Impact Factor: 0.727).** **Appendix 2.3**

CHAPTER- 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Diabetes Mellitus

1.1.1. Introduction

Diabetes Mellitus is a metabolic disorder of multiple etiology characterised by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in either insulin secretion or insulin action, or both. Several pathogenic processes are involved in the development of Diabetes Mellitus. These include processes which destroy the beta cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action. The detrimental effects of Diabetes Mellitus include dysfunction and failure of various organs, including renal and cardiovascular system. The symptoms of chronic and uncontrolled Diabetes Mellitus include excessive thirst, polyuria, blurring of vision, delayed wound healing, weight loss and feeling tired. The complications of Diabetes Mellitus include retinopathy, neuropathy, nephropathy, vasculopathy, dermatopathy, cardiomyopathy and in latter stage encephalopathy.

1.1.2. Diagnosis of Diabetes Mellitus

Diabetes Mellitus is characterised by hyperglycemia and diagnosis is based on the presentation of subjects with high glucose. However, diagnosis is more difficult in subjects who are asymptomatic and demonstrate marginally elevated glucose concentration. Until the underlying molecular pathophysiology of the disease is identified, plasma glucose concentrations are likely to remain an essential

diagnostic modality. The widely accepted diagnosis criteria have been introduced by the World Health Organization (WHO, 1999) that is shown in Table 1.1.

Table 1.1: Diagnostic criteria for Diabetes Mellitus

Classification	Fasting plasma glucose level, mg/dL	2-h oral glucose tolerance test, mg/dL*	Random plasma glucose, mg/Dl
Normal	<100	<140	
Pre-diabetes impaired fasting glucose level	100-125		
Impaired glucose tolerance		140-199	
Diabetes	126†	200†	>200‡

*75-g glucose dose.
†Abnormal test must be repeated on subsequent day.
‡Must occur with hyperglycemic symptoms such as polydipsia, polyphagia, polyuria, blurred vision.
Source: Executive summary; standards of medical care in diabetes -2008. Diabetes Care. 2008: 31(Suppl 1): S5-S11

1.1.2.1. Glycosylated Haemoglobin (HbA1c)

Glycosylated haemoglobin (HbA1c) is a form of haemoglobin that is measured in order to find the average glucose concentration in plasma over a three month-period. The HbA1c is formed as a result of a non-enzymatic glycation pathway by haemoglobin's exposure to plasma glucose. Normal levels of glucose can produce a normal amount of glycated haemoglobin. The amount of HbA1c increases when the average amount of plasma glucose is elevated, indicating the presence of Diabetes Mellitus and poor control of blood glucose levels. This condition is associated with cardiovascular disease, nephropathy, and retinopathy (Larsen et al., 1990).

1.1.3. Types of Diabetes Mellitus

Diabetes Mellitus is classified into four broad categories: type 1, type 2, gestational diabetes and "other specific types" (Shoback, 2011). The term "diabetes" usually refers to diabetes mellitus. The rare disease diabetes insipidus has similar symptoms as Diabetes Mellitus, but without disturbances in the sugar metabolism (*insipidus* means "without taste" in Latin).

The term "type 1 Diabetes Mellitus (T1DM)" has replaced several former terms, including childhood-onset diabetes, juvenile diabetes, and insulin-dependent diabetes mellitus (IDDM). The term "type 2 diabetes mellitus (T2DM)" has replaced several former terms, including adult-onset diabetes, obesity-related diabetes, and non-insulin-dependent diabetes mellitus (NIDDM). Beyond these two types, there is no agreed-upon standard nomenclature.

1.1.3.1. Type 1 Diabetes Mellitus

The T1DM occurs as a result of loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas and insulin deficiency. This type can be further classified as immune-mediated or idiopathic. The majority of T1DM is of the immune-mediated nature in which the loss of beta cells is a T-cell mediated autoimmune attack (Rother, 2007). There is no known preventive measure against T1DM, which contributes to approximately 10% of Diabetes Mellitus. The T1DM can affect children or adults, but was traditionally termed "juvenile diabetes" because the majority of T1DM occur in children.

1.1.3.2. Type 2 Diabetes Mellitus

The T2DM is the most common type of Diabetes Mellitus. The T2DM is characterized by insulin resistance, which may be combined with relatively reduced insulin secretion (Shoback, 2011). The defective responsiveness of body tissues to insulin is believed to involve the insulin receptor. However, the specific defects are obscure. In the early stage of T2DM, the predominant abnormality is a reduction in insulin sensitivity. At this stage, hyperglycemia can be managed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the liver.

1.1.3.3. Gestational Diabetes Mellitus

Gestational Diabetes Mellitus (GDM) resembles T2DM in several aspects, involving a combination of relatively inadequate insulin secretion and responsiveness. It occurs in about 2%–5% of all pregnancies and may improve or disappear after delivery (Upadhyay and Dwivedi, 2011). GDM is fully treatable, but requires careful medical supervision throughout the pregnancy. About 20%–50% of affected women develop T2DM later in life (Upadhyay and Dwivedi, 2011). Though it may be transient, untreated GDM can affect the health of the foetus or mother. Risk to the infant includes macrosomia (high birth weight), congenital cardiac and central nervous system anomalies, and skeletal muscle malformations. In severe cases, perinatal death may occur, most commonly as a result of poor placental perfusion due to vascular impairment. A 2008 study completed in the U.S. (Lawrence et al., 2008) found that the number of American women having pregnancy with pre-existing Diabetes Mellitus is increasing. In

fact, the rate of diabetes mellitus in expectant mothers has been more than twice in the past 6 years (Lawrence et al., 2008).

1.1.4. Prevalence of Diabetes Mellitus

The total number of people with Diabetes Mellitus has been estimated to increase from 366 million in the year 2011 to 552 million by the year 2030 (International Diabetes Federation-IDF, 5th edition). Top 10 countries with highest prevalence of Diabetes Mellitus are shown below (Table 1.2) as reported by the International Diabetes Federation (International Diabetes Federation-IDF, 5th edition; Balakumar, 2013).

Table 1.2: Listed are the top 10 countries with high prevalence of Diabetes Mellitus in 2011 and 2030 (Projected value) as reported by the International Diabetes Federation (IDF), 5th edition; Balakumar, 2013).

Country with Rank	2011 (Millions)	Country with Rank	2030 (Millions)
1. China	90.0	1. China	129.7
2. India	61.3	2. India	101.2
3. USA	23.7	3. USA	29.6
4. Russian Federation	12.6	4. Brazil	19.6
5. Brazil	12.4	5. Bangladesh	16.8
6. Japan	10.7	6. Mexico	16.4
7. Mexico	10.3	7. Russian Federation	14.1
8. Bangladesh	8.4	8. Egypt	12.4
9. Egypt	7.3	9. Indonesia	11.8
10.Indonesia	7.3	10. Pakistan	11.4

The number of people with T2DM is increasing in every country. Around 80% of people with Diabetes Mellitus live in low- and middle-income countries. The greatest number of people with Diabetes Mellitus is between 40 to 59 years of age. One hundred and eighty three million people (50%) with Diabetes Mellitus are undiagnosed. Diabetes Mellitus caused 4.6 million deaths in 2011. Diabetes Mellitus caused at least US $465 billion in healthcare expenditures worldwide in 2011; 11% of total healthcare expenditures in adults (20-79 years). Seventy eight thousand (78,000) children develop T1DM every year (International Diabetes Federation-IDF, 5th edition). The Malaysian National Health Morbidity Survey III conducted in 2006 that involved 34,539 respondents of age > or =18 years in all states of Malaysia revealed that the overall prevalence of Diabetes Mellitus was 11.6% where Indians had the highest prevalence (19.9%), followed by Malays (11.9%) and Chinese (11.4%) (Letchuman et al., 2010).

1.1.5. Complications of Diabetes Mellitus

All forms of chronic and uncontrolled Diabetes Mellitus are known to cause long-term complications. In fact, Diabetes Mellitus could double the risk of cardiovascular disease (Sarwar et al., 2010). Diabetes Mellitus causes "microvascular" complications, which include damage to the small blood vessels (Boussageon et al., 2011). Diabetic retinopathy affects blood vessel formation in the retina of the eye and can lead to visual abnormalities. Chronic Diabetes Mellitus could affect the renal structure and function leading to the development of diabetic nephropathy. Diabetic Mellitus could damage the neuronal function leading to diabetic neuropathy. Together with vascular disease in the legs,

neuropathy contributes to the risk of diabetes-associated foot problems known as diabetic foot ulcers.

The main “macrovascular” complications of Diabetes Mellitus are related to atherosclerosis of larger arteries that includes ischaemic heart diseases such as angina pectoris and myocardial infarction, and stroke and peripheral vascular diseases.

1.1.6. Pathophysiology of Diabetes Mellitus

Insulin is the principal hormone that regulates the uptake of glucose from the circulation into most cells. Therefore, deficiency of insulin or insulin resistance play a central role in Diabetes Mellitus. Humans can digest foods containing carbohydrates like starch and some disaccharides such as sucrose, which are converted to monosaccharides like glucose, the principal carbohydrate energy source used by the system. Insulin is released into the blood by beta cells of islets of Langerhans in the pancreas, in response to rising levels of blood glucose from a meal. Insulin is used by about two-thirds of the body's cells in order to absorb glucose from the circulation to be used as fuel (Patra, 2012). Insulin is the principal control signal as well for the conversion of glucose into glycogen for internal storage in the liver and muscle cells. Reduced availability of glucose levels results both in the reduced release of insulin from the beta cells of islets of Langerhans and in the reverse conversion of glycogen to glucose to manage hypoglycemia. This physiological event is considered to be mainly regulated by glucagon, the hormone that acts in an opposite manner to that of insulin. Higher

insulin levels increase some anabolic ("building up") processes such as cell growth and duplication, and protein synthesis.

If the amount of insulin is insufficiently available, if cells respond poorly to the effects of insulin (insulin insensitivity or resistance), or if the insulin itself is defective, then glucose will not have its usual effect because it would not be absorbed properly by those body cells that require it, nor will it be stored appropriately in the liver and muscles. The net effect is persistent high levels of blood glucose, poor protein synthesis, and other metabolic derangements, which collectively lead to various complications of Diabetes Mellitus. When the glucose concentration in the blood is raised beyond its renal threshold of about 10 mmol/L, the reabsorption of glucose in the proximal renal tubules could be incomplete, and part of the glucose remains in the urine, known as glycosuria. This increases the osmotic pressure of the urine and inhibits reabsorption of water by the kidney, resulting in increased urine production (polyuria) and increased fluid loss. The loss of blood volume could be replaced osmotically from water held in body cells and other body compartments, and this could cause dehydration and increased thirst.

1.1.7. Animal Models of Diabetes Mellitus

T1DM is typically an immune-mediated destruction of the pancreatic beta cells of islets of Langerhans while T2DM occurs as a result of insulin resistance and impaired insulin secretion. Animal models have been used extensively in the field of diabetic study. The currently available animal models of T1DM and T2DM are shown in Table 1.3 (Rees and Alcolado, 2005).

Table 1.3: Animal models of T1DM and T2DM

T1DM	Streptozotocin-induced rats Bio breeding (BB) rat, Chinese hamster Celebes black ape, Keeshand dog Long Evans Tokushima Lean (LETL) rat Non-obese diabetic (NOD) mouse
T2DM	CBA/Ca mouse, db/db mouse, Diabetic Torri rat GotoKakizaki (GK) rat , Israeli sand rat, KK mouse, New Zeland obese mouse, Nagoya-Shibata-Yasuda (NSY) mouse Ob/Ob mouse, Otsuka Long-Evans Tokushima fatty (OLETF) rat

1.2. Diabetic Nephropathy: An Overview

Diabetic nephropathy is one of the common complications of Diabetes Mellitus that has become the leading cause of end-stage renal failure in many countries (Chen et al., 2005). In general, about 1 out of 3 patients with T1DM or T2DM proceed to developing significant diabetic nephropathy (Zipp and Schelling, 2003). It is believed that the pathophysiologic mechanisms of renal disorder are similar in both types of Diabetes Mellitus (Kern et al., 1999). The pathogenesis and clinical course of diabetic nephropathy can be monitored by renal structural and hemodynamic changes. The earliest change is an increase in glomerular filtration rate (GFR), also called as "hyperfiltration" stage, which is followed by detectable glomerular lesions with normal albumin excretion rate. This could follow with the development of microalbuminuria. Once microalbuminuria persists, both changes in glomerular structure, such as mesangial expansion and basement membrane thickening, and permeability could occur. At this stage,

prominent proteinuria, hypertension, and renal insufficiency progressed. The pathological alterations in this stage are glomerular basement membrane thickening, mesangial expansion, resulting in diffuse and/or nodular glomerulosclerosis, afferent and efferent arteriolar hyalinosis, and tubulointerstitial fibrosis (Cooper and Gilbert, 2003). After several years of persistent proteinuria, progression to end-stage renal disease could occur (Caramori and Mauer, 2001). Advanced diabetic glomerulopathy is commonly characterized by diffuse glomerulosclerosis and may sometimes exhibit a distinctive morphological appearance, namely, the nodular form of glomerulosclerosis, as first described by Kimmelstiel and Wilson in 1936 (Kimmelstiel and Wilson, 1936; Kern et al., 1999). The stages of diabetic nephropathy are shown in Table 1.4 (Vora and Ibrahim, 2003).

The current strategies to treat diabetic nephropathy include intensive glycaemic control, antihypertensive treatment with a particular focus on the interruption of renin-angiotensin aldosterone system (RAAS), restriction of dietary protein, and treatment of hyperlipidemia. There are several hypothetical approaches to the treatment of diabetic nephropathy based on an ever growing mechanistic understanding of the causes of diabetic nephropathy. These approaches include pharmacologic inhibitions of advanced glycation end products (AGEs) formation, protein kinase C (PKC), oxidative stress, and transforming growth factor β (TGF-β) in the kidney (Lee et al., 2003). Some medicinal herbs have been used widely for the treatment of diabetes and diabetic complications for hundreds of years (Alarcon et al., 1998; Li et al., 2004).

Table 1.4: Stages of diabetic nephropathy in T1DM

Stage	Renal Manifestation
1	Renal hyperfiltration (GFR↑) Renal hypertrophy
2	Silent stage Renal hyperfiltration (GFR↑); normal urine albumin excretion rate (UAER) Early histologic changes; non-specific increase in basement membrane thickness, increase mesangial matrix
3	Microalbuminuria (UAER 30-300mg/24h) or incipient nephropathy GFR may elevate or reduced into normal range Histology: mesangial expansion, glomerular basement membrane thickening, arteriolar hyalinosis
4	Established or overt nephropathy (proteinuria, nephrotic syndrome) GFR decline, hypertension
5	End stage renal disease(ESRD)

1.2.1. Features of Diabetic Nephropathy

1.2.1.1. Glomerular Basement Membrane Thickening

Glomerular basement membrane (GBM) thickening is a characteristic early change in T1DM (Mauer et al., 1984; Osterby et al., 1987; Drummond and Mauer, 2002) and T2DM (White and Bilous, 2000) and occurs with duration of disease (Perrin et al., 2006). Glomerular basement membrane thickening is a consequence of extracellular matrix (ECM) accumulation, with increased deposition of normal ECM components such as collagen, laminin, and fibronectin (Falk et al., 1983;

Kim et al., 1991). Such accumulations could result from either increased production of these proteins or their decreased degradation, or both. Glomerular basement membrane thickening might already be present in T1DM patients, who are normoalbuminuric (Drummond and Mauer, 2002; Perrin et al., 2006).

1.2.1.2. Mesangial Expansion

Mesangial cells are found in a part of the kidney called the glomerulus involved in filtration of plasma. Water, waste, and excess nutrients are filtered from the blood through the capillary walls into the surrounding Bowman's capsule. As shown in Figure 1.1, mesangial cells are found between the capillaries and help regulate the filtration process while providing support for the glomerular structure. They are also involved in the kidney's response to injury and disease. Intra-glomerular mesangial cells have an irregular shape and are related to smooth muscle cells. They do have similar proteins such as myosin and actin, and have the ability to contract. Chronic diabetes can give rise to progressive expansion of mesangial matrix that ultimately occludes glomerular capillaries.

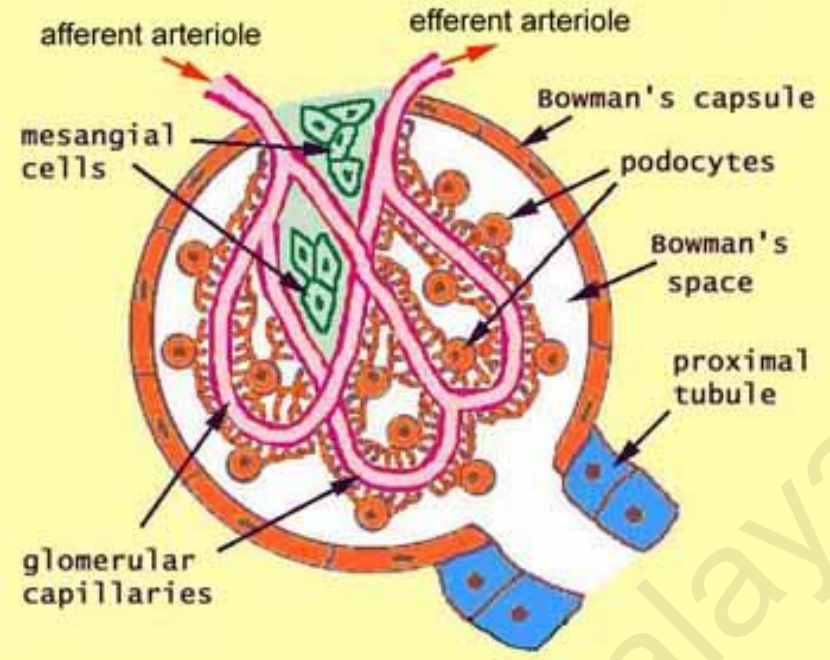


Figure 1.1: Location of mesangial cells in the glomerulus. Adapted from: http://www.siumed.edu/~dking2/crr/rnguide.htm#mesangial

Diabetic nephropathy is characterized by the progressive expansion of mesangial matrix that ultimately occludes glomerular capillaries. Diabetes Mellitus could contribute to the development of increased amounts of mesangial matrix. Glucose stimulates an increase in synthesis of collagens and matrix glycoproteins expressed within the mesangium. Abnormal glycosylation of matrix proteins interferes with their degradation and turnover. In Diabetes Mellitus, abnormal increases in glucose conspire to produce a relentless increase in accumulation of mesangial matrix, with altered composition and function (Abrass, 1995).

1.2.1.3. Pathogenesis of Diabetic Nephropathy

As described previously, diabetic nephropathy is a major cause of renal failure. Several factors have been implicated in the pathogenesis of diabetic nephropathy that include association of genetic factors, haemodynamic factors, vasocoactive factors, growth factors, renal structural factors, and hyperlipidemia. Among these, it has been confirmed that chronic and uncontrolled hyperglycemia plays a significant role and it is widely recognized that hyperglycaemic-induced oxidative

stress (figure 1.2), is the major culprit in the development of diabetic nephropathy (Arora et al., 2013).

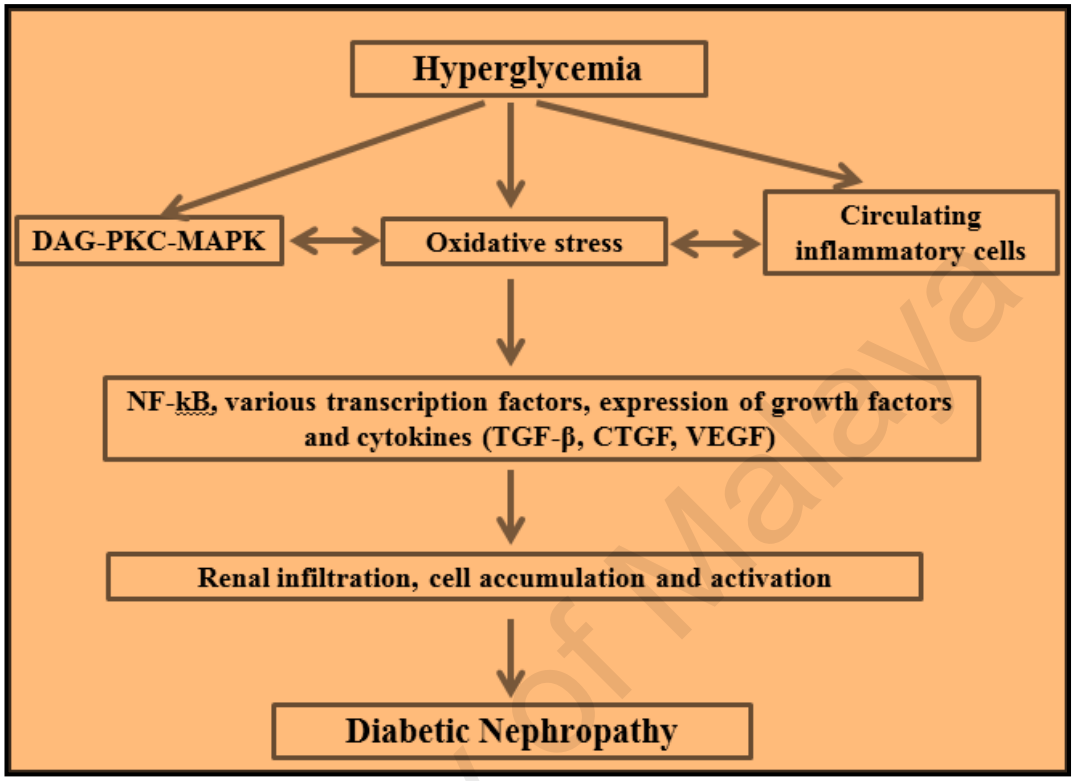


Figure 1.2: The induction and progression of diabetic nephropathy. Adapted from Soetikno et al., (2012).

Abbreviations: DAG, diacylglycerol; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; TGF-β, transforming growth factor-beta; CTGF, connective tissue growth factor; VEGF, vascular endothelial growth factor; NF-kB, nuclear factor kappa beta.

Oxidative stress

In 1956, Denham Harman, father of the free radical theory, suggested that free radicals produced during aerobic respiration cause cumulative oxidative damage (Wickens, 2001). Free radicals are generally considered harmful byproducts of oxidative metabolism (Oberley, 1988), causing organ damage in living systems.

This concept has implications in numerous biological phenomena such as cellular aging, mutagenesis, inflammation, and other pathologies.

Oxidative stress is defined as the excess formation or insufficient removal by antioxidant defenses of highly reactive molecules including reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Fig. 1.3. A). Examples of ROS include free radicals, superoxide ($O_2^{\cdot -}$), hydroxyl (HO·), peroxyl ($O_2^{\cdot}$) and hydroperoxyl ($HO_2^{\cdot}$). Examples of RNS include peroxynitrite ($ONOO^-$) and alkyl peroxynitrates (RONOO). The major free radical implicated in diabetic complications is $O_2\cdot^-$, which can be produced by various sources, including the mitochondrial electron transport chain (ETC) during oxidative phosphorylation, NADPH oxidase, xanthine oxidase, cyclooxygenase, lipoxygenase, cytochrome P-450, and nitric oxide synthase in certain contexts (Fig. 1.3. B) (Droge, 2002). In the presence of transition metals such as iron and copper, H_2O_2 can be converted to the highly reactive HO· radical via the Fenton reaction. Excess $O_2\cdot^-$also can react with NO· to form $ONOO^-$(Fig. 1.3. B) (Droge, 2002).

In normal conditions, $O_2\cdot^-$is eliminated rapidly by antioxidant defense mechanisms. Superoxide dismutase (SOD) can catalyze the dismutation of $O_2\cdot^-$to H_2O_2. SOD has 3 major isoforms: cytosolic CuZnSOD (SOD1), mitochondrial MnSOD (SOD2), and extracellular SOD (SOD3). H_2O_2 is converted to H_2O and O_2 via catalase in lysosomes or glutathione peroxidase (GPx) in the mitochondria and cytosol.

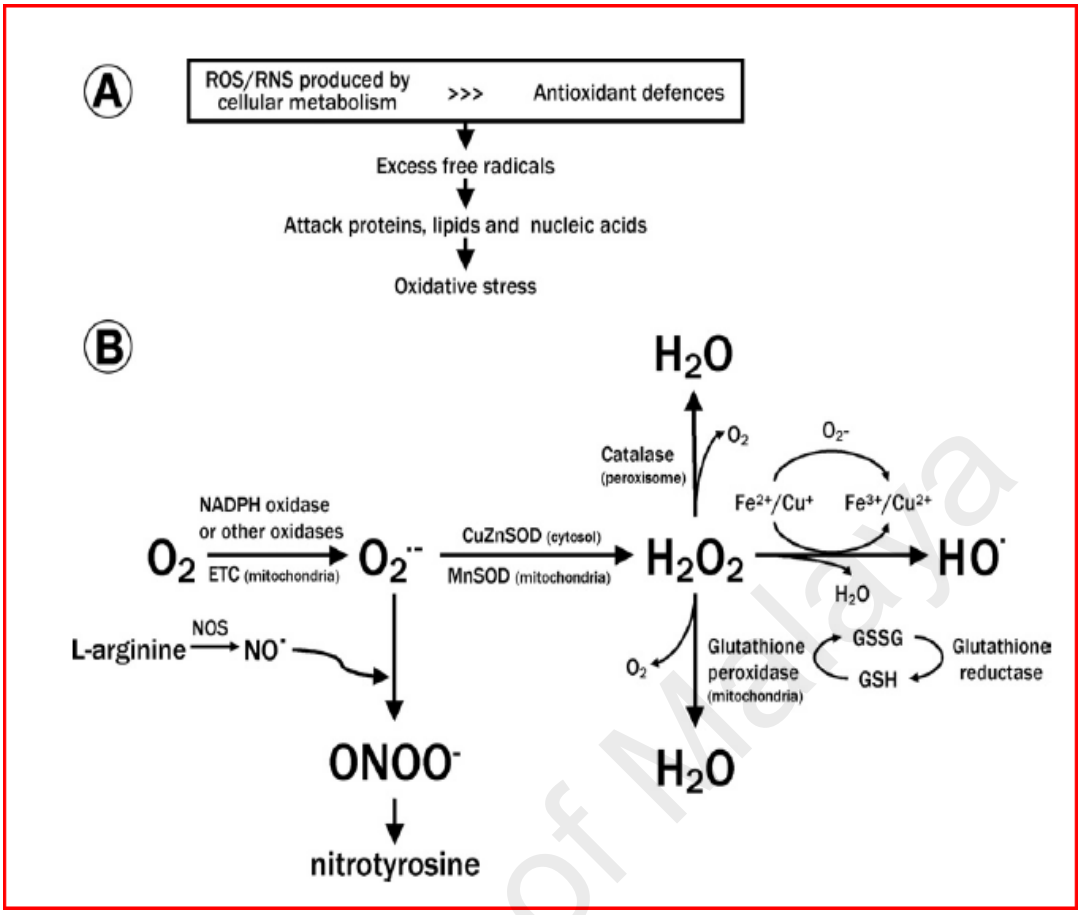


Figure 1.3: A and B. Oxidative stress and the generation of reactive species. (A) Oxidative stress results when highly reactive molecules including ROS and RNS are not sufficiently removed by antioxidant defences. (B) Generation of reactive species may occur when oxygen is converted to $O_2^{\cdot-}$, which then is dismutated to H_2O_2 by SOD. H_2O_2 may be converted to H_2O by catalase or glutathione peroxidase, or to $HO\cdot$ by reaction with copper (Cu) or iron (Fe). In addition, $O_2\cdot$-- also can react rapidly with $NO\cdot$ to form $ONOO^{-\cdot}$ (Adapted from Tan et al., 2007)

1.2.1.4. Non-enzymatic Sources of Oxidative Stress

Non-enzymatic sources of oxidative stress induced by Diabetes Mellitus include glucose auto-oxidation, advanced glycation, the polyol pathway, and the mitochondrial ETC (Johansen et al., 2005) (figure 1.4). It has been suggested that the primary initiating event in the development of diabetic complications is $O_2\cdot^-$ formation by mitochondria (Nishikawa et al., 2000). Hyperglycemia induces changes in the mitochondrial voltage gradient by increasing electron donors of the

ETC (Nishikawa et al., 2000). Diabetic rats have mitochondrial enlargement in renal proximal tubules associated with disturbed ATP metabolism (Kaneda et al., 1992). Furthermore, diabetic rats have altered mitochondrial permeability transition in the kidney (Oliveira et al., 2004).

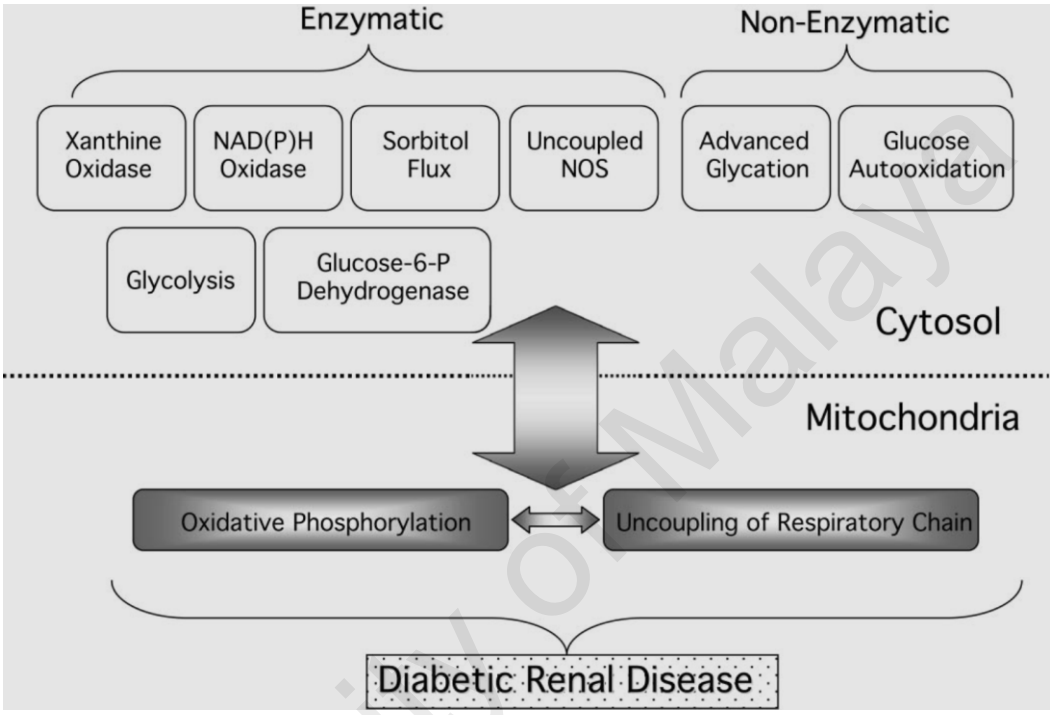


Figure 1.4: Cytosolic and mitochondrial sources of ROS implicated in the pathogenesis of DN. (adapted from Forbes et al., 2008)

1.2.1.5. Enzymatic sources of oxidative sources -NADPH Oxidase

Nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidases are known to generate reactive oxygen species (ROS). NADPH oxidase (NOX)-1 and NOX-2 are major sources of ROS, and are shown to be involved in the pathogenesis of endothelial dysfunction in early diabetic nephropathy (Li and Shah, 2003).

NADPH oxidases are composed of six subunits including two membrane-associated components (p22 phagocytic oxidase (phox) and gp91phox) and four cytosolic components (p47phox, p40phox, p67phox, and small GTPase Rac). NADPH oxidase is activated by membrane translocation of three cytosolic proteins (p47phox, p67phox, and small GTPase Rac). NADPH oxidases catalyse the transfer of electrons from the cytosol across biological membranes and into various intracellular and extracellular compartments (Lassegue and Griendling, 2010). At the biological membrane, these proteins assemble with gp91phox-p22phox heterodimer and induce a conformational change of gp91phox, resulting in superoxide production (Taura et al., 2009). A large and rapidly expanding body of experimental evidence implicates NADPH oxidases in the constitutive cells of the artery wall, as underlying causes of oxidative stress in various micro-vascular diseases, including nephropathy (Gorin et al., 2005; Kashihara et al., 2010; Capellini et al., 2010).

The NADPH oxidase activation is a key pathogenic mechanism involved in the induction and progression of diabetic nephropathy (Chakkarwar and Krishan, 2011). An increased understanding of the role of oxidative stress in the pathogenesis of diabetic nephropathy has led to the exploration of a number of anti-oxidant strategies to treat diabetic nephropathy.

1.3. Molecular Mechanisms of Oxidative Stress in Diabetic Nephropathy

Four major biochemical pathways are considered to lead to the development of diabetic complications associated with hyperglycemia: (a) the polyol pathway, glucose is converted to sorbitol and then metabolized to fructose (b) the

hexosamine pathway, fructose-6-phosphate is converted to glucosamine intermediates and the production of ROS is increased, (c) the protein kinase C (PKC) pathway, glucose is converted to glyceraldehyde-3-phosphate and leads to the formation of diacylglycerol (DAG). The elevation of intracellular DAG levels activate PKC, and then activate NADPH oxidase to induce ROS formation, (d) the formation of advanced glycation end products (AGEs), interaction of AGEs with the receptors of advanced glycation end-products (RAGE) results in ROS activation (Figure 1.5 and figure 1.6) (Koya et al., 2003; Stirban et al., 2006; Shah et al., 2009; Forbes et al., 2008; Brownlee 2005; Kanwar et al., 2008; Singh et al., 2011;Lazo-de-la-Vega-Monroy and Fernandez-Mejia, 2013).

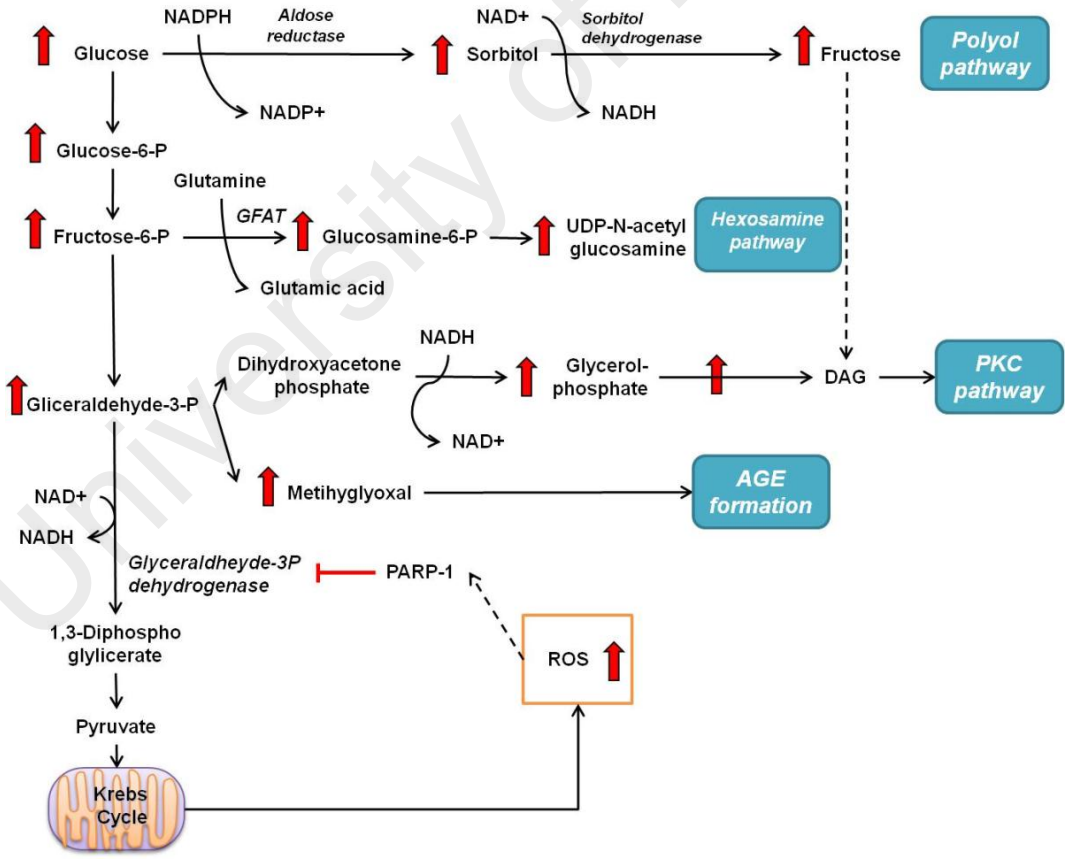


Figure 1.5: Potential mechanism by which hyperglycemia-induced mitochondrial superoxide overproduction activates four pathways of hyperglycemic damage. Adapted from Lazo-de-la-Vega-Monroy and Fernandez-Mejia (2013).

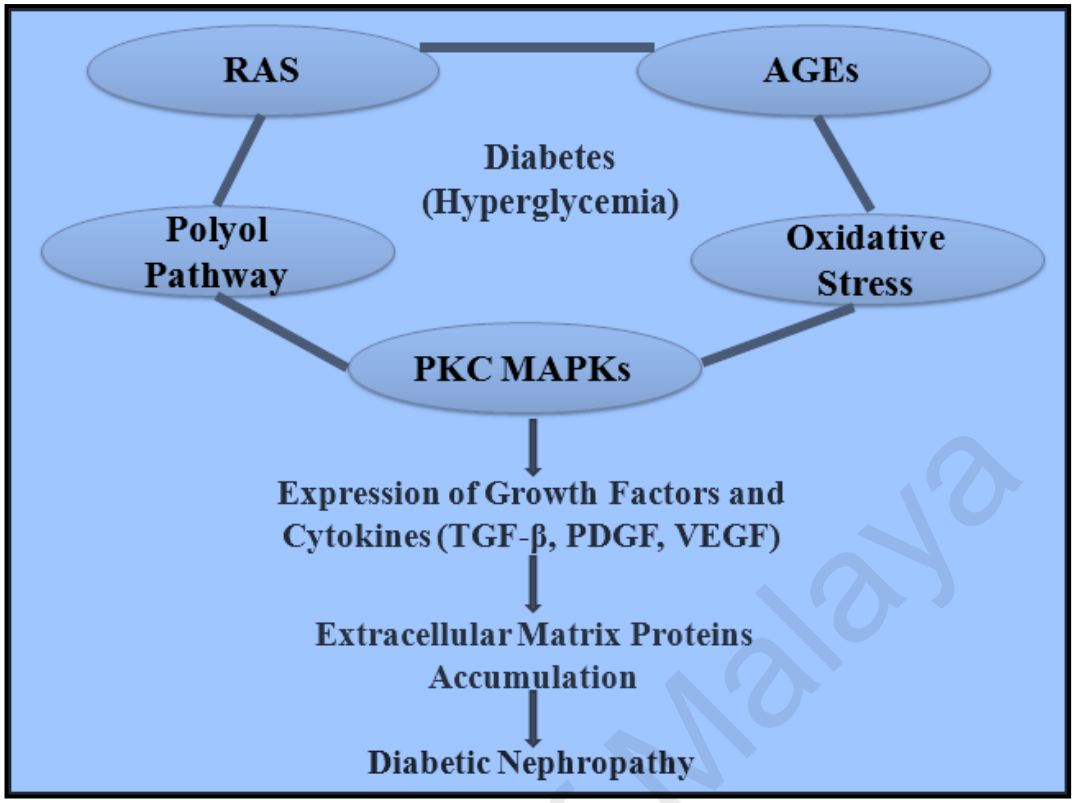


Figure 1.6: Biochemical hypothesis for diabetic nephropathy. Adapted from Koya et al., (2003)

Increased oxidative stress has been widely involved in the development and progression of Diabetes Mellitus and its complications including diabetic nephropathy (Maritim et al., 2003). ROS are generated in glomerular mesangial and tubular epithelial cells by high glucose, AGE, and cytokines (Park et al., 1999). Excessive amount of free radicals induce damage to cellular proteins, membrane lipids, nucleic acids, and then cell death (Maritim et al., 2003). Besides, increased ROS can cause vascular endothelium abnormalities, reacting directly with nitric oxide (NO) to produce cytotoxic peroxynitrite and increasing reactivity to vasoconstrictors and modification of ECM proteins (Schnackenberg, 2002). ROS can also damage endothelial cells indirectly by stimulating expression of various genes involved in inflammatory pathway (Baldwin, 1996). Previous

study finds that high glucose induces ROS and then up-regulates TGF-β1 and ECM expression in the glomerular mesangial cell (Lee et al., 2003). Ha et al. (2002) reported that ROS mediates high glucose-induced activation of NF-κB and NF-κB dependent monocyte chemoattractant protein (MCP)-1 expression. NF-κB, a nuclear transcription factor, can initiate the transcription of genes associated with inflammatory response. It is induced by various cell stress-associated stimuli including growth factors, vasoactive agents, cytokines, and oxidative stress (Kuhad and Chopra, 2009). Increased steady-state mRNA levels of inflammatory genes have been shown to associate with interstitial fibrosis and progressive human diabetic nephropathy (Kuhad and Chopra, 2009).

Transforming growth factor-β, a fibrotic cytokine that is up-regulated by ROS, plays an important role in the development of renal hypertrophy and accumulation of ECM components in diabetes mellitus (Wolf and Ziyadeh, 1999). The expression of TGF-β was found increased in diabetic nephropathy (Park et al., 1997; Yamamoto et al., 1993; Sharma et al., 1997; Shankland et al., 1994). Treatment with anti-TGF-β antibody has been documented to attenuate the effect of high glucose-induced cellular hypertrophy *in vitro* and in streptozotocin-induced diabetic mice (Wolf et al., 1992; Ziyadeh et al., 1994; Sharma et al., 1996). TGF-β is also the key regulator of ECM remodeling in mesangium causing mesangial expansion and inducing the process of epithelial-mesenchymal transition (EMT) causing tubulointerstitial fibrosis (Ziyadeh et al., 2000; Oldfield et al., 2001). There are also evidences that antioxidants can effectively inhibit high glucose-induced TGF-β1 and fibronectin up-regulation (Ha et al., 1997). Various signalling events induced by exposure of renal cells are shown in figure 1.7.

Although strict glycemic control is very important in diabetic patients, many of the current standard therapeutic approaches may also ameliorate oxidative stress as pleiotropic effects (Singh et al., 2011), such as angiotensin-2 converting enzyme (ACE) inhibitors (Kobayashi et al., 2006), angiotensin-2 receptor blockers (ARB) (Ogawa et al., 2006) and aldosterone blocker (spironolactone) (Takebayashi et al., 2006). They activate eNOS to increase bioavailability of NO, and inhibit synthesis of Ang-II and TGF-β and to decelerate or prevent tubulointerstitial fibrosis in diabetic nephropathy, accompanied with control of systemic and intra-renal blood pressure.

AGE products contribute to the pathogenesis of diabetic nephropathy via receptor mediated mechanisms and indirectly via the generation of ROS. Circulating levels of AGE products in diabetic patients were elevated with decreased renal function (Kubba et al., 2003). In addition, AGE accumulation in tissues correlated with the severity of organ injury, particularly with glomerular lesions (Kanauchi et al., 2001; Shimoike et al., 2000). In the development of diabetic nephropathy, excesses of AGEs such as pentosidine and carboxymethyllysine have been identified in the expanded mesangial area and thickened glomerular capillary wall (Horie et al ., 1997a; 1997b). *In vitro*, AGE products have been shown to increase TGF-β1 and ECM expression in glomerular endothelial and mesangial cells in hyperglycemic conditions (Chen et al., 2001; Doi et al., 1992; Skolnik et al., 1991; Scivittaro et al., 2000).

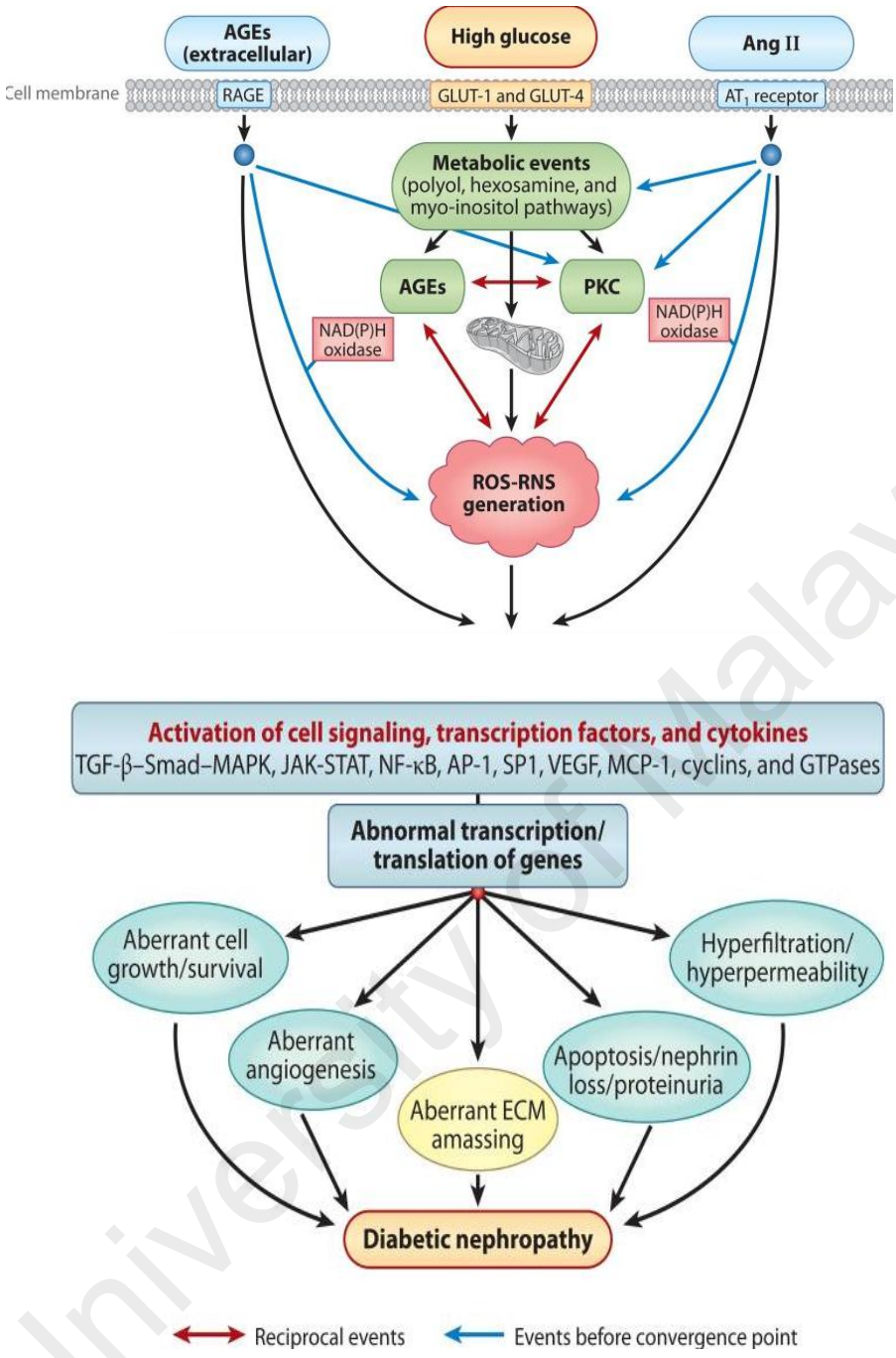


Figure 1.7: An overview of different signaling events induced by exposure of renal cells to high glucose concentrations, with resulting altered expression of various genes and cellular abnormalities leading to diabetic nephropathy. Adapted from Kanwar et al., (2011).

Abbreviations: Ang II, angiotensin II; AP-1, activator protein 1; AT1, Ang II receptor; ECM, extracellular matrix; GLUT, glucose transporter; JAK-STAT, Janus kinase–signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein 1; NF-κB; nuclear factor κB; RNS, reactive nitrogen species; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor.

Dietary AGE products are also thought to contribute to the development of diabetic nephropathy. Moreover, diets high in AGE content were known to impair insulin sensitivity (Hofmann et al., 2002). Various agents, including LR-90 (Figarola et al., 2003), aminoguanidine (Youssef et al., 1999) and alagebrium chloride (ALT-711) (Forbes et al., 2003) were potent in reducing AGE accumulation in renal tissues in experimental diabetic nephropathy, and subsequently improving renal function. Benefits were also seen in the clinical context with agents such as metformin, which decrease toxic dicarbonyls and AGE products in addition to its anti-hyperglycemic action (Ouslimani et al., 2007). Furthermore, benfotiamine (liposoluble vitamin B1 derivative), decreased AGE accumulation, inflammation and improved vascular function in T2DM patients consuming diets high in AGE content (Stirban et al., 2006).

Although numerous signaling mechanisms have been identified to be involved in the pathogenesis of diabetic nephropathy, a precise signaling pathway with a complete picture is yet to be identified. Current studies are therefore focused identifying major signaling culprits prominently involved in the pathogenesis of diabetic nephropathy. Studies till date support with strong evidences that renal oxidative stress due to activation of NADPH oxidase plays a pivotal role in the pathogenesis of diabetic nephropathy.

1.3.1. Role of NADPH Oxidase in the Pathogenesis of Diabetic Nephropathy

It has been suggested that free radicals are implicated in the development of diabetic microangiopathy and macroangiopathy (Barnett, 1991). Excessive free radical production has been reported in diabetics with chronic renal failure treated

by haemodialysis (Loughrey et al, 1994). Consequently, free radical mechanisms have been implicated in the pathogenesis of tissue damage in diabetes (Oberley, 1988; Packer, 1993; Wolff, 1993; Halliwell and Gutteridge, 1998).

Diabetic nephropathy is characterized by excessive deposition of ECM in the kidney, leading to glomerular mesangial expansion and tubulointerstitial fibrosis (Sedeek et al., 2010). Activation of NADPH oxidase induces oxidative stress, which is involved in the pathogenesis of various vascular diseases (Cai et al., 2003).

Oxidative stress-mediated vascular endothelial dysfunction could play a central role in the pathogenesis of diabetic nephropathy (Balakumar et al., 2009). Oxidative stress is apparent during the stage of glucose intolerance long before clinically apparent Diabetes Mellitus. The NADPH oxidase pathway constitutes the most important source of ROS in individuals with Diabetes Mellitus (Cave et al., 2006). Likewise, hyperglycemia-induced oxidative stress has been shown in patients with clinically established Diabetic Mellitus (Stentz et al., 2004). Importantly, NADPH oxidase is a cytosolic enzyme complex located in the plasma membrane of various renal cell types, including mesangial and proximal tubular cells, vascular smooth muscle cells, endothelial cells and fibroblasts (Griendling et al., 1994; Geiszt et al., 2000). This indicates the involvement of NADPH oxidase in the detrimental diabetic renal milieu.

Hyperglycemia can trigger the activation of the RAAS in patients with early T1DM (Miller, 1999). Angiotensin-II (Ang-II), an executive peptide of the

RAAS, is one of most potent inducers of NADPH oxidase and markedly contributes to ROS generation in diabetic nephropathy (Li and Shah, 2003). Hence, it may be considered that angiotensin II can directly activate NADPH oxidase in diabetic nephropathy. In the kidney, the primary role of the NADPH complex is as a signalling molecule (Figure 1.8). The enhanced protein kinase C (PKC)-mediated activation of NADPH oxidase by Ang -II or transforming growth factor β (TGF-β) leads to excessive generation of free radicals, which induce renal hypertrophy in rats and other detrimental effects on renal cell (Gorin et al., 2005).

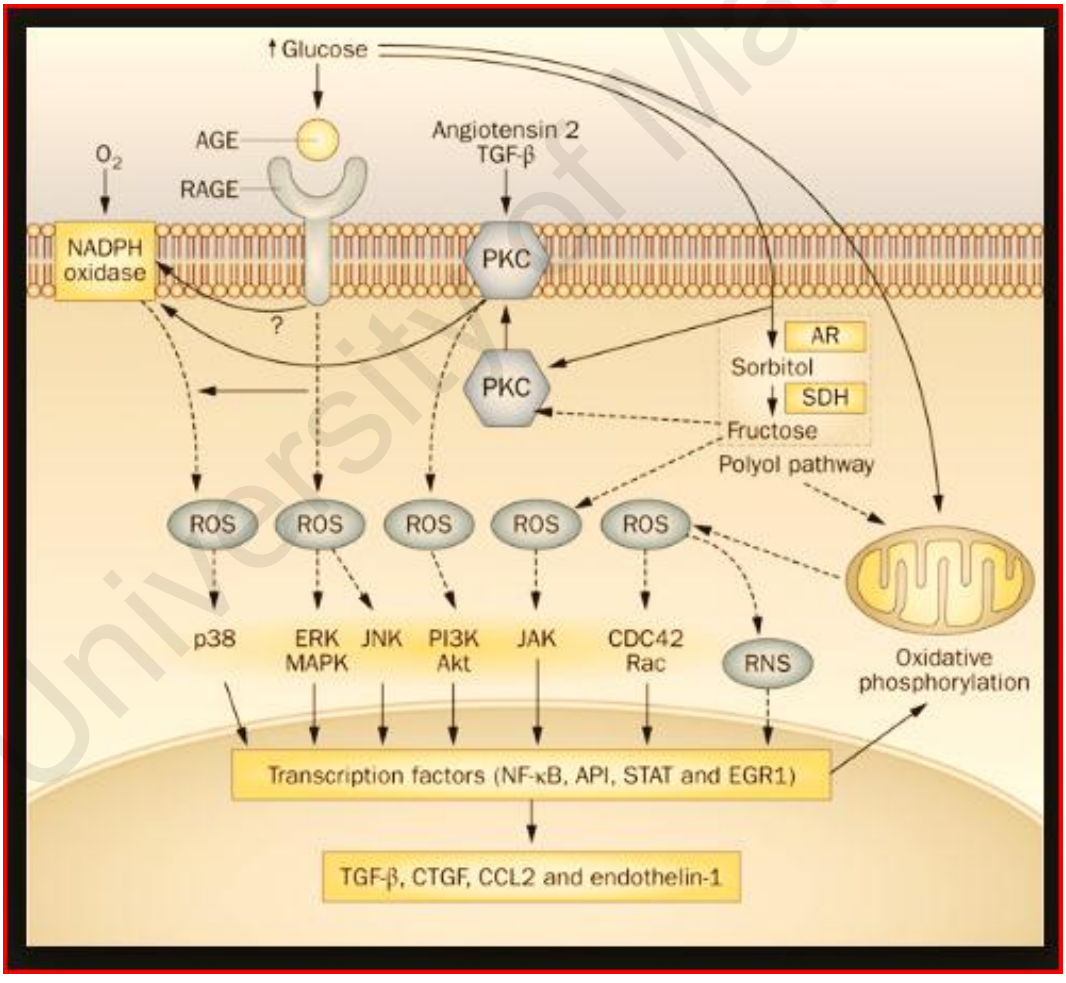


Figure 1.8: Organ damage can be triggered by both extracellular and intracellular hyperglycemia. Adapted from Singh et al., (2011).

The aforementioned evidences provide a rationale for the use of pharmacological inhibitors of NADPH oxidase to reduce oxidative stress and its associated vascular pathologies in diabetic nephropathy. Among various available inhibitors, apocynin and diphenyleneiodonium (DPI) are the most widely studied inhibitors. Other than apocynin and DPI, taurine is also noted as important NADPH oxidases inhibitor. The frequent albuminuria and development of glomerulopathy in diabetic rabbits was significantly attenuated by the treatment with taurine. Thus, the nephroprotective effect of taurine could be attributed to its NADPH oxidase inhibitory property (Winiarska et al., 2008). It is noteworthy that apocynin restored endothelial dysfunction in diabetic rats through regulation of nitric oxide synthase and NADPH oxidase expression (Olukman et al., 2010). Further, apocynin effectively blocked high glucose-induced ROS generation in mesangial cells (Lee et al., 2003). Moreover, treatment with apocynin markedly attenuated the renal oxidative stress induced via NADPH oxidase and prevented the development of diabetic nephropathy by decreasing the renal expression of fibronectin and collagen-I in diabetic rats (Asaba et al., 2005). Furthermore, inhibition of NADPH oxidase using apocynin attenuated the progression of diabetic nephropathy in rats by reducing the occurrence of albuminuria and preventing the development of glomerulosclerosis (Thallas-Bonke et al., 2008). The other injurious role of NADPH oxidase in diabetic nephropathy via occurrence of albuminuria by renal oxidative stress and glomerular expression of vascular endothelial growth factors in Otsuka Long Evans Tokushima Fatty (OLETF) rats was significantly attenuated with the use of apocynin (Nam et al., 2009).

Under diabetic conditions NADPH oxidase played a key role in methylglyoxal-induced renal fibrosis via superoxide generation (Ho et al., 2007). Herbal drugs have wide spread utility and are used as antioxidant in various microvascular diseases. Continuous administration of green tea, an antioxidant, downregulated NADPH oxidase in diabetic spontaneously hypertensive rats and significantly attenuated the development of nephropathy (Ribaldo et al., 2009). Since Diabetes Mellitus-mediated activation of NADPH oxidase induces oxidative stress, which is involved in renal damage, targeting NADPH oxidase is considered as an important therapeutic option to prevent development of diabetic nephropathy.

1.4. Antioxidants

Antioxidants such as vitamin C, vitamin E, β-carotene, α-lipoic acids and honey have been shown to ameliorate hyperglycemia through increased beta cell mass and insulin secretion (Rafighiet al., 2013; Hegde et al., 2013; Xu et al., 2013; Gariballa et al., 2013). In patients with type 1 diabetes, supplementation with vitamins E and/or C combination ameliorated oxidative stress and improved endothelium-dependent vasorelaxation (Johansen et al., 2005, Rahimi et al., 2005). A study found that supplementation with combined chromium (Cr) and vitamins C and E ameliorated oxidative stress, reduced fasting blood glucose, HbA1c and insulin resistance in type 2 diabetes (Lai, 2008). Similarly, a recent study reported that vitamin E supplementation significantly reduced malondialdehyde (MDA) levels and increased the concentrations of GSH in type 1 diabetic patients (Gupta et al., 2011). In patients with type 1 Diabetes Mellitus, vitamins C and E supplementation ameliorated oxidative stress markers, improved

vascular dysfunction, retinal blood flow and creatinine clearance (Scott and King, 2004).

α-lipoic acid (LA) supplementation markedly protected beta cells, reduced cholesterol levels, and attenuated albuminuria and glomerular mesangial expansion in diabetic mice. Renoprotection by LA was equally effective regardless of whether the dietary supplementation was started 4 weeks before, simultaneously with, or 4 weeks after the induction of diabetes by STZ. LA supplementation significantly improved DN and oxidative stress in the diabetic mice (Yi et al., 2011).

Green tea, which is well-researched for its antioxidant and anti-inflammatory properties, has been shown to be renoprotective. Catechin (CTN), a component of green tea, is responsible for the renoprotection. The therapeutic potential of CTN in STZ-induced diabetic rats was comparable with the effects of an angiotensin-converting enzyme inhibitor (ACEi) enalapril for the treatment of albumin excretion. After 12 weeks of 35 mg/d CTN treatment in drinking water, urinary albumin excretion and plasma creatinine concentrations in all the diabetic treatment groups were reduced, compared with the diabetic group with no treatment. Urine creatinine and creatinine clearance were higher in diabetic groups treated with CTN and ACEi compared with the diabetic group with no treatment. CTN has renoprotective properties comparable with ACEi, and co-administration of CTN and enalapril might be useful in reducing albumin excretion as well as improving endothelial function (Chennasamudram et al., 2012).

However, at the moment, less evidence is available to recommend antioxidants for the prevention of diabetic nephropathy. Instead, efforts are being made to

understand the role of ROS and oxidative stress in the pathogenesis and progression of diabetic nephropathy. Since oxidative stress is implicated in the pathogenesis of diabetes and its complications, there could be a place for antioxidants in the treatment of Diabetes Mellitus and its complications. However, previous clinical trials using antioxidants in the treatment of diabetic nephropathy have yielded both promising and inconsistent results (Mann et al., 2004; Vignini et al., 2011). Although the data from the large scale clinical trials are inconclusive, many of those clinical trials were linked with inappropriate study designs and several limitations (Johansen et al., 2005, Penckofer et al., 2002, Wierzba, 2005, Robinson et al., 2006, Willcox et al., 2008). These include (1) trials did not address specific diabetic populations; (2) some studies included both healthy and unhealthy subjects; (3) no data establishing the occurrence of oxidative stress in the patients before treatment and comparing such data with those obtained after treatment; (4) the short duration of treatment; (5) most of the trials were performed with vitamins A, C and E without consideration for other antioxidants; (6) the use of vitamin E supplementation without the concurrent use of vitamin C (Johansen et al., 2005, Penckofer et al., 2002, Wierzba, 2005, Robinson et al., 2006, Willcox et al., 2008). Other limitations include (1) some trials are gender-specific (comprising either men or women); (2) lack of pharmacokinetic data of antioxidants employed, before and after treatment, so as to ascertain if these antioxidants reached the target cells/tissues in adequate concentrations; (3) no data to show that effects of different doses of each antioxidant were investigated so as to obtain and select optimal dose; (4) vitamins inappropriately administered relative to meal ingestion; (5) poor patient compliance. The aforementioned points are some of the issues that could have contributed to the failure of antioxidants in

clinical studies using antioxidants in the treatment of diabetic nephropathy (Johansen et al., 2005, Penckofer et al., 2002, Wierzba, 2005, Robinson et al., 2006, Willcox et al., 2008). Hence, limited research and findings are available on the effects of antioxidants in diabetic patients. However, available evidences in small or medium sample-sized diabetic studies, both experimental and clinical, suggest antioxidants might play a role in diabetic nephropathy (Soory, 2012; Martini et al., 2010; Rahimi et al 2005; Zatalia and Sanusi, 2013).

These studies indicate that antioxidants could play a role in the management of Diabetes Mellitus and its renal complications. However, considering that Diabetes Mellitus is a disorder with multiple etiology and metabolic derangements, antioxidant supplementation alone is likely to be less effective.

1.5. Antioxidant act as a Pro-oxidant

Pro-oxidant can be defined as any endobiotic or xenobiotic that induces oxidative stress either by generation of ROS or by inhibiting antioxidant systems. In fact pro-oxidant can form all reactive, free radical containing molecules in cells or tissues. Although antioxidants function against oxidative damage induced by free radicals, they might also exhibit pro-oxidant action, leading to oxidative damage [Galati et al., 2006; Heim et al., 2002].

Ascorbic acid is a strong antioxidant, and it has been shown to reduce the oxidative indices against ischemia/reperfusion injury. Paradoxically, ascorbic acid has also been shown to have pro-oxidant property (Seo and Lee, 2002). The pro-oxidant action of ascorbic acid was revealed in a study by Seo and Lee (2002) who demonstrated that ascorbic acid acts as an antioxidant in low dose while

surprisingly it exhibits pro-oxidant action in high dose (Seo and Lee, 2002). Likewise, Park and Lee (2008) reported that ascorbic acid might act not only as an antioxidant but also as a pro-oxidant during cold ischemia/reperfusion in the liver (Park and Lee, 2008). In detail, cold ischemia/reperfusion was noted to decrease the reduced to oxidized glutathione ratio, whereas it increased the level of lipid peroxidation and mitochondrial swelling (Park and Lee, 2008). These changes were noted to be prevented by exposing the liver to 0.5 mM ascorbic acid but were however augmented at 2 mM ascorbic acid (Park and Lee, 2008), confirming that ascorbic acid might be an antioxidant or pro-oxidant depending upon the dose employed for a therapeutic purpose.

It is important to note that flavonoids such as myricetin, baicalein, and quercetin as well as ascorbic acid were noted to cleave plasmid pBR322 DNA and calf thymus DNA potently. However, addition of catalase was noted to protect the DNA from the strand breaks caused by flavonoids (Yoshino et al., 1999). The authors of this study concluded that the mutagenic and carcinogenic action of flavonoids might be due to pro-oxidant effects of the compounds (Yoshino et al., 1999).

Yoshino et al. (2002) reported that gallic acid and its alkylesters (polyphenolic compounds with anti-oxidative activity) also acted as a pro-oxidant, causing a copper-dependent DNA damage. Treatment of DNA from plasmid pBR322 and calf thymus with gallic acid plus copper ion was shown to cause a strand scission and the formation of 8-hydroxy-2'-deoxyguanosine in DNA. However, addition of catalase was shown to protect the DNA from the gallic acid/copper-dependent strand breaks and the formation of 8-hydroxy-2'-deoxyguanosine (Yoshino et al.,

2002). This study concluded that the cytotoxic effect of gallate compounds might be due to their pro-oxidant action dependent on the reducing activity (Yoshino et al., 2002). The anticancer and apoptosis inducing properties of green tea are mediated by its polyphenolic constituents particularly catechins (Azam et al., 2004). Green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) is considered as an effective chemopreventive and apoptosis-inducing agents. Plant polyphenols are naturally occurring antioxidants; however it is important to note that they might also have pro-oxidant properties (Azam et al., 2004). The copper oxidized catechins were suggested to be more efficient pro-oxidants as compared to their un-oxidized forms (Azam et al., 2004).

Yoshino et al. (2004) reported that curcumin, a well-known antioxidant in a principal ingredient of turmeric, acted as a pro-oxidant causing a copper-dependent DNA damage and apoptosis. Treatment of DNA from plasmid pBR322 and calf thymus with curcumin plus copper ion was shown to cause the strand scission and the formation of 8-hydroxy-2'-deoxyguanosine in DNA. However, addition of catalase was shown to protect DNA from the curcumin-dependent injuries (Yoshino et al., 2004). This study concluded that the pro-oxidant action of curcumin might be related to the conjugated beta-diketone structure of this compound (Yoshino et al., 2004).

1.6. Oil Palm

Palm leaves are abundant and under-utilised in tropical region like Malaysia, Indonesia, Thailand, Africa and South America. Palm leaves have been used for decades as ruminant feed without any reports of toxicity. The oil palm (*Elaeis guineensis*) leaves methanolic extract (OPLE) is rich in flavonoids and catechins

(Jaffri et al., 2011b). The OPLE is a potential economically viable, new source of catechins. The health benefits of catechins for the prevention of oxidative stress and hypercholesterolemia have been documented (Naghma and Hasan, 2007). The OPLE has been shown to have potent antioxidant property (Jaffri et al., 2011b; Han and May, 2010). The finding of the good *in-vivo* antioxidant property of OPLE forms the basis of this study, which is to evaluate the potential modulatory effects on diabetic nephropathy, in which oxidative stress plays a key role in the disease pathogenesis.

Catechins are flavonoid phytochemical compounds that are also present in green tea, black tea, grapes, wine, and chocolate. The polyphenol catechins present in green tea are gallocatechin (GC), epigallocatechin (EGC), epicatechin (EC), and epigallocatechin gallate (EGCG) (Waltner-Law et al, 2002). Due to their potent antioxidant capabilities, catechins, often referred to as "tea flavonoids," have been investigated for their ability to prevent cardiovascular and renal diseases. (Chyu et al., 2004; Tijburg et al., 1997; Miura et al., 2001; Jaffri et al 2011b; Runnie et al., 2003)

Chronic and uncontrolled diabetes mellitus is generally accompanied with nephropathy due to microvascular dysfunction or impairment. In normal kidney tissue, the production of thromboxane A2 (TXA2) and prostacyclin (PGI2) is controlled, and the balance between them is important to maintain homeostasis *in-vivo*. A modification of the PGI2:TXA2 ratio accelerates thrombogenesis in the renal tubules, increasing the risk of impaired function and atherosclerosis. The production of these compounds depends on the activity of phospholipase A2

(which is higher in the case of kidney disorders). Streptozotocin-induced diabetes increases the synthesis of TXA2 and decreases PGI2. Treatment with catechins derived from green tea in rats made diabetic by streptozotocin decreases the synthesis of TXA2 and increases PGI2 levels and subsequently restores the balanced ratio (Rhee et al., 2002a; 2002b).

Catechins, a subclass of compounds in the flavonoid family, have been found to have several biologically beneficial properties, including potent antioxidative effect. Flavonoids act as scavengers of free radicals or ROS, leading to cytoprotective effects against oxidative stress. More than two decades ago, it was reported that (+)-catechin inhibited protein and basal membrane synthesis in isolated glomeruli from diabetic rats (Hasslacher, 1980). Treatment with catechins supplementation reduced albuminuria in diabetic rats (Rhee et al., 2002a; 2002b).

Catechin was found to reverse the renal dysfunction as assessed by serum creatinine and blood urea nitrogen, however lower dose of catechin restored only the increased serum creatinine levels (Anjaneyulu et al., 2003). Likewise, EGCG had beneficial effects on diabetic nephropathy by suppressing hyperglycemia and related oxidative stress in kidney. Another catechin compound, epicatechin has been shown to prevent the progression of cisplatin-induced kidney injury by protecting mitochondria. Epicatechin was administered after the induction of cisplatin injury in the mouse kidney. Cisplatin significantly induced renal dysfunction and tubular injury along with an increase in oxidative stress. The renal damages and mitochondrial injuries were significantly prevented by epicatechin treatment. Such a protective effect of epicatechin might be attributed

to decreased oxidative stress. Epicatechin exhibited protective effects due in part to its ability to prevent the progression of mitochondrial injury in mouse cisplatin nephropathy. Epicatechin was therefore suggested to be a novel option to treat renal disorders associated with mitochondrial dysfunction (Tanabe et al., 2012).

1.7. Rationale of the Study

Diabetic nephropathy is one of the major complications in Diabetes Mellitus that needs immediate therapeutic intervention. The increasing prevalence and incidences of end-stage renal disease in Diabetes Mellitus contribute to increased cost and decreased quality of human life. Pre-clinical and clinical studies have demonstrated the implication of oxidative stress in the induction and progression of diabetic nephropathy. The oxidative stress can be due to several inter-related factors in the pathogenesis of Diabetes Mellitus. Hyperglycemic condition is one of the major factors that contribute to the oxidative stress in Diabetes Mellitus and its complications including diabetic nephropathy. Treatment with antioxidants such as green tea and antioxidant vitamins have produced marginal benefit in preventing the progression of diabetic renal complications (Lee et al., 2003) and human studies have shown that high doses of vitamin E failed to impart positive effect (Kanwar et al., 2008; Mustata et al., 2005). The outcomes of these studies lay emphasis on the significance of developing good novel antioxidant treatments for reducing the incidence and attenuating the progression of diabetic complications such as DN. Since the oil palm leaves extract (OPLE) has considerable amount of catechins and ferulic acid, we hypothesized that ethanolic extract of OPLE might have a modulatory effect on Diabetes Mellitus-induced

nephropathy. To address this issue, we have investigated the effect of ethanolic extract of OPLE treatment for 4 and 12 weeks in diabetic rats with nephropathy. According to previous work done in our laboratories (Muniandy, 2007) proteinuria and minor histological changes were observed at 4 weeks after diabetes induction. At 12 weeks, animals developed major histological changes in addition to massive proteinuria; hence 4 and 12 weeks were chosen to assess the renal functional studies and histological changes in the kidney.

1.8. Hypothesis:

Considering the presence of considerable amount of catechins and ferulic acid in the oil palm leaves extract (OPLE), we hypothesized that OPLE might have a potent anti-oxidant property, which in turn might have a modulatory effect on Diabetes Mellitus-induced nephropathy.

The extract of these leaves is rich in flavonoids and catechins. Catechins are polyphenolic compounds which possess antioxidant activity that are several folds higher than that of vitamins C and E.

1.9. Aims and Objectives

1. To assess the alteration in renal architecture and renal function due to chronic hyperglycemia-induced oxidative stress in an animal model
2. To investigate whether OPLE can attenuate/ prevent the progression of diabetic nephropathy in rats

3. To investigate the mechanisms of the antioxidant effect of OPLE in rats with diabetic nephropathy
4. To investigate the antioxidant and pro-oxidant effects of chronically administering high dose of OPLE in an animal model of diabetic nephropathy

1.10. Benefits of the Study

This project has been undertaken to study the therapeutic potential of ethanolic extract of oil palm leaves (OPLE) in rats with diabetic nephropathy. Conclusions arising from this study would be of therapeutic importance in understanding further the detrimental role of renal oxidative stress during the development of diabetic nephropathy. In addition, this study explored whether the anti-oxidant potential of OPLE would be beneficial or not in ameliorating the disease pathogenesis of nephropathy in chronic diabetic condition. Moreover, the output of this study would provide useful insights in understanding the mechanisms of oil palm leaves extract in terms of physiological and morphological changes in diabetic nephropathy.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Experimental Compounds	Supplier
OPLE Extract	Nova Laboratories, Malaysia
Streptozotocin	Sigma-Aldrich, USA

2.1.2. Phytochemical Studies

Equipment	Supplier
Orbital shaker	Boeco, Germany
UV-Vis spectrophotometer (mini 1240)	Shimadzu, USA
Spectrophotometer UV/VISIBLE	Perkin Elmer, USA
Vortex	Boeco, Germany

Reagents	Supplier
Aluminum trichloride	Sigma-Aldrich, USA
Dimethyl sulphoxide	Gaylord Company, UK
Folin-Ciocalteu reagent	Sigma-Aldrich, USA
Gallic acid	Sigma-Aldrich, USA
Methanol	Merck, Germany
Sodium bicarbonate	British Drug House, Germany
Quercetin	Sigma-Aldrich, USA

2.1.3. *In-vitro* study

Reagents	Supplier
Acetate buffer solution	Sigma-Aldrich, USA
Ascorbic acid	Sigma-Aldrich, USA
Butylated hydroxy toluene	Cayman Chemicals, USA
Ethanol	HmbG Chemicals, Germany
Ferric chloride	Sigma-Aldrich, USA
Ferrous sulpahte	Sigma-Aldrich, USA
Hydrochloric acid	Univar, USA
Trolox	Cayman Chemicals, USA
Quercetin	Sigma-Aldrich, USA
2,4,6-tripyridyl-s-triazine (TPTZ)	Sigma-Aldrich, USA

2.1.4. *In-vivo* study

Equipment	Supplier
Advantage glucometer	Roche, Switzerland
Angle poise lamp	Philips, Malaysia
Blood pressure transducer	Gould P23 ID,USA
Cannula	Smith Industries, UK
PP100 i.d. 0.86mm, o.d. 1.52mm	
PP50 i.d. 0.97mm, o.d. 0.58mm	
Fiber-optic light source	Euromex, Netherlands
Glucometer strip	Roche, Switzerland
Needles	Terumo, USA
25G X 1”, 23G X 1”, 21G X 1½’’	

Perfusor	B Braun, Germany
Powerlab 2/25	ADI, USA
Renal blood flow probe	Carolina Medical, USA
Surgical set	Red-Cross, USA
Surgical sutures	Britannica, UK
Square-wave electromagnetic flowmeter	Carolina Medical, USA
Syringes (1mL, 5 mL, 10 mL, 20 mL)	Terumo, USA

Reagents	**Supplier**
D-Glucose (dextrose)	BDH, Germany
Diethyl ether	BDH, Germany
Heparin	B Braun, Germany
Inulin	Sigma-Aldrich, USA
Sodium pentobarbitone (Nembutal)	CevaSante Animale, France
Sodium chloride	BDH, Germany

2.1.5. Biochemical Analyses

Equipment	**Supplier**
Centrifuge	Eppendorf 5415 D, USA
Flame photometer	Sherwood Model 420, USA
Hotplate and magnetic stirrer	Vision Scientific, Korea
Orbital shaker	Boeco, Germany
UV/Visible spectrophotometer (mini 1240)	Shimadzu, USA
Vortex	Boeco, Germany
100 ^{o}C water bath	Mermmett, Germany

Protein Assay

Total protein biuret reagent	Sigma-Aldrich, USA
Protein standard	Sigma-Aldrich, USA

Electrolyte Assay

Potassium chloride	BDH, Germany
Sodium chloride	BDH, Germany

Inulin Assay

Absolute ethanol	HmbG Chemicals, Germany
96% ethanol	HmbG Chemicals, Germany
Diphenylamine	Sigma-Aldrich, USA
Inulin	Sigma-Aldrich, USA
Sodium hydroxide (NaOH)	BDH, Germany
Sulphuric acid (H_2SO_4)	Univar, USA
Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)	BDH, Germany

2.1.6. Enzyme-Linked Immunosorbent Assay (ELISA)

Equipment	**Supplier**
Centrifuge	Biofuge Pico Heraeus, USA
Elisa plate reader (KC Junior)	PowerWave$_x$340, USA
Homogenisation Glass Tube	GlasCol^{TM}, USA
Orbital Shaker	Boeco, Germany
Sonicator	Vir Tis, USA
Tissue homogenizer	Gilson, USA

8-channel multipipette	Eppendorf, USA
96-well plate, flat bottom with lid	Nunc Maxisorp, USA

Kits	**Supplier**
8-hydroxy-2-deoxy Guanosine EIA Kit	Cayman Chemical, USA
Glutathione Assay Kit	Cayman Chemical, USA
Lipid Hydroperoxide (LPO) Assay Kit	Cayman Chemical, USA
TGF-β1 Assay Kit	Abnova Manufacturer, USA

Other Reagents for Above Kits

Ethylene diamine tetraacetic acid (EDTA)	Sigma-Aldrich, USA
Metaphosphoric acid	Sigma-Aldrich, USA
Phosphate buffer saline	Bio-Rad, USA
Triethanolamine	Sigma-Aldrich, USA
2-Vinylpyridine	Sigma-Aldrich, USA
Chloroform	Merck, Germany
Methanol	Merck, Germany

2.1.7. Histology

Haematoxylin and Eosin Staining

Equipment	**Supplier**
Automated tissue processor	Leica, Germany
Coplin jars	Labsystem, USA
Embedding machine	Leica, Germany
Light microscope	Olympus CH-2, USA

Microscope glass slides	Sigma-Aldrich, USA
Microscope slide coverslips	Hirschmann, USA
Microtome	Leica, Germany
Microtome blade	Feathers, USA
40 ^{o}C water bath	Leica, Germany

Reagents	**Supplier**
Absolute alcohol	HmbG Chemicals, Germany
Dextropropoxyphene (DPX)	BDH, Germany
Eosin	BDH, Germany
Formalin	Surgipath, USA
Harris' Haematoxylin	BDH, Germany
Paraffin wax	Paraplast, UK
Sodium acetate	BDH, Germany
Sodium cacodylate	Agar Scientific, UK
Xylene	BDH, Germany

2.1.8. Immunohistochemistry Staining Assay

Equipment	**Supplier**
Automated tissue processor	Leica, Germany
Coplin jars	Labsystem, USA
Embedding machine	Leica, Germany
Light microscope	Olympus CH-2, USA
Microscope slide coverslips	Hirschmann, USA
Microtome	Leica, Germany

Microtome blade	Feathers, USA
40^{o}C water bath	Leica, Germany
Poly-L-lysineglass slides	Sigma-Aldrich, USA
Decloaking chambers	Biocare Medical, USA
Plastic Staining jar	Sigma-Aldrich, USA

Dako ARK^{TM} (Animal Research Kit),	**DAKO, USA**

Other Reagents for DAKO Kits	**Supplier**
Absolute alcohol	HmbG Chemicals, Germany
Dextropropoxyphene (DPX)	BDH, Germany
Formalin	Surgipath, USA
Harris' Haematoxylin	BDH, Germany
Paraffin wax	Paraplast, UK
Phosphate buffer saline	Bio-Rad, USA
Potassium acetate	BDH, Germany
Sodium acetate	BDH, Germany
Sodium cacodylate	Agar Scientific, UK
Xylene	BDH, Germany

2.1.9. Western Blotting Assay

Equipment	**Supplier**
Autoclave	All American Sterilizer
Centrifuge	Biofuge Pico Heraeus, USA
Chemiluminescence	UVP, Bio Spectrum, USA

Elisa plate reader (KC Junior)	PowerWave$_x$340, USA
Electrophoresis power supply E844	Consort, USA
Mini-Protean tetra cell	Bio-Rad, USA
Mini trans-blot electrophoretic transfer cell	Bio-Rad, USA
Orbital Shaker	Boeco, Germany
Spectrophotometer	Perkin Elmer, USA
Thermo block TDB	Boeco, Germany
Tissue Homogenizer	Gilson, USA

Other Reagents	**Supplier**
Absolute methanol	HmbG, Germany
Bio-Rad Precision Protein Western C	Bio-Rad, USA
ECL Detection reagent	GE Health care, USA
Laemmli sample buffer	Bio-Rad, USA
Lysis solution	Sigma-Aldrich, USA
Mercaptoethanol	Sigma-Aldrich, USA
Mini-PROTEAN TGX Gel, 4-20%	Bio-Rad, USA
Non fat dry milk	Bio-Rad, USA
Phosphate buffer saline (PBS)	Bio-Rad, USA
Phosphatase cocktail	Sigma-Aldrich, USA
Protease inhibitor cocktail	Sigma-Aldrich, USA
PVDF membrane	GE Health care, USA
Sodium azide	Sigma-Aldrich, USA
Tweezer	Sigma-Aldrich, USA
10X Tris/Glycine/SDS	Bio-Rad, USA

10X Tris/Glycine	Bio-Rad, USA

Bicinchoninic Acid (BCA) Protein Assay Kit	**Bio-Rad, USA**

NADPH Oxidase Subunits

p22phox	Santa Cruz Biotechnology, USA
p67phox	Gene Tex,USA
β-actin	Santa Cruz Biotechnology, USA
HRP-conjugated goat antirabbit IgG	Santa Cruz Biotechnology, USA

2.2 Methods

2.2.1. Study Overview

The study was divided into three parts viz. phytochemical studies, *in vitro* studies and *in vivo* studies. In phytochemical studies, total phenolic content and total flavonoid content were analyzed. Diphenyl picryl hrdrazine assay (DPPH) and ferric reducing antioxidant power assay (FRAP) were carried out *in vitro*. *In vivo* studies included pharmacological evaluation of OPLE in diabetes-induced experimental nephropathy in rats. All the experimental procedures were approved (FAR/20101106/NAA-R) and complied with the guidelines of the care and use of laboratory animals at the University of Malaya, Kuala Lumpur, Malaysia.

2.2.2. Phytochemical Studies

2.2.2.1. Total Phenolic Content (TPC)

The TPC of OPLE was estimated according to Folin-Ciocalteu method (Singleton et al., 1965). OPLE solution (5µL) (10 mg/mL in methanol) was transferred to a 96-well microplate (TPP, USA) in triplicate. Folin-Ciocalteu reagent (80 µL) (1:10) was subsequently added and mixed well. After 5 min, 160 µL sodium bicarbonate solution (7.5%) was added and the mixtures were incubated for 30 min with intermittent shaking. Absorbance was measured at 765 nm using a microplate reader (Molecular Devices, Sunnyvale, USA). The TPC was expressed as gallic acid equivalent (GAE) in mg/g extract, obtained from the standard curve of gallic acid. The gallic acid standard plot ($y=0.0018x + 0.0039$; $R^2=0.9975$) was madeby plotting the curve of concentration (mg/mL) versus absorbance (nm).

2.2.2.2.Total Flavonoid Content (TFC)

The TFC was determined by aluminium trichloride ($AlCl_3$) method using quercetin as standard (Eghdami and Sadeghi, 2010) with slight modification. OPLE was dissolved in dimethyl sulphoxide (DMSO, 1 mg/mL) and the sample solution (1.0 mL) was mixed with 1.0 mL of $AlCl_3$ (0.15 mol/L). Following incubation for 10 min at ambient temperature, the absorbance of the supernatant was measured at 435 nm using Shimadzu UV-Vis spectrophotometer (Mini 1240). The samples were prepared in triplicate. The total flavonoid content was expressed as quercetin equivalent (QE) in mg/g extract. The standard plot ($y=0.0019x+0.0358$; $R^2=0.994$) of quercetin was made by plotting the curve of concentration (mg/mL) versus absorbance (nm).

2.2.3. *In vitro* Antioxidants Assays of OPLE

2.2.3.1. Assessment of Free Radicals Scavenging Capacity Using DPPH Assay

The scavenging activity of OPLE on diphenylpicrylhydrazyl (DPPH) was determined according to the method described by Choi et al. (2002). This method measures the reduction of purple DPPH to yellow colour diphenyl picrylhrdrazine. The reaction mixture comprised of 1 mL of freshly prepared 0.3 mM DPPH in ethanol and 2.5 mL of 25 µg/mL of OPLE sample solution. Blank sample comprised of 1 mL DPPH solution and 2.5 mL ethanol. Standard antioxidants such as ascorbic acid and butylated hydroxytoluene (BHT) were used as positive controls. All reactions mixtures were kept in dark condition for 30 min at room temperature, and absorbance was measured at 518 nm. The absorbance values were converted into percentage inhibition using the following equation:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \text{ X } 100$$

Where A_B denotes absorbance of blank sample and A_A denotes absorbance of test sample. To assess the antioxidant capacity of OPLE, the IC_{50} of DPPH scavenging activity of OPLE was determined.

2.2.3.2. Ferric Reducing / Antioxidant Power Assay (FRAP Assay)

Modified method described by Benzie and Strain (1996) was employed for the determination of total anti-oxidant activity (FRAP assay) of OPLE. The stock solutions included 300 mM acetate buffer solution (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM HCL, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3.6H_2O$, and the solution temperature was raised to 37 °C before use. Samples (10 µL) were allowed to react with 300 µL of the FRAP solution after 4 min in dark condition. The intensities of the coloured product (ferrous tripyridyltriazine complex) were measured at 593 nm. The standard curve was linear between 100 and 1000 µM $FeSO_4$. Results were expressed in µM Fe (II)/g dry mass and compared with that of quercetin and trolox.

2.2.4. Experimental Design

The *in vivo* study was carried out for both 4-week and 12-week duration after the induction of experimental diabetes mellitus (Figures 2.1 and 2.2). This study permitted a controlled environment and complete anaesthesia for invasive parameter measurements. Figure 2.3 illustrates a flowchart of the study design for the both durations. The metabolic parameters, renal haemodynamic and excretory functions were analysed. At the end of the study, kidneys were incised for

morphological and histopathological studies, and for assessment of oxidative stress parameters. In addition, blotting techniques were carried out to assess the expression pattern of the NADPH oxidase subunits such as p22phox and p67phox. Experimental parameters that were measured in this study are shown in Table 2.1.

Protocol 1

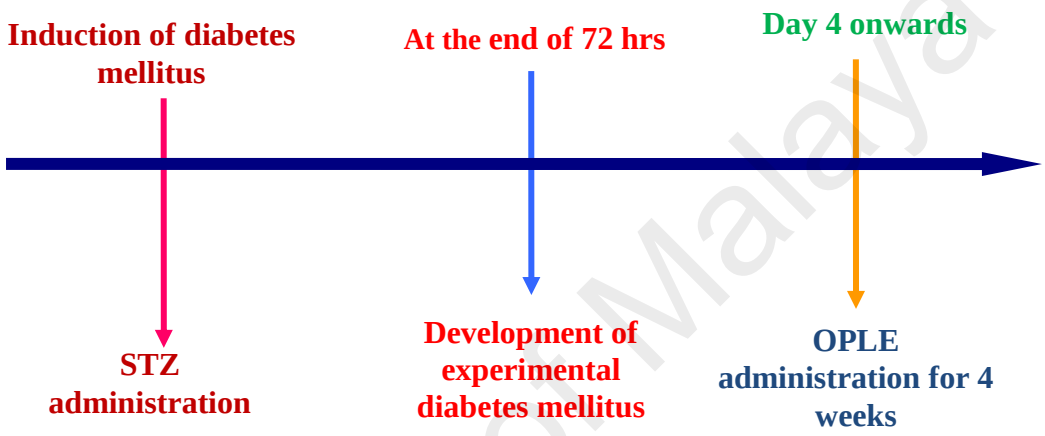


Figure 2.1: Schedule of the *in vivo* studies

Protocol 2

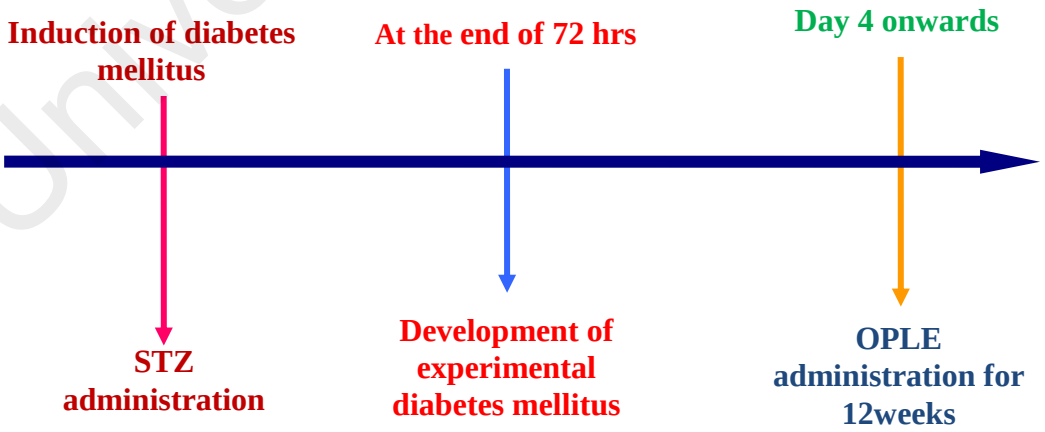


Figure 2.2: Schedule of the *in vivo* studies

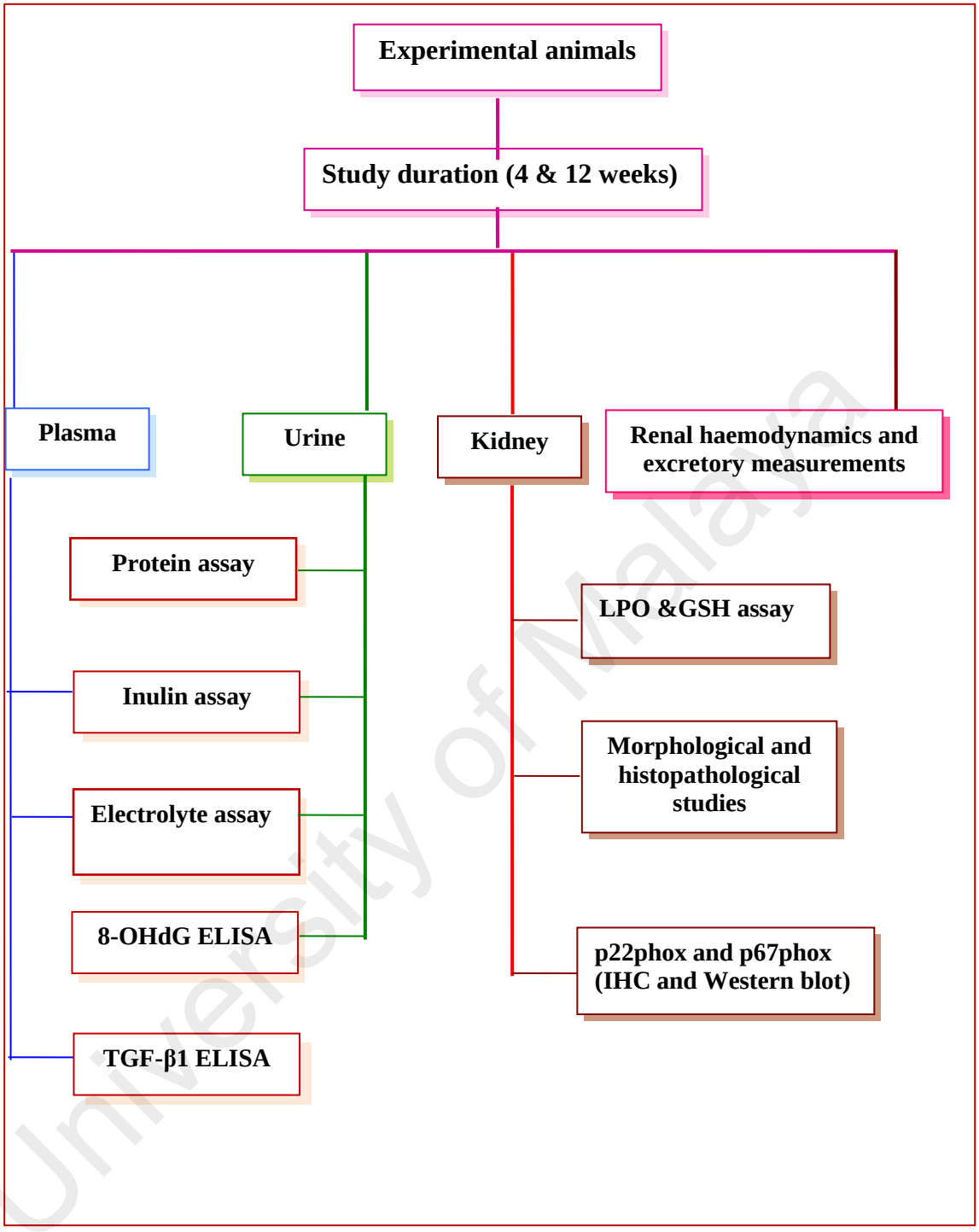


Figure 2.3: Flowchart illustrating the outline of the study

Table 2.1: Renal parameters that were assessed in this study

Assays / Measurements	Renal Parameters
Protein assay	Total protein excretion (mg/2h)
Electrolyte assay	Urine Na^+ and K^+concentration (µmol/L) Plasma Na^+concentration (µmol/L) Absolute Na^+ and K^+excretion (µmol/min/kg) Fractional Na^+excretion (%)
Inulin assay	Urine inulin concentration (mg/mL) Plasma inulin concentration (mg/mL) Inulin clearance (mL/min/kg)
Other renal haemodynamics and excretory measurements	Urine flow rate (µL/min/g kidney) Systemic blood pressure (mmHg) Renal blood flow (mL/min/g kidney)

2.2.5. Induction of diabetes mellitus in rats

2.2.5.1. Principle

Streptozotocin (STZ) is an antibiotic derived from *Streptomyces achromogenes* and is structurally a glucosamine derivative of nitrosourea. Rakieten and his associates first demonstrated the diabetogenic action of the STZ in dogs and rats in the year 1963 (Rakieten et al., 1963). STZ causes hyperglycemia by the virtue of its deletetious cytotoxic action on the pancreatic beta cells of islets of Langerhans. Its nitrosourea moiety plays a key role in inducing pancreatic beta cells toxicity where as its deoxyglucose moiety could facilitate its transport across

the cell membrane. Generation of ROS and subsequent induction of high oxidative stress in the pancreatic beta cells play a primary role in STZ-associated diabetogenic action. Moreover, STZ could cause DNA strand breakage, which could activate the DNA repairing enzyme like poly-ADP-ribose polymerase (PARP). However, overactivation of PARP depletes cellular storage of NAD and ATP leading to necrosis and pancreatic beta cells loss (Srinivasan and Ramarao, 2007).

2.2.5.2. Method of Induction

Sprague Dawley rats (*Rattus norvegicus*) weighing between 270-330 g were used in the present study (source: University of Malaya Animal Facility). Diabetes mellitus was induced in rats via a single intraperitoneal injection of STZ (Sigma-Aldrich, USA) at the dose of 60 mg/kg body weight. The STZ was dissolved in 150 mM sodium chloride (BDH Chemicals) prior to injection. Random blood glucose level was monitored 72 hrs after STZ administration. The tail was pricked with a needle and a small volume of blood was drawn to measure blood glucose level using Advantage^{TM} glucometer. A value of 15 mmol/L and above was accepted as successful diabetes mellitus induction.

2.2.6. *In vivo* study

2.2.6.1. Animals Classification and Duration

One hundred and twenty five healthy male Sprague Dawley (SD) rats, weighing between 270-330 g were used in this study. The animals were weight-matched and divided into 10 groups; (A-E) for 4 week study duration (each group comprised 10 rats, n= 10) and (F-J) for 12 week study duration (each group comprised 15 rats,

n=15). Table 2.2 and table 2.3 show the list of the experimental groups and their respective treatments. Overall mortality rate of the present study was noted to be 8%.

Three doses of OPLE (200 mg/kg, 500 mg/kg and 1000 mg/kg) were tested in our animals based on the antioxidant studies in previous research (Jaffri et al., 2011; Ibraheem et al., 2011; Rosalina Tan et al., 2011)

Protocol 1

A. Normal control rats were maintained on free acess to water and food. No treatment was given. Normal saline was administered once intraperitoneally.
B. Diabetic control: Rats were administered STZ (60 mg/kg i.p.) for the induction of diabetic mellitus
C. Diabetes + OPLE 200 mg/kg: After confirmation of diabetes i.e. after 3 days of STZ administration the diabetic rats were treated with OPLE (200 mg/kg/day p.o.) for 4 weeks
D. Diabetes + OPLE 500 mg/kg: After confirmation of diabetes i.e. after 3 days of STZ administration the diabetic rats were treated with OPLE (500 mg/kg/day p.o.) for 4 weeks
E. Diabetes + OPLE 1000 mg/kg: After confirmation of diabetes i.e. after 3 days of STZ administration the diabetic rats were treated with OPLE (1000 mg/kg/day p.o.) for 4 weeks

Table 2.2: Experimental animal groups and treatment for protocol 1

	Experimental groups	Drugs and chemicals	Dose, route and duration
A	Normal control	-------	
B	Diabetic control	STZ	60 mg/kg *i.p.*
C	Diabetes + OPLE 200 mg/kg	STZ + OPLE	60 mg/kg *i.p.* + 200 mg/kg *p.o.*for 4 weeks
D	Diabetes + OPLE 500 mg/kg	STZ + OPLE	60 mg/kg *i.p.* + 500 mg/kg *p.o.*for 4 weeks
E	Diabetes + OPLE 1000 mg/kg	STZ + OPLE	60 mg/kg *i.p.* + 1000 mg/kg *p.o.*for 4 weeks

Protocol 2

G. Normal control rats were maintained on free acess to water and food. No treatment was given. Normal saline was administered once intraperitoneally.

H. Diabetic control: Rats were administered STZ (60 mg/kg i.p.) for the induction of diabetic mellitus

I. Diabetes + OPLE 200 mg/kg: After confirmation of diabetes i.e. after 3 days of STZ administration the diabetic rats were treated with OPLE (200 mg/kg/day p.o.) for 12 weeks

J. Diabetes + OPLE 500 mg/kg: After confirmation of diabetes i.e. after 3 days of STZ administration the diabetic rats were treated with OPLE (500 mg/kg/day p.o.) for 12 weeks

K. Diabetes + OPLE 1000 mg/kg: After confirmation of diabetes i.e. after 3 days of STZ administration the diabetic rats were treated with OPLE (1000 mg/kg/day p.o.) for 12 weeks

Table 2.3: Experimental animal groups and treatment for protocol 2

	Experimental groups	Drugs and chemicals	Dose, route and duration
F	Normal control	-------	
G	Diabetic control	STZ	60 mg/kg *i.p.*
H	Diabetes + OPLE 200 mg/kg	STZ + OPLE	60 mg/kg *i.p.* + 200 mg/kg *p.o.*for 12 weeks
I	Diabetes + OPLE 500 mg/kg	STZ + OPLE	60 mg/kg *i.p.* + 500 mg/kg *p.o.*for 12 weeks
J	Diabetes + OPLE 1000 mg/kg	STZ + OPLE	60 mg/kg *i.p.* + 1000 mg/kg *p.o.*for 12 weeks

2.2.7. Surgical Procedure

At the end of the 4th and 12th weeks of treatment protocols, rats were anaesthetised with sodium pentobarbitone, 60 mg/kg intraperitoneally. The fur at the neck (ventral), left thigh (ventral) and lower left dorsal of the rat was removed. All areas of incision were cleansed with 70% ethanol. The rat was positioned on a surgical platform with the ventral facing up, and was kept warm throughout the surgical procedure using an angle poise lamp. A tracheotomy was carried out using a PP100 cannula to facilitate respiration (Figure 2.4). Subsequently, the left jugular vein was identified and cannulated using a PP50 cannula for infusion of anaesthesia (sodium pentobarbitone at 12.5 mg/kg/hr) and inulin at 10 mg/mL concentration. The infusion was administered at a flow rate of 3.0 mL/h using a perfusor pump throughout the experimental procedure. The left carotid artery was then cleared and cannulated with a PP50 cannula pre-filled with heparinised saline

(50 units/mL).The cannula was then connected to a pressure transducer linked to a Powerlab® system for arterial blood pressure measurement. The left femoral artery was cannulated with a PP50 cannula for blood collection. The cannula was also pre-filled with heparinised saline to avoid formation of blood clots. The bladder was catheterised using a PP50 cannula for urine collection. All surgical openings were covered with cotton gauze soaked in saline.

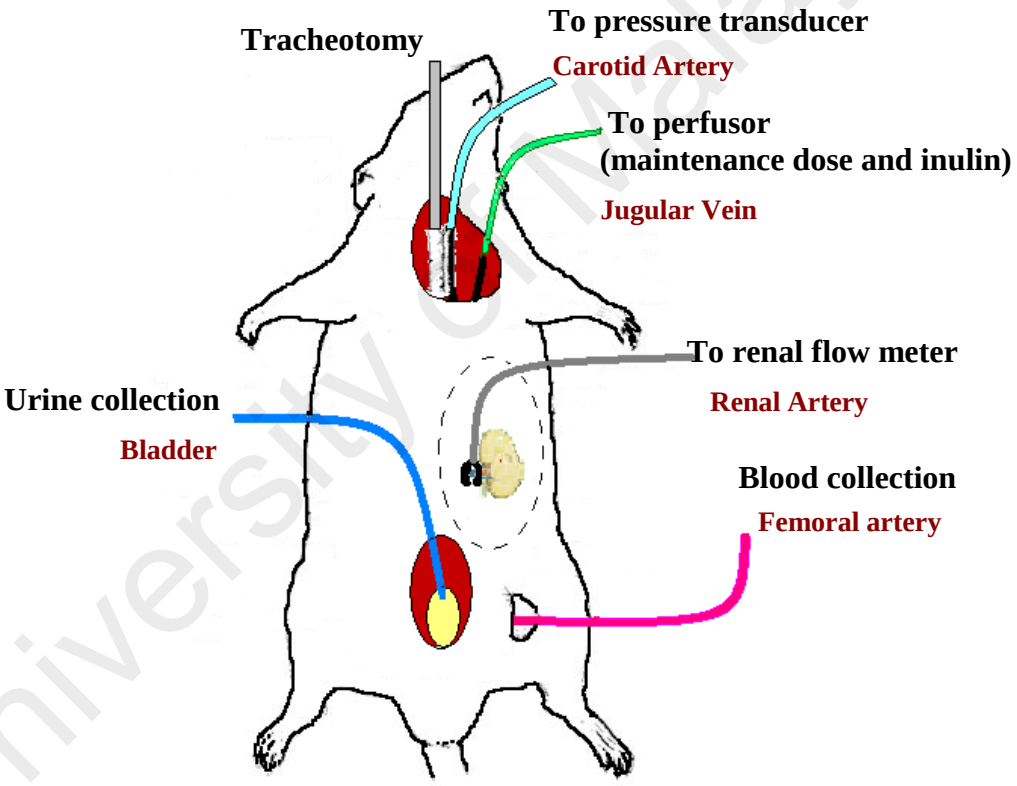


Figure 2.4: *In vivo* study

The animal was repositioned such that the left dorsal plane was facing up and all initial cannulations remain unobstructed. A flank incision was performed to expose the left kidney and six knots were made along the periphery of the opening

using surgical sutures. Each extension of the sutures was tied to a metal ring frame fixed to a retort stand in order to widen the view of the surgical area (Figure 2.5). The renal artery was isolated from the renal vein and an electromagnetic flow probe was fitted around the artery for the measurement of renal blood flow. The flow probe was connected to a flowmeter linked to the Powerlab®system for recording.

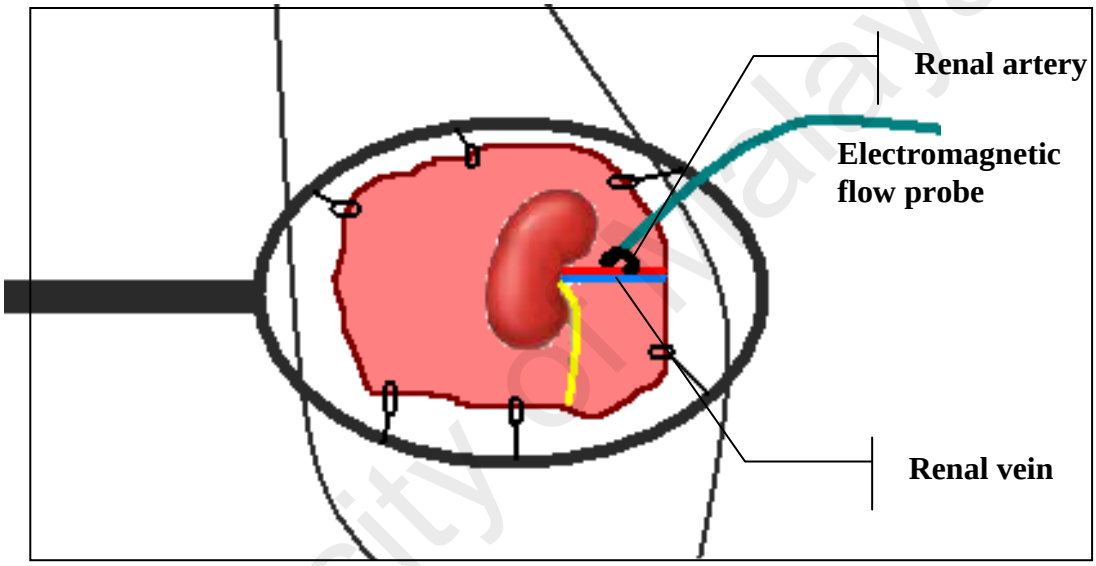


Figure 2.5: Flank incision to expose the left kidney

2.2.8. Experimental Procedure

A priming dose of 2 mL of inulin in saline (10 mg/mL) was administered via the jugular vein upon completion of the surgical procedure and the animal was allowed to stabilize for 60 to 90 min (Figure 2.6).

After the stabilization period, 0.6 mL of blood was withdrawn via the femoral artery using a 1 mL syringe. The blood was transferred into a 1.5 mL Eppendorf tube and immediately centrifuged at 6000 rpm for 2 min to separate the plasma.

Clear plasma was transferred into a clean 1.5 mL eppendorf tube whilst the packed blood cells were resuspended in an equal volume of normal saline and was infused back to the animal via the femoral artery. The animal was allowed to rest for 5 to 10 min until the baseline blood pressure was achieved following blood withdrawal and reinfusion procedures. Then a 30-min urine collection was started (one clearance period). Urine was collected in a clean 1.5 mL pre-weighed eppendorf tube. The difference between the initial and final weight of the Eppendorf tube indicated the volume of the urine.

The experimental procedure consisted of four clearance periods of urine (urine was collected at every 30-min interval for four times) and three times blood collections (the blood was withdrawn at 0 hr, 1 hr and 2 hr). All urine and plasma samples were stored at -20 ^{o}C until further analysis. Mean arterial blood pressure and renal blood flow values were also recorded throughout the experimental procedure.

At the end, the rat was sacrificed by a rapid intravenous injection of 1 mL sodium pentobarbital and the right kidney was removed, dissected into cortex and medulla, and frozen in the liquid nitrogen (stored at -80 °C) for measurement of GSH and LPO. The left kidney was perfused first with ice-cold phosphate buffered saline until cleared of blood and then fixed with 10% formalin for histological studies.

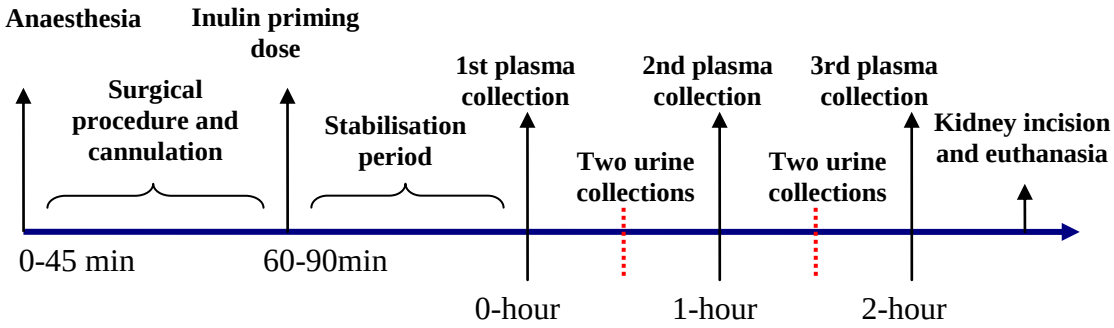


Figure 2.6: The time frame of the *in vivo* study

2.3. Biochemical Analyses

2.3.1. Protein Assay

2.3.1.1. Principle

Protein molecules consist of peptide bonds that are unique to protein. The biuret reagent contains copper ions, which can specifically interact with these bonds. The resultant purple complex has a maximum absorbance at 540 nm, and the intensity of the colour is directly proportional to the total protein concentration present in the sample. Thus, plotting a standard curve with variable concentrations of protein standards can be employed to quantify the concentration of total protein present in samples.

2.3.1.2. Method

Total protein concentration in urine samples was measured according to the method of Doumas et al. (1981). Total protein reagent (Sigma Diagnostics®) was used in the assay. Protein standards were prepared in a concentration range of 0-80 mg/mL from a protein stock of 80 mg/mL concentration. Twenty microlitres (20 µL) of standard or urine sample was added to 1.0 mL of total protein reagent

in a test tube. A blank was prepared by substituting the standard with distilled water. The contents were mixed-well and incubated at room temperature for 10 min. Upon incubation, the mixture was transferred into a cuvette for the measurement of absorbance at 540 nm against the blank using the spectrophotometer. A standard curve of absorbance versus concentration was prepared to determine the protein concentration in the urine samples.

2.3.2. Electrolyte Assay

2.3.2.1. Principle

Sodium and potassium concentrations in urine and plasma samples were measured using flame photometry technique. Samples in the form of aerosols are aspirated into a low temperature flame. The flame photometer is then able to excite electrons of metal ions to a higher energy orbital. When the excited electrons return to the ground state (original energy level), a discrete amount of energy, which is the difference between the excited and ground orbital, will be emitted out at a specific wavelength. The emission of energy is discreted and differs in various atom types. Thus, this criterion is exploited to detect the type of metal ions present in a sample as well as the corresponding concentration of the ions. The emitted light is then filtered through an optical filter and detected via a photodetector. The electrolyte concentration is calculated against a pre-calibrated range using standard solutions of sodium and potassium chloride.

2.3.2.2. Method

Preparation of Standard and Samples

Urine and plasma samples were prepared in a dilution of 1:499 and 1:199 ratio, respectively. To prepare a 1:499 dilution, 10 µL of urine were added to 4990 µL of distilled water to make a final volume of 5 mL. To prepare a 1: 199 dilution, 25 µL of plasma was added to 4975 µL of distilled water to make a final volume of 5 mL.

Stock solutions of the electrolyte standards for urine and plasma were prepared at different concentrations. The urine standard electrolyte stock solution contained 100 mM sodium chloride and 100 mM potassium chloride (100 mM/100 mM Na^+/K^+) whilst the plasma standard electrolyte stock solution contained 100 mM sodium chloride and 5 mM potassium chloride (100 mM/5 mM Na^+/K^+).

Preparation of urine electrolyte stock solution (100 mM/100 mM Na^+/K^+):

Sodium chloride (NaCl)	0.585g
Potassium chloride (KCl)	0.745g

Dissolved in 100 mL of distilled water

Preparation of plasma electrolyte stock solution (100 mM/5 mM Na^+/K^+):

Sodium chloride (NaCl)	0.585g
Potassium chloride (KCl)	0.037g

Dissolved in 100 mL of distilled water

Electrolyte working solutions for urine and plasma were prepared by diluting the stock solutions by 1: 99 (1 mL stock solution + 99 mL distilled water).

Calibration

The flame photometer was allowed to stabilize using distilled water prior to the calibration. For the measurement of urine sodium and potassium concentration, urine working standard solution was used to calibrate the instrument. The calibration will allow a linear range to be set for subsequent measurements. Similarly, plasma working standard was used to calibrate the flame photometer prior to the measurement of plasma electrolyte concentration.

Measurement

Upon calibration, the flame photometer tube was allowed to aspirate distilled water to remove previous residue of the standard solution. Removal of residue is complete when both channels (Na^+ and K^+) appear zero on the screen. This procedure was repeated after the measurement of each sample and at the end of the experiment. Once the residue has been cleared, the tube was immersed inside the sample and the values on both channels were recorded.

Calculation

Recorded readings were used to calculate sodium and potassium concentrations in urine and plasma samples based upon their respective standard solutions. Subsequently, absolute and fractional excretions of electrolytes were calculated as stipulated below.

Assume that:

NaU = Reading obtained from the Na^+ channel for urine sample

KU = Reading obtained from the K^+ channel for urine sample

NaP = Reading obtained from the Na^+ channel for plasma sample

UFR = Urine flow rate

GFR = Glomerular filtration rate

Urine sodium concentration, U_{Na} $= \left(\frac{NaU \times 1}{100} \times 500 \right)$ mmol/L

$= 5\ NaU \times 10^3$ µmol/L

100 is the calibration value

1 is the concentration of sodium in working standard solution

500 is the dilution factor for urine

Urine potassium concentration, U_K $= \left(\frac{KU \times 1}{100} \times 500 \right)$ mmol/L

$= 5\ KU \times 10^3$ µmol/L

100 is the calibration value

1 is the concentration of potassium in working standard solution

500 is the dilution factor for urine

Plasma sodium concentration, P_{Na} $= \left(\frac{NaP \times 1}{100} \times 200 \right)$ mmol/L

$= 2\ NaP \times 10^3$ µmol/L

100 is the calibration value

1 is the concentration of sodium in working standard solution

200 is the dilution factor for plasma

Absolute sodium excretion = Urine Na^+concentration x UFR

= 5 NaU x 10^3 µmol/L x UFR µL/min/kg

= 5 (NaU) (UFR) µmol/min/kg

Absolute potassium excretion = Urine K^+ concentration x UFR

= 5 KU x 10^3 µmol/L x UFR µL/min/kg

= 5 (KU) (UFR) µmol/min/kg

Fractional sodium excretion = $\frac{\text{Absolute } Na^+\text{excretion}}{\text{Plasma } Na^+\text{concentration x GFR}}$ x 100%

GFR calculated from inulin clearance

2.3.3. Inulin Assay

2.3.3.1. Principle

Prior to the assay, all samples (urine and plasma) are deproteinised in order to precipitate out the proteins and other non-sugar substances which interfere with the inulin assay. Subsequently, the samples are subjected to acid hydrolysis. Acid hydrolysis of inulin, a poly fructose molecule, releases the monomers, a non-reducing sugar called fructose. Fructose forms a purple complex upon interacting with diphenylamine in acid alcohol. The formed complex has a maximum absorbance at 650 nm, and the absorbance is directly proportional to the amount of complex formed, corresponding to the amount of inulin present. A standard curve is prepared using variable concentrations of inulin ranging from 0 to 100 mg/dL. Concentrations of inulin in samples were calculated based on the standard curve plotted.

2.3.3.2. Method

Preparation of Reagents

(A) Deproteinisation Solutions

Two stock solutions viz.Solution I and Solution II were prepared for this reaction. Solution I contained zinc sulphate dissolved in sulphuric acid while Solution II was made of 0.75N sodium hydroxide. To prepare Solution I, 1.25g of zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) was mixed carefully into 12.5 mL of 0.25N sulphuric acid (H_2SO_4). Distilled water was slowly added to make up a volume of 100 mL. This solution was further diluted 1: 3.25 (35 mL Solution I + 113.75 mL distilled water) to give Solution Ia, the working solution.

Solution II was prepared by dissolving 15g of sodium hydroxide in 500 mL of distilled water. Solution II was further diluted 1: 4 (100 mL Solution I + 400 mL distilled water) to make Solution IIa, the working solution.

(B) Colour Reagent

Diphenylamine mixture was prepared by dissolving 4g diphenylamine in 20 mL of absolute ethanol (1: 5, weight: volume). A diluent solution was prepared by adding 600 mL of sulphuric acid (H_2SO_4) carefully into 2000 mL of absolute ethanol, in an ice bath. The colour reagent was prepared by diluting the diphenylamine mixture with the prepared diluent by a 1: 21.66 ratio (20 mL diphenylamine mixture + 433.2 mL diluent).

(C) Preparation of Standards

Inulin standards were prepared in the range of 0 to 100 mg/dL. To prepare a 100 mg/dL solution, 100 mg of inulin was dissolved in 100 mL of distilled water. The solution was mixed using a magnetic stirrer at temperature 55 ^{o}C. Standard solutions were stored at room temperature.

Preparation of Samples

Both urine and plasma samples were assayed for inulin. Plasma samples were used undiluted while urine samples were diluted according to the volume of urine collected during their respective clearance period. The dilution is shown in Table 2.4.

Table 2.4: Dilution guideline for urine samples for inulin assay

Urine Volume	Dilution Ratio	Dilution Volume
<500	1: 199	25 µL urine + 4975 µL distilled H_2O
500-1500 µL	1: 99	25 µL urine + 2475 µL distilled H_2O
>1500 µL	1: 49	25 µL urine + 1225 µL distilled H_2O

Deproteinisation

Urine and plasma samples were deproteinised according to the method developed by Somogyi (1930). Aliquots of samples (50 µL) were prepared in a 5 mL disposable tube (LP3). Solutions Ia and IIa were added to the samples at volumes of 1700 µL and 250 µL, respectively. The tubes were capped and mixed well using a vortex mixer. Subsequently, the mixtures were allowed to stand for 10min at room temperature. Once the proteins precipitated out, the mixtures were

centrifuged at 1500 g for 15 min at 4 ^{o}C. The supernatant (1 mL) was transferred into a clean glass test tube for subsequent procedures.

Acid Hydrolysis and Diphenylamine Reaction

The determination of inulin concentrations in deproteinised samples were carried out (Bojesen, 1952). The colour reagent (5 mL) containing sulphuric acid and diphenylamine (dissolved in absolute ethanol) was added to the glass test tubes containing 1mL of deproteinised samples. The mouths of the tubes were closed using marbles. The tubes were then kept inside the water bath at 100 ^{o}C for 15 min, after which the tubes were transferred into an ice/water mix bath for 10 to 15 min to stop the reaction.

Measurement

The mixtures were brought to room temperature before spectrometric analysis. The samples were read at 650 nm against a blank. Blank was prepared by using distilled water in place of sample, and was assayed parallel with the samples. Inulin standards were also assayed similarly and a standard curve was plotted. Inulin concentrations in the samples were calculated based on the standard curve.

Calculation

$$\text{Inulin Clearance, } C_{In} = \left(\frac{\text{Inulin Concentration in Urine} \times \text{UFR}}{\text{Inulin Concentration in Plasma}} \right) \text{ mL/min/g kidney}$$

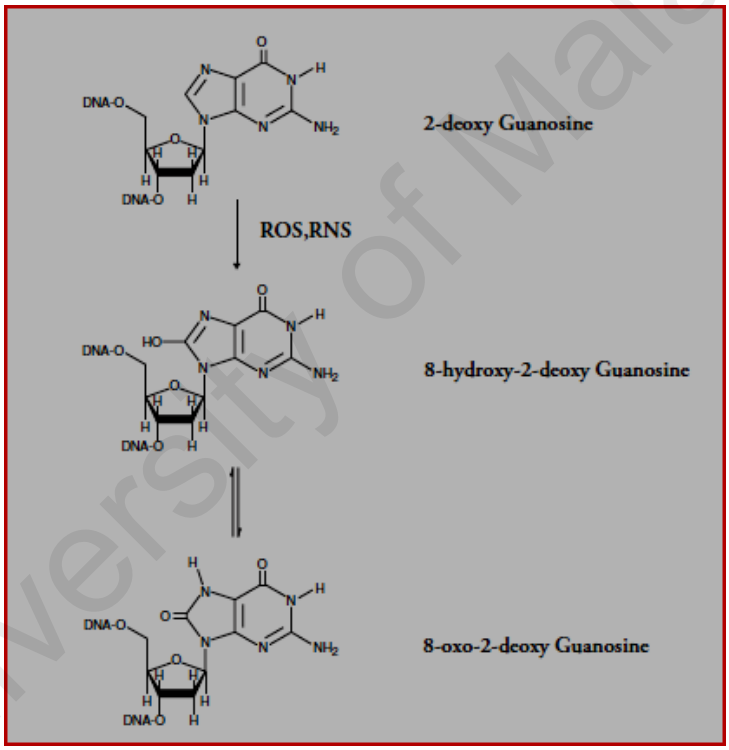
UFR = Urine flow rate

2.4. Oxdiative Stress Parameters

2.4.1. 8-Hydroxy-2-deoxy Guanosine EIA

2.4.1.1. Background

8-Hydroxy-2-deoxy Guanosine (8-OH-dG) is produced by the oxidative damage of DNA (Scheme 2.1) by reactive oxygen and nitrogen species and serves as an established marker of oxidative stress (Floyd et al., 1990; Spencer et al., 1995; Epe et al., 1996; Beckman et al., 1997).



Scheme 2.1: Oxidation of guanosine - obtained from the systematic protocol given by the manufacturer Cayman Chemicals.

Description of ACE™ Competitive EIAs

This assay (Maclouf et al., 1987; Pradelles et al., 1985) is based on the competition between 8-hydroxy-2-deoxy guanosine (8-OH-dG) and 8-OH-dG-acetylcholinesterase (AChE) conjugate (8-OH-dG Tracer) for a limited amount of

8-OH-dG monoclonal antibody. Because the concentration of 8-OH-dG tracer is held constant while the concentration of 8-OH-dG varies, the amount of 8-OH-dG tracer that is able to bind to 8-OH-dG monoclonal antibody will be inversely proportional to the concentration of 8-OH-dG in the well. This antibody-8-OH-dG complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of 8-OH-dG tracer bound to the well, which is inversely proportional to the amount of free 8-OH-dG present in the well during the incubation. A schematic representation of this process is shown in Figure 2.7.

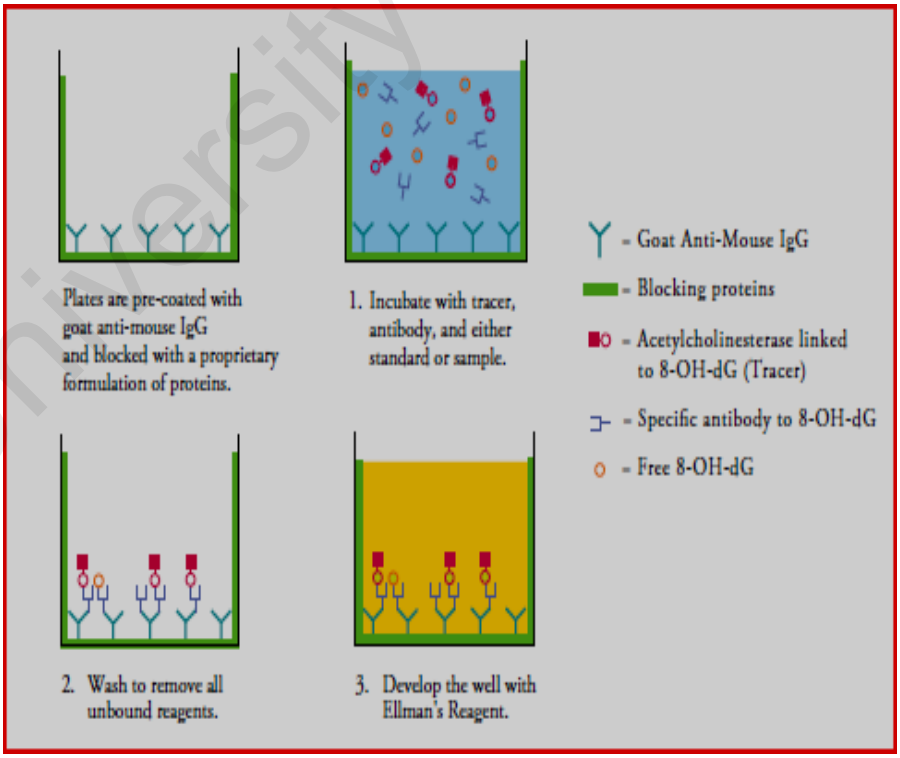


Figure 2.7: Schematic of the ACE™ EIA - obtained from the systematic protocol given by the manufacturer Cayman Chemicals.

2.4.1.2. Measurement of 8-OHdG in Urine Samples

8-OHdG levels were measured in urine samples using ELISA kit from Cayman Chemicals and in accordance to manufacturer's protocol. The 8-OHdG standards (0.5-80 ng/mL) or 50 µL of urine were allowed to incubate with monclonal antibody against 8-OHdG and 8-OHdG tracer in a microtiter plate precoated with anti-mouse IgG, for 18 h at 4 ºC. After washing the antibodies bound to 8-OHdG in the sample, Ellaman's reagent ((5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB)) was added to each well and incubated for 90 min. The colour that developed by the addition of the reagent was measured by absorbance at 420 nm using spectrophotometer. Urinary 8-OHdG was expressed at total amount excreted in 2 hour.

2.4.2. Lipid Hydroperoxide (LPO) Assay

2.4.2.1. Background

Quantification of lipid peroxidation is essential to assess the role of oxidative injury in an organ/tissue during pathophysiological conditions (Cross et al., 1987; Porter et al., 1995; Halliwell et al.,1996). Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. Traditionally, lipid peroxidation is quantified by measuring malondialdehyde (MDA) and 4-hydroxy nonenal (4-HNE), the degradation products of polyunsaturated fatty acids (PUFAs), and hydroperoxides (Esterbauer et al., 1991; Pryor et al., 1990; Janero et al., 1990). Sensitive colorimetric assays have been developed to measure these aldehydes (Esterbauer et al., 1991). However, these assays seem to be non-specific and often lead to an error showing an over-estimation of lipid peroxidation. Moreover, the by-product formation is

highly inefficient and varies according to the transition metal ion content present in the sample. Only hydroperoxides derived from PUFAs give rise to these by-products. For example, 4-HNE is formed only from ω-6 PUFA hydroperoxides and is catalyzed by transition metal ions like ferrous (Pryor et al., 1990). Decomposition of hydroperoxides derived from abundant cellular lipids such as cholesterol and oleic acid does not produce MDA or 4-HNE. These factors can lead to an under-estimation of lipid peroxidation. MDA is also produced in ng/mL concentrations by the platelet enzyme thromboxane synthase during whole blood clotting and platelet activation (Diczfalusy et al., 1977). This could lead to gross over-estimation of lipid peroxidation. Estimation of lipid hydroperoxide levels range from 0.3-30 µM depending on the method used. However, direct methods of estimation suggest that the concentration in normal human plasma is approximately 0.5 µM (Warsoet al., 1984; Yamamoto et al., 1987). Given the limitations of the indirect methods, direct measurement of hydroperoxides is the obvious choice.

Principle

Lipid Hydroperoxide Assay measures the hydroperoxides directly utilizing the redox reactions with ferrous ions (Scheme 2.3) (Mihaljevic et al., 1996). Hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using thiocyanate ion as the chromogen.

$$ROOH + Fe^{2+} \longrightarrow RO^{\bullet} + Fe^{3+}$$

$$RO^{\bullet} + Fe^{2+} + H^{+} \longrightarrow ROH + Fe^{3+}$$

$$Fe^{3+} + 5SCN^{-} \longrightarrow Fe(SCN)_5^{2-}$$

Scheme 2.2: Reduction /oxidation reactions

Since this method relies on the measurement of ferric ions generated during the reaction, ferric ions present in the sample are a potential source of error. Also, many biological samples contain hydrogen peroxide which readily reacts with ferrous ions to give an over-estimation of lipid hydroperoxides. These problems are easily circumvented by performing the assay in chloroform.

An easy to use, quantitative extraction method was developed to extract lipid hydroperoxides into chloroform and the extract is directly used in the assay. This procedure eliminates any interference caused by hydrogen peroxide or endogenous ferric ions in the sample and provides a sensitive and reliable assay for lipid peroxidation.

2.4.2.2. Measurements of Lipid Peroxides in Kidney

LPO in the renal cortex was measured colourimetrically using a Cayman's assay kit. Renal cortex was homogenized in HPLC-grade water and LPO was extracted from the homogenates into chloroform according to the manufacturer's protocol. LPO was measured directly by redox reactions with ferrous ions using the kit, and the resulting ferric ions were detected using thiocyanate ion as the chromogen. The colour that developed was measured by absorbance at 500 nm.

2.4.3. Glutathione Assay

2.4.3.1. Background

Glutathione (GSH) is a tripeptide (γ-glutamylcysteinylglycine) distributed widely in both plants and animals (Foyer et al., 1994; Arias et al 1976). GSH is an

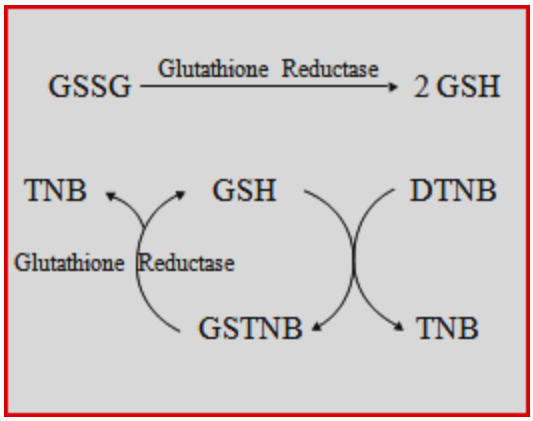
endogenous antioxidant that serves as a nucleophilic co-substrate to glutathione transferases in the detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxides (Arias et al., 1976; Baillie et al., 1991). GSH is also involved in amino acid transport and maintenance of protein sulfhydryl reduction status (Inoue et al., 1987; Inoue, 1985). Concentration of GSH ranges from a few micromolar in plasma to several millimolar in tissues such as liver, kidney etc (Lash et al., 1985; Wendel et al., 1980).

Principle

GSH assay utilizes a carefully optimized enzymatic recycling method, using glutathione reductase, for the quantification of GSH (Scheme 2.4) (Tietze et al., 1969; Eyer et al., 1986; Baker et al., 1990). The sulfhydryl group of GSH reacts with DTNB (5,5'-dithio-bis-2-(nitrobenzoic acid), Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405-414 nm provides an accurate estimation of GSH in the sample.

GSH is easily oxidized to the disulfide dimer Glutathione disulfide (GSSG). The GSSG is produced during the reduction of hydroperoxides by glutathione

peroxidase. The GSSG is reduced to GSH by glutathione reductase and it is the reduced form that exists mainly in biological systems.



Scheme 2.3. GSH recycling

2.4.3.2. Measurement of reduced GSH in kidney

Cytosolic reduced GSH in renal cortex was measured using a glutathione assay kit from Cayman Chemicals according to the manufacturers protocol. Renal cortex homogenate (100-150 µg) was deproteinized using metaphosphoric acid, and the amount of the yellow coloured 5-thio-2-nitrobenzoic acid produced in the supernatant was measured at 410 nm a spectrophotometer (UV/Vis λ Perkin Elmer, USA)

2.5. Transforming Growth Factor-β (TGF-β 1) (RAT) ELISA Kit

2.5.1. Measurements of Plasma TGF-β1

Plasma samples were collected from the experimental animals on the 4th and 12th week. Samples had been stored at -20 °C without thawing before use in this assay. ELISA kit was used to measure plasma TGF-β1 concentration according to manufacturers protocol. Each 50 µl of plasma sample was first acid activated by

incubation with 1 µl of 1.0 N HCl for 15 min at room temperature and neutralized by 1 µl of 1 N NaOH for the activation of the plasma TGF-β1 to the immunoreactive form. The TGF-β1 standards or plasma sample were added to microwells precoated with TGF-β1 specific antibodies and then incubated at room temperature. After washing, a Biotin conjugated anti-rat TGF-β1 detection antibody is added and then incubated at room temperature. Following washing, Streptavidin horseradish peroxidase (HRP) conjugate is added to each well, incubated at room temperature then washed again. Tetramethylbenzidine (TMB) is added and than catalysed by HRP that changed colour after adding acidic stop solution. The colour was measured by absorbance at 450 nm.

2.6. Histology

2.6.1. Principle

Histological preparation of renal tissues follows several procedures. The initial step is the fixation of the tissue in a suitable fixative such as formalin. This step is crucial to preserve the internal structures and prevent decomposition of the tissue. Upon fixation, the tissue is trimmed down to obtain a section of interest. The tissue is then processed in an exchange of reagents to dehydrate the tissue and ease paraffin embedding. In the first few exchanges of reagents, the tissue goes through a series of increasing percentages of alcohol until it is finally transferred into absolute alcohol. At this stage, water is completely removed from the tissue. Then, the tissue is transferred into a few exchanges of xylene and finally into warm molten paraffin. The final step of immersing the tissue in the paraffin wax allows thorough infiltration of the paraffin inside the tissue. The tissue is then embedded in molten paraffin wax and the metal cassette is transferred onto a cold

surface of the instrument to harden the paraffin. Solid paraffin blocks are used to prepare thin sections of the tissue (Thompson and Samuel, 1966).

2.6.2. Haematoxylin and Eosin Staining

Haematoxylin and Eosin (H & E) is the most commonly used stain to study the histology of tissues. Haematoxylin is a blue dye that stains the nucleus of cells whilst eosin (a xanthine dye) is used as a background stain, which stains the cytoplasm pink. Haematoxylin is a cationic dye that has high affinity for the nucleus, a negatively charged structure. Upon oxidisation, haematoxylin will form haematein that gives a blue colour to the nucleus. Conversely, eosin, an anionic stain that has high affinity to the positively charged cytoplasm, stains the background pink (Thompson and Samuel, 1966).

2.6.3. Periodic Acid Schiff's Stain (PAS)

The Clin-Teach Periodic Acid-Schiff (PAS) is a staining method used in histology and pathology. This method is primarily used to identify glycogen in tissues. This reaction of periodic acid selectively oxidizes the glucose residues, creating aldehydes that react with the Schiff reagent producing a purple-magenta color. A suitable basic stain is often used as a counterstain. PAS staining is mainly used for staining structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, and proteoglycans), typically found in connective tissues, mucus, and basal laminae

2.6.4. Masson's Trichrome

Differential visualisation of tissue elements are achieved by two similar acid dyes. The dye which has smaller molecule size (acid fuchsin) is used first to stain all tissue elements in the section. The red stain are selectively removed from the unwanted areas by differentiating with phosphomolybdic acid, which will act as a mordant dye for the next step. The dye with a large molecule (light green) is then progressively applied to the section. A celestin blue/haematoxylin dye is used to stain the nuclei as it is more resistant than alum haematoxylins. This method differentiates the oedema from fibrosis and visualises immunological deposits.

2.6.5. Histo pathological Study

Tissue sections were cut at 5 μm thickness using a microtome, dewaxed and stained with Haemtoxylin and eosin (H & E), Periodic acid-Schiff (PAS) or Masson's trichrome stains. Renal morphology changes within the glomeruli and interstitial areas were assessed with the aid of a Nikon Eclipse 80i light microscope, and using a semi quantitative scoring method (Saito et al., 1987 and Taneda et al., 2003).

2.7. Immunohistochemistry

Principle

Immunohistochemistry is the localization of antigens in tissue sections by the use of labeled antibody as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold.

In principle, the specimens are first incubated with Peroxidase Block for 5 min to quench endogenous peroxidase activity. The specimens are then incubated for 10 min with a protein block to suppress nonspecific binding of subsequent reagents, followed by 1 hr incubation with an appropriately characterized, and diluted mouse primary antibody or negative control reagent. This is followed by sequential 30-min incubations with anti-mouse immunoglobulins-HRP, fluorescyl-tyramide hydrogen peroxide (amplification reagent) and anti-fluorescein-HRP. Staining is completed by a 5-min incubation with 3,3' diaminobenzidine tetrahydrochloride (DAB)/ hydrogen peroxide, which results in a brown precipitate at the antigen site.

2.7.1. Immunohistochemistry Evaluation

Renal tissue was sectioned into 5 µm thickness using a rotary microtome and placed onto poly-L-lysine coated slides. For antigen retrieval, the poly-L-lysine specimen slides were transferred to 10 mmol/Lcitrate buffer solution (pH 6.0), and then heated in decloaking chamber at 120 °C for 20 min. Subsequently, the sections were incubated with Dako Real^{TM} Peroxidase blocking solution for 10 min and rinsed with phosphate buffer saline (PBS) (pH 7.4). The sections were incubated with primary antibodies recognising p22phox (1:200) and p67phox (1:100) for 1 hr at room temperature. The sections were rinsed with PBS (pH 7.4) and were incubated with HRP rabbit/mouse secondary antibody (Dako Real^{TM} Envision^{TM}) for 30 min at room temperature. For coloration, the slides were incubated with a mixture of Dako Real^{TM}DAB Chromogen and Dako Red^{TM} substrate buffer (1:50) for 5 min at room temperature. Sections were finally counterstained with hematoxylin. Representative areas of renal morphology

changes within the glomeruli and interstitial areas were photographed using Nikon Eclipse 80i light microscope. All slides were examined under the light microscope.

2.8. Western Blotting Assay

2.8.1. Overview of Western Blotting

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin et al. in 1979, and is now a routine technique for protein expression analysis. The specificity of the antibody-antigen interaction enables a target protein to be identified in the midst of a complex protein mixture. Western blotting can produce qualitative and semiquantitative data about that protein expression.

The first step in a Western blotting procedure is to separate the macromolecules using gel electrophoresis. After electrophoresis, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. Most commonly, the transferred protein is complexed with an enzyme-labelled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic precipitate on the membrane for a colorimetric detection. The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a by-product. The light output can be captured using film, a CCD camera

or a phosphorimager that is designed for chemiluminescent detection. Detailed procedures for detection of a Western blot vary widely. One common variation involves direct vs. indirect detection. With the direct detection method, the primary antibody that is used to detect an antigen on the blot is labelled with an enzyme or fluorescent dye. This detection method is not widely used as most researchers prefer the indirect detection method for a variety of reasons. In the indirect detection method, a primary antibody is added first to bind to the antigen. This is followed by a labelled secondary antibody that is directed against the primary antibody. Labels include biotin, fluorescent probes such as fluorescein or rhodamine, and enzyme conjugates such as horseradish peroxidase or alkaline phosphatase. The indirect method offers many advantages over the direct method.

2.8.2. Assay Protocol

2.8.2.1. Sample lysis

A. Preparation of lysate from renal tissues

Renal cortex tissue was dissected with clean tools on ice and as quickly as possible to prevent degradation by proteases. The tissue was placed in eppendorf tubes and immersed in liquid nitrogen to "snap freeze". Samples were stored at -80 °C for later use or kept on ice for immediate homogenization. For a ~5 mg piece of tissue, ~300 µL lysis buffer was rapidly added to the tube, homogenized with an electric homogenizer, the blade was rinsed twice with another 2 x 300 µL lysis buffer, the homogenates was then maintained on constant agitation (for example, on an orbital shaker) for 2 hrs at 4 °C.

Tubes containing homogenate were centrifuged for 20 min at 15,000 x g at 4 °C in a microcentrifuge. The tubes were gently removed from the centrifuge and placed

on ice. The supernatant was then saved for western blotting. Total protein in supernatant was carried out using bicinchoninic acid (BCA) method, developed by Smith et al., 1985.

Western Blotting Assay

Thirty microgram (30 µg) of protein extracts from renal cortex were loaded on pre-cast 4-20% sodium dodecyl sulphate (SDS-PAGE) gels and the proteins were transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% non-fat milk followed by primary antibodies recognising p22phox and p67phox (1:500), and incubated at 4 ^{o}C overnight. The membranes were washed and incubated with HRP-conjugated goat antirabbit IgG. Band densities were normalised to the total amount of protein loaded in each well, as determined by densitometric analysis of PVDF membranes stained with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare). The proteins were visualised by chemiluminescence (UVP, Bio Spectrum, USA) and the densities of specific bands were quantitated by densitometry using Vision Work LS software (Version: 7.1 RC3.10). Housekeeping protein β-actin (1:1000) was used as loading control.

2.9. Statistical Analysis

Data are expressed as mean ± SEM. The data obtained from various groups were statistically analysed using one way analysis of variance (ANOVA), followed by Tukeys Multiple Comparison Test using Graph Pad Prism Statistical Software. A *p* value of less than 0.05 was considered as statistically significant.

CHAPTER 3

RESULTS

3.1. Phytochemical studies: TPC and TFC Content of OPLE

The chemical constituents of OPLE such as TPC and TFC were determined to be 180.16 ± 3.52 mg GAE/g and 5.21 ± 1.63 mg QE/g extract, respectively.

3.2. DPPH Scavenging Activity of OPLE

OPLE was noted to exhibit a dose-dependent inhibition of DPPH activity with an IC_{50} value of 33.23 ± 2.60 (Fig.3.1) in comparison with ascorbic acid and BHT IC_{50} values of 15.87 ± 1.31 and 18.63 ± 1.11, respectively.

3.3. FRAP Assay

FRAP assay was used for evaluating total antioxidant activity. Figure 3.2 describes the capacity of the reduction of ferric ions to ferrous ion due to the presence of reducing substances in the ethanolic extract of OPLE as compared with the standards such as quercetin and trolox by UV-spectrophotometric method at 593 nm.

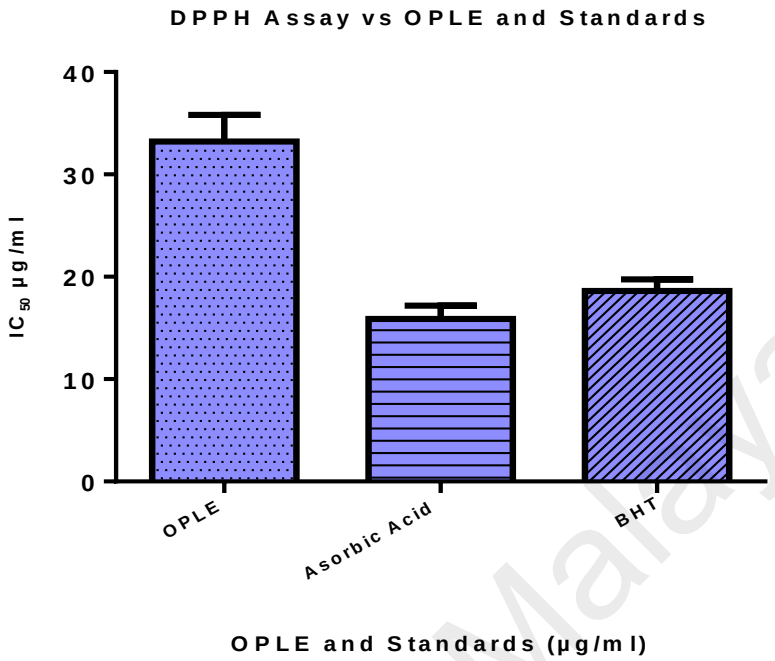


Figure 3.1. DPPH free-radical scavenging activity of OPLE. Ascorbic acid and BHT were used as positive controls.

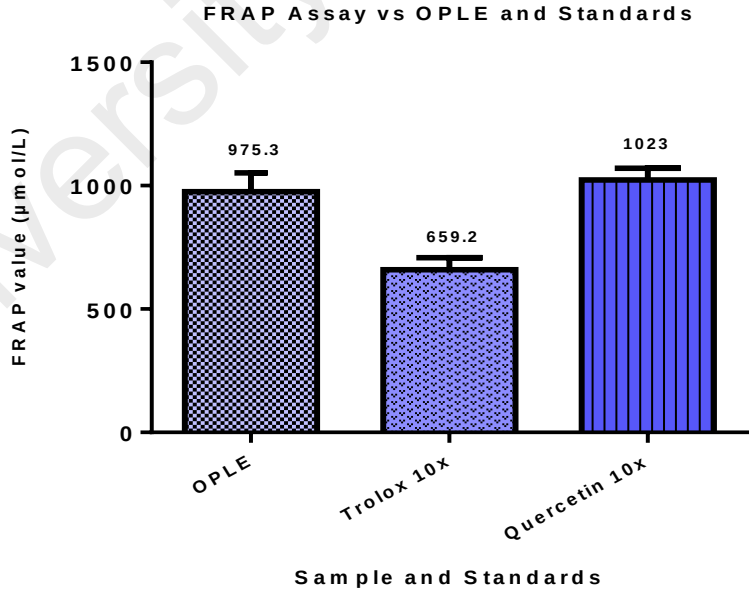


Figure 3.2. Total antioxidant (FRAP) activity in OPLE. Trolox and quercetin were used as positive controls.

3.4. Body Weight Profile

Body weight of all experimental animals was recorded every week. The weekly body weight profiles of all experimental groups are shown in Figure 3.3(a,b). The mean body weight in diabetic rats was 1.2-fold and 1.8-fold lower than in non-diabetic rats at 4 and 12 weeks after induction of diabetes, respectively; however, the difference was only significant at 12 weeks ($P < 0.001$). OPLE did not affect the mean body weight in diabetic rats at the tested doses of 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day for the 4-week experimental duration.

However, in the 12-week study, as shown in Figure 3.3(b), diabetic animals receiving OPLE 500 mg/kg and 1000 mg/kg showed a non-significant attenuation in their body weights reduction as compared to the other diabetic treated group. In summary, all diabetic animals irrespective of treatment showed a reduction in their body weights with significance ($P < 0.001$) being achieved at the end of the 12-week experimental duration as compared to the non-diabetic control.

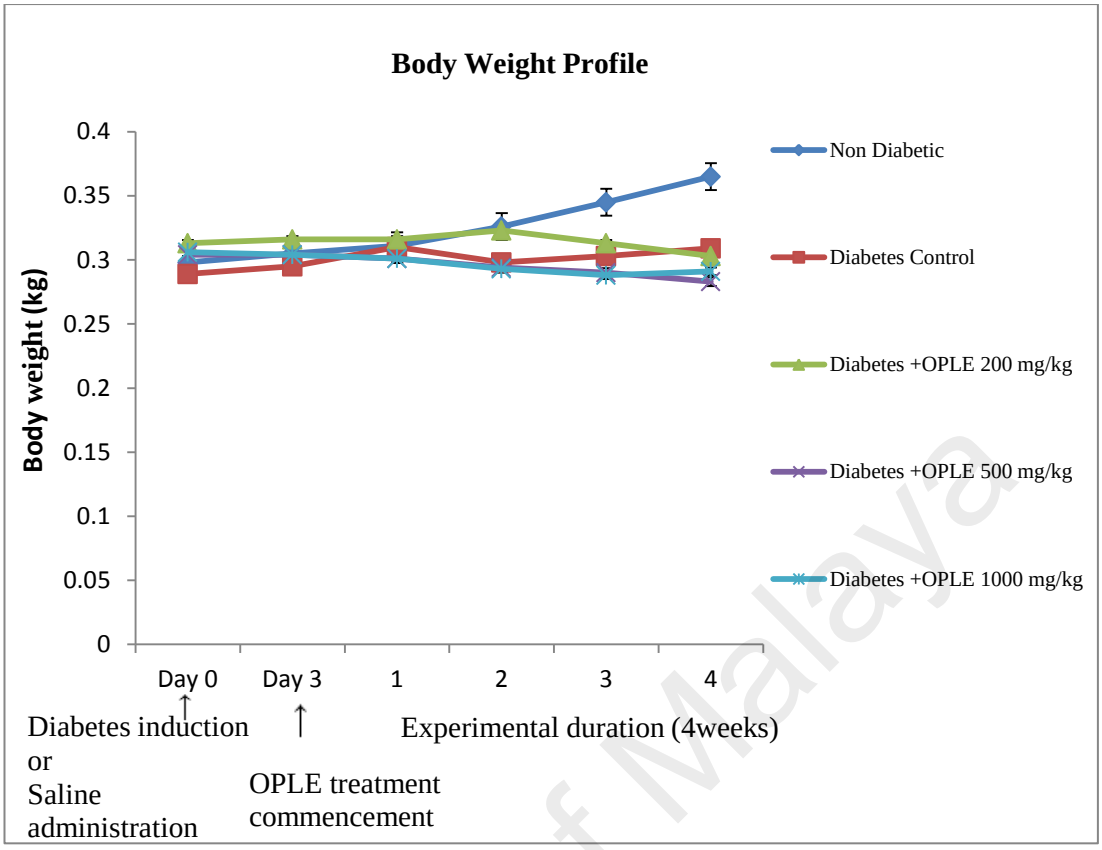


Figure 3.3(a). Effects of OPLE on body weight profile in 4 weeks experimental model**.** Data are expressed as mean ± SEM (*n*=6 per group).

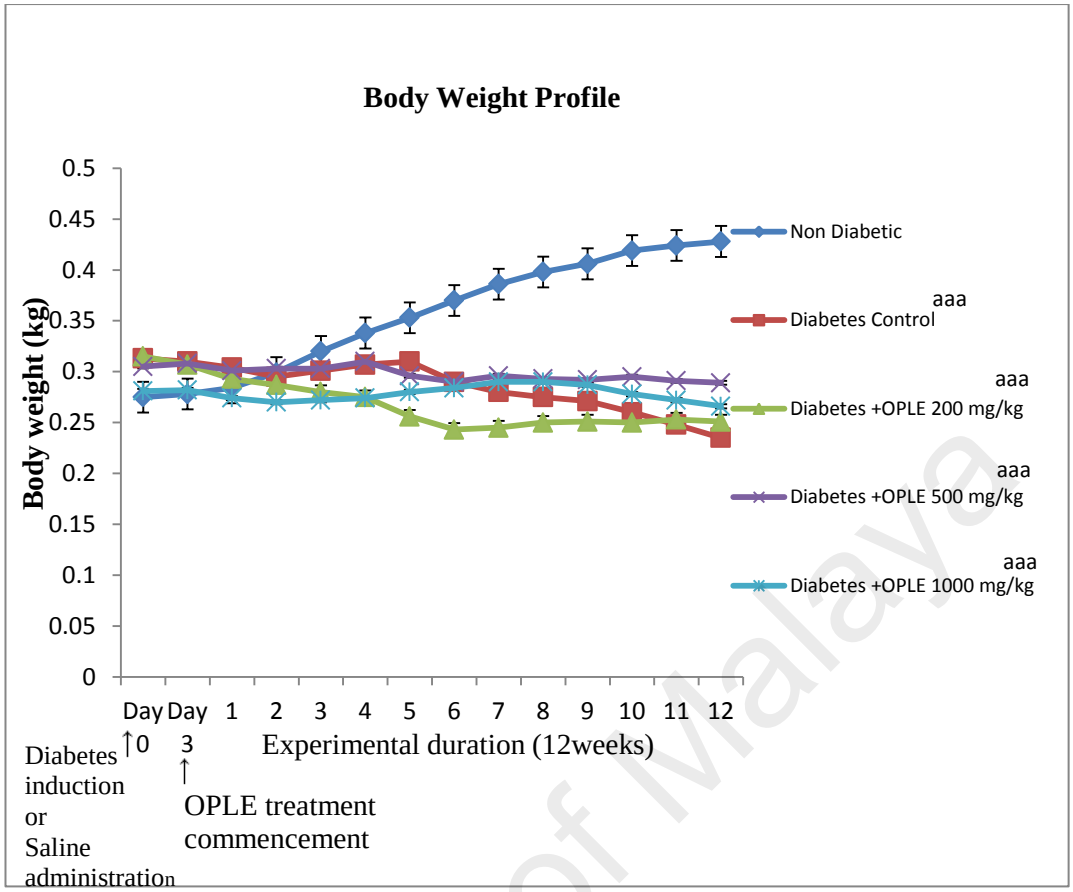


Figure 3.3(b). Effects of OPLE on body weight profile in 12 weeks experimental model**.** Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P< 0.001$ vs. non-diabetic.

3.5. Total Kidney Weight

Kidney to body weight ratio is to assess renal hypertrophy and ECM expansion. Figures 3.4. (a and b), show the kidney weight normalized by 100g body weight in all experimental groups. In the 4 weeks study, the diabetic control animals when compared with the non-diabetic control animals showed a significant difference by 1.4 fold increases (0.57±0.03 gm/100gm body weight vs 0.40±0.01 gm/100gm body weight, $P < 0.05$). In the 4 weeks model, diabetic animals treated with OPLE 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day did not show any improvement in the kidney to body weight ratio (0.53±0.04 gm/100gm body weight, 0.59±0.04 gm/100gm body weight and 0.56±0.04 gm/100gm body weight, respectively) when compared with diabetic control. In the 12 weeks study, kidney to body weight ratio has increased in diabetic control rats when compared with non-diabetic control rats by 2.1 fold (0.69±0.02 gm/100 gm body weight vs 0.32±0.02 gm/100 gm body weight, $P< 0.001$). OPLE 500 mg/kg/day showed a slight tendency to reduce renal enlargement (0.59±0.02 gm/100 gm body weight) in rats with 12 weeks diabetes when compared with diabetic control group but the difference was not statistically significant.

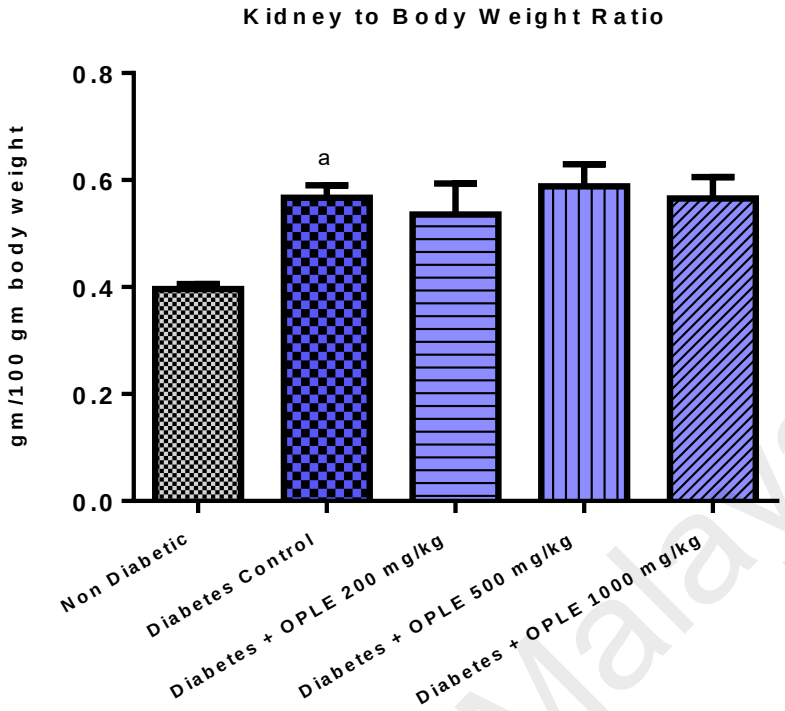


Figure 3.4(a). Effects of OPLE on kidney to body weight ratio in 4 weeks experimental model. Data are expressed as mean ± SEM (*n*=6 per group). $^{a}P < 0.05$ vs. non-diabetic.

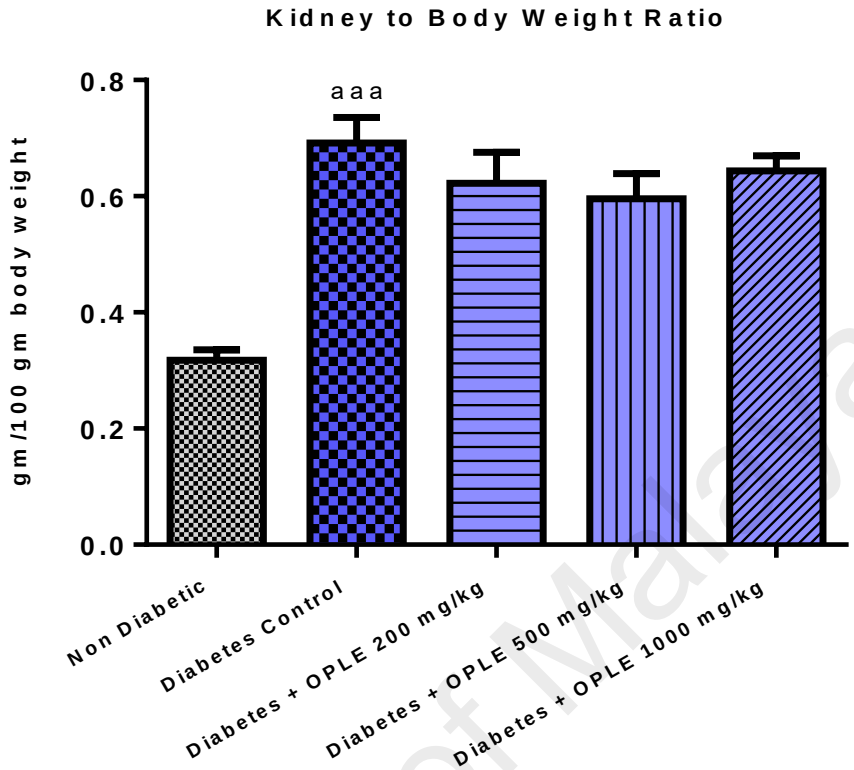


Figure 3.4(b). Effects of OPLE on kidney to body weight ratio in 12 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P <$ 0.001 vs. non-diabetic.

3.6. Random Blood Glucose Level

Hyperglycemia can be measured via two ways; either by measuring a random or fasting blood glucose level. However, as our experimental animals were generally weak and not suitable for fasting, a random measurement was employed. Random blood glucose levels were monitored weekly and the results are as shown in Figure 3.5 (a,b). We confirmed the occurrence of hyperglycemia in the rats after 72 hours of STZ administration. Diabetic animals demonstrated a clear elevation in random blood glucose levels at both 4 and 12 weeks as mean of 30.7±01.7mmol/L and 31±1.2mmol/L whilst non-diabetic saline control showed as mean of 4.7±0.1mmol/L and 6.3±0.2 mmol/L, respectively, throughout the experimental duration.

Random blood glucose levels of all diabetic animals were significantly different (P<0.001) from the non-diabetic saline control. Random blood glucose level of OPLE 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day receiving group was not significantly different from diabetic control. Thus, the treatment of OPLE with 3 dosage concentrations did not show any significant effect on the level of random blood glucose in the experimental animals in both the 4-week and 12-week treatment groups.

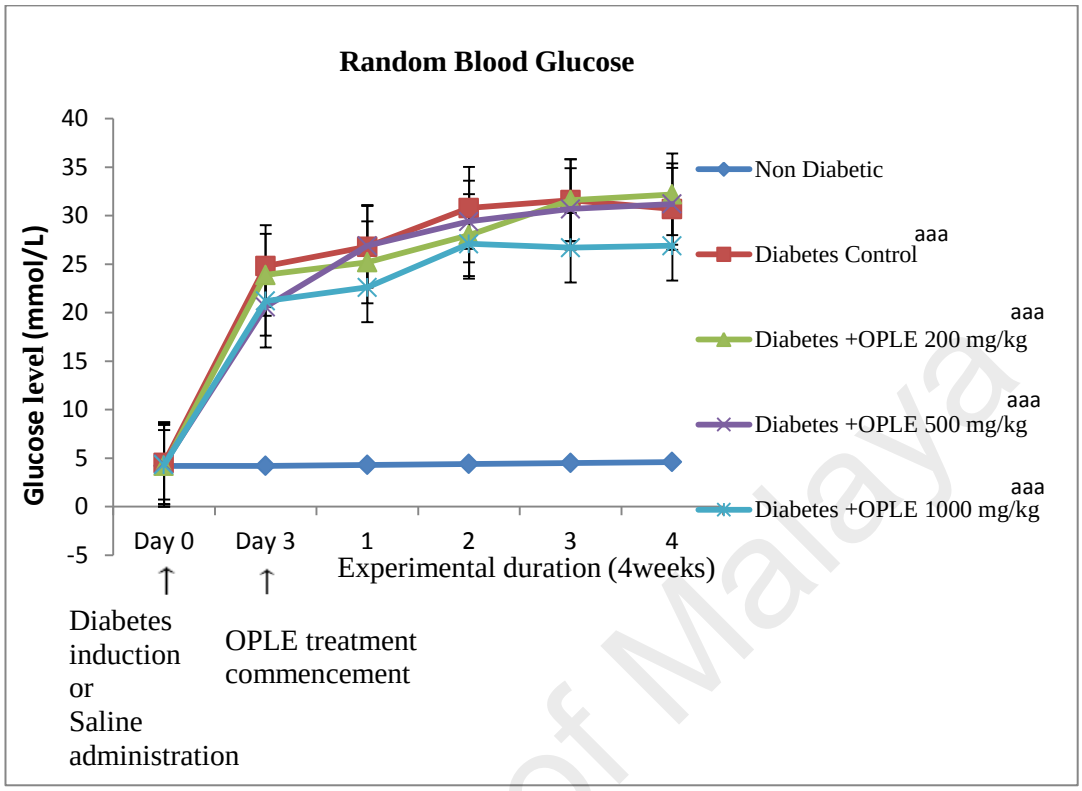


Figure 3.5(a). Effects of OPLE on random blood glucose level in 4 weeks experimental model**.** Data are expressed as mean ± SEM (*n*=6 per group). $^{aaa}P <$ 0.001 vs. non-diabetic.

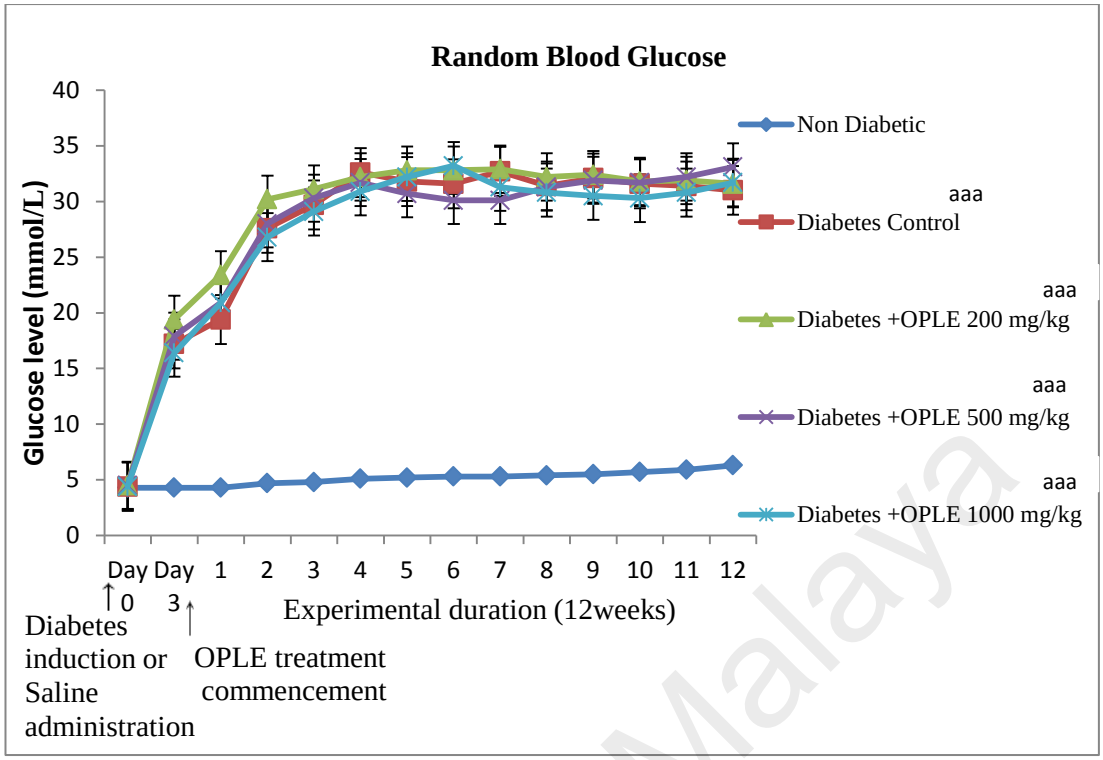


Figure 3.5(b). Effects of OPLE on random blood glucose level in 12 weeks experimental model**.** Data are expressed as mean ± SEM (n=6 per group). ^{aaa}P < 0.001 vs. non-diabetic.

3.7. Mean Arterial Blood Pressure

Mean arterial blood pressure (MABP) was measured at the end of the experimental study of both 4 and 12 weeks. Three MABP values were noted at the beginning, after two clearances and at the end of the study (i.e. an hour interval between each reading). Average of the three values was taken as the mean of ABP of each rat. Mean values of 6 animals of each group are plotted in Figure 3.6 (a,b). In general, there were no significant differences in MABP between the experimental groups of 4 week and 12 weeks study.

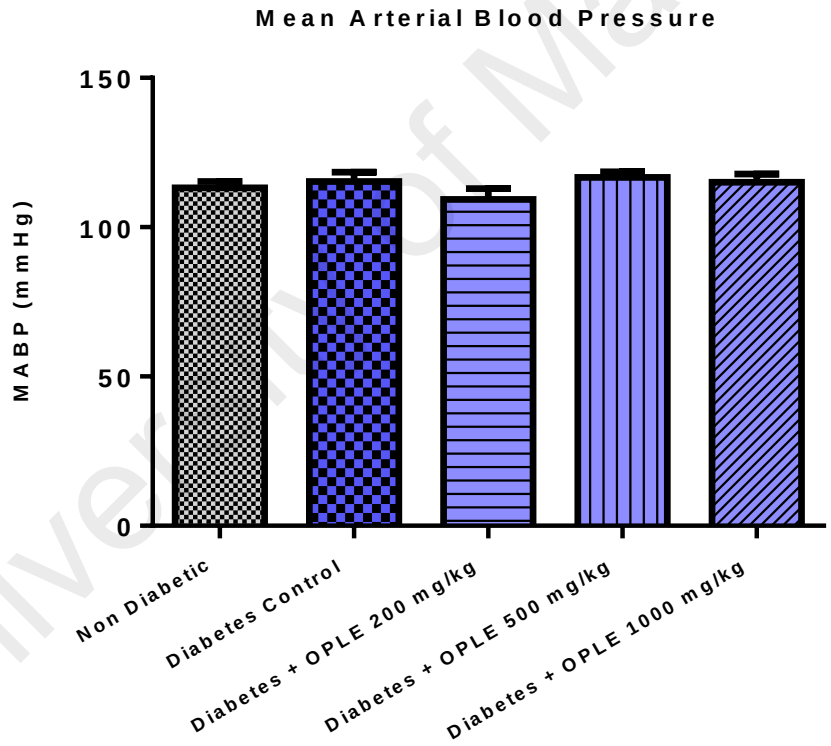


Figure 3.6(a). Effects of OPLE on mean arterial blood pressure in 4 weeks experimental model**.** Data are expressed as mean ± SEM (*n*=6 per group). No significant difference between all groups was noted.

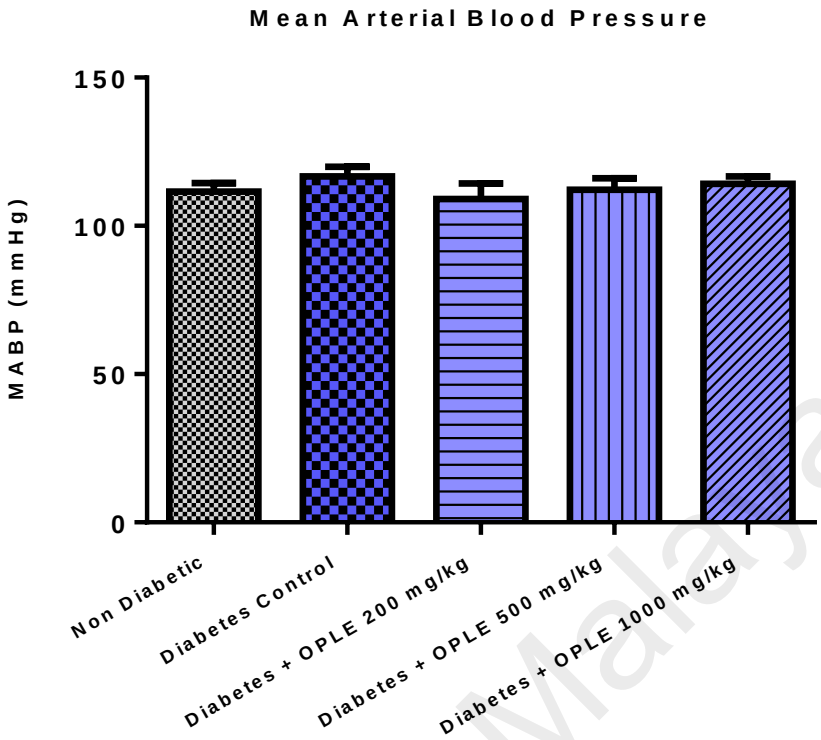


Figure 3.6(b). Effects of OPLE on mean arterial blood pressure in 12 weeks experimental model**.** Data are expressed as mean ± SEM (*n*=6 per group). No significant difference between all groups was noted.

3.8. Renal Blood Flow

Renal blood flow (RBF) of various groups is shown in Figure 3.7 (a & b). In the 4 weeks study, the diabetic control animals when compared with the non-diabetic control animals showed a significant increased in the RBF (1.5-fold, $P < 0.05$) Figure 3.7 (a), and diabetic animals treated with OPLE 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day show reduction in the RBF, but not statistically different when compared with diabetic control. In the 12 weeks study, RBF was significantly increased in diabetic rats compared with non-diabetic rats at 12 weeks study (1.5-fold, $P < 0.001$) Figure 3.7 (b). The increase in RBF was normalised by treatment with OPLE 200 mg/kg/day and 500 mg/kg/day ($P < 0.01$ and $P < 0.001$, respectively). On the other hand, treatment with OPLE 1000 mg/kg/day did not normalize the RBF when compared to diabetic control group rats in the 12 weeks study.

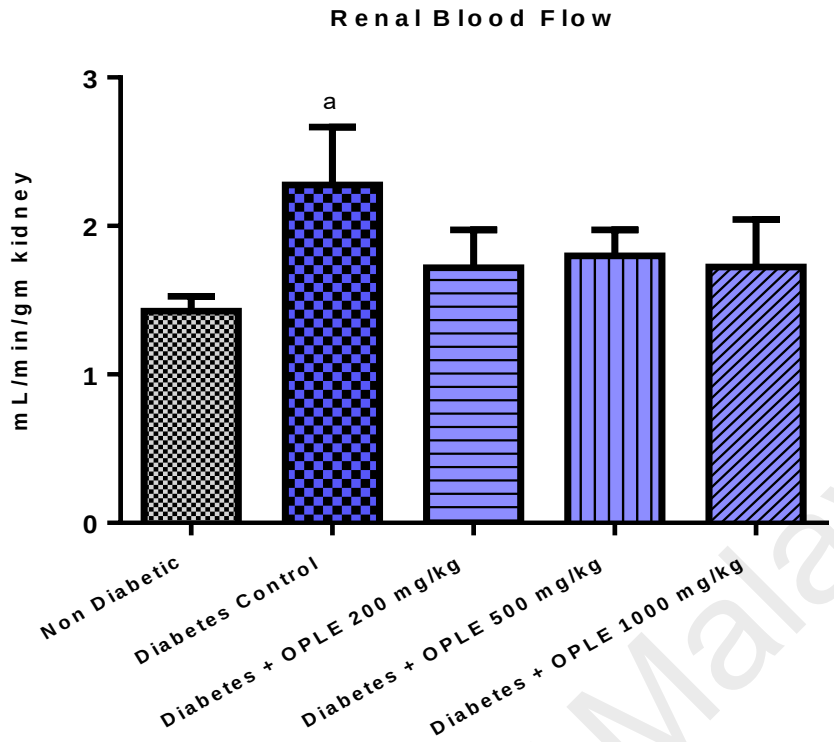


Figure 3.7(a). Effects of OPLE on renal blood flow in 4 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{a}P < 0.05$ vs. non-diabetic.

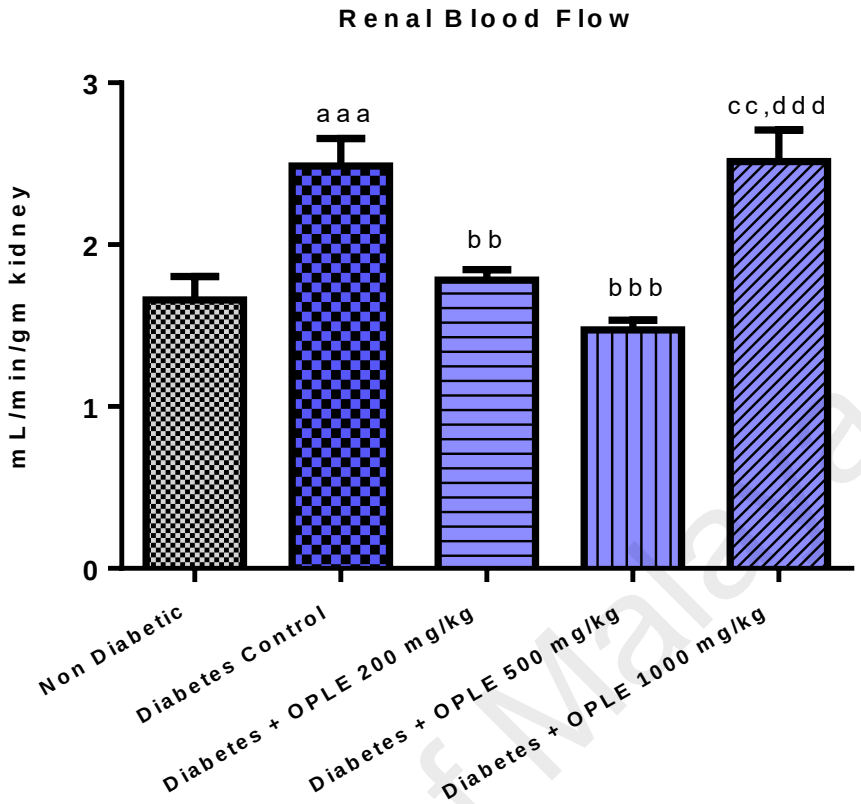


Figure 3.7(b). Effects of OPLE on renal blood flow in 12 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P < 0.001$ vs. non-diabetic; $^{bb}P < 0.01$; $^{bbb}P < 0.001$ vs. diabetic control; $^{cc}P < 0.01$ vs diabetes + OPLE 200 mg/kg; $^{ddd}P < 0.001$ vs. diabetes + OPLE 500 mg/kg.

3.9. Urine Flow Rate

Four cycles of urine collections (each with 30 min duration) were collected directly from the bladder of the rats under anaesthesia. The average urine flow rate (UFR) was then calculated, and the mean values of six animals are shown in Figure 3.8 (a,b). As evident from the figure, UFR of the diabetic control group rats was noted to be remarkably increased as compared to the non-diabetic group rats on both the 4th week (10.68±1.30 vs 3.75±0.59 µL/min/gm kidney, $P < 0.001$)

and 12th week (7.84±0.70 vs 2.89±0.34 µL/min/gm kidney $P < 0.001$) which may be the outcome of hyperfiltration. Diabetic animals treated with OPLE 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day significantly reduced the UFR elevations to 5.27±0.45, 5.80±0.80 and 5.40±0.67 µL/min/gm kidney, $P < 0.01$, respectively, and more over they almost prevented the elevated urine flow rate at 4 weeks in the diabetic rats. Similarly for the 12 weeks study, as shown in Figure 3.8 (b), diabetic animals treated with OPLE 500 mg/kg/day showed significant reduction in the UFR (5.29±0.70 µL/min/gm kidney, $P < 0.05$) when compared to diabetic control animals. Comparably 200 mg/kg/day OPLE reduced the UFR elevation (6.42±0.50 µL/min/gm kidney), but this reduction was not significant. However, treatment with OPLE, 1000 mg/kg/day administered over a period of 12 weeks to diabetic animals did not show any alteration to the increase in UFR (10.9±0.80 µL/min/gm kidney, $P < 0.05$), unlike with the other lower doses of OPLE.

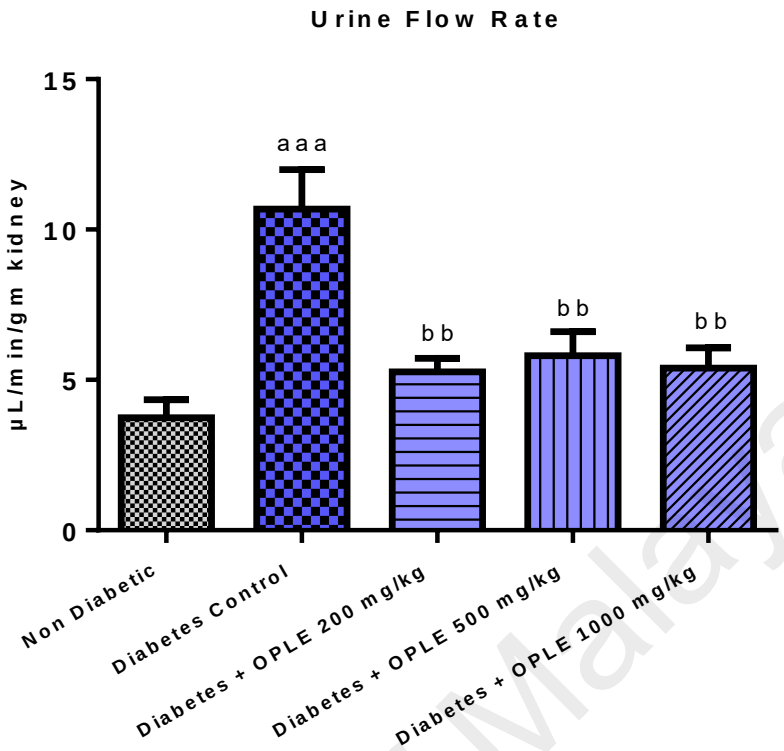


Figure 3.8(a). Effects of OPLE on urine flow rate in 4 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P < 0.001$ vs. non-diabetic; $^{bb}P < 0.01$ vs. diabetic Control.

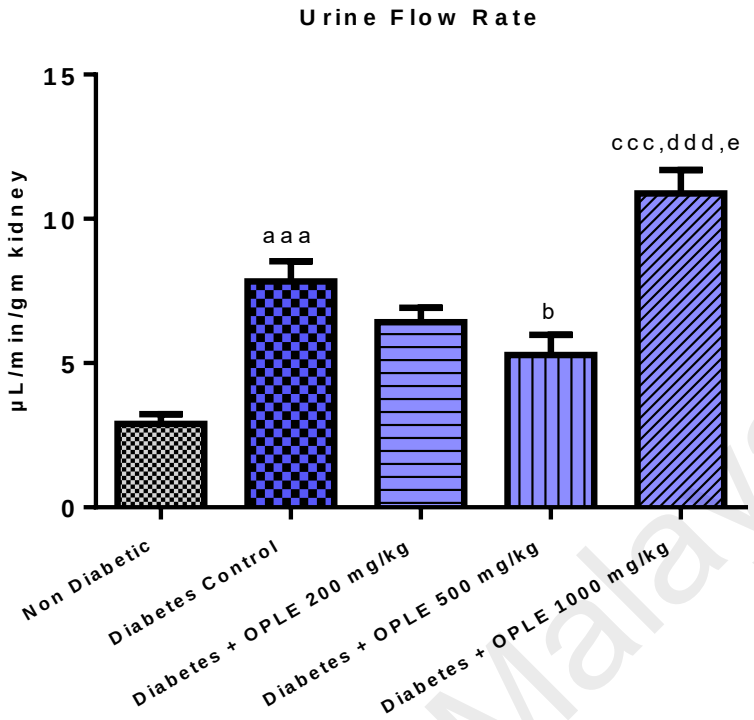


Figure 3.8(b). Effects of OPLE on urine flow rate in 12 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P < 0.001$ vs. non-diabetic; $^{b}P < 0.05$ vs. diabetic control; $^{ccc}P < 0.001$ vs diabetes + OPLE 200 mg/kg; $^{ddd}P < 0.001$ vs. diabetes + OPLE 500 mg/kg; $^{e}P < 0.05$ vs diabetic control.

3.10. Proteinuria

Total protein excretion was measured to indicate the level of proteinuria in experimental animals. To substantiate the beneficial effect of OPLE treatment on renal dysfunction in diabetic rats, we measured urinary protein excretion (Figure 3.9 a and b). Urinary protein excretion increased significantly in diabetic rats compared with non-diabetic rats at both 4 weeks (6.83±0.30 mg/2hr vs 1.33±0.03 mg/2hr, $P < 0.001$) and 12 weeks (4.66±0.73 mg/2hr vs 1.13±0.05 mg/2hr, $P < 0.001$), indicating a modification in glomerular barrier characteristics. Proteinuria induced by diabetes was significantly reduced by treatment with OPLE at 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day concentrations tested at 4 weeks (2.87±0.17 mg/2hr, 1.89±0.34 mg/2hr and 1.77±0.17 mg/2hr, $P < 0.001$, respectively); furthermore the higher dose reduced the excretion to almost non-diabetic value. At 12 weeks, OPLE at 200 mg/kg/day and 500 mg/kg/day concentrations tested ameliorated the increase in urinary protein excretion in diabetic rats (2.92±0.26 mg/2hr, $P < 0.05$ for 200 mg/kg/day and 2.68±0.21 mg/2hr, $P < 0.01$ for 500 mg/kg/day). However, treatment of diabetic animals with OPLE at 1000 mg/kg/day in the 12-week study the incidence of proteinuria was aggravated ($P < 0.01$), indicating renal dysfunction and renal damage. This finding shows that treatment with OPLE at high dose (1000 mg/kg/day) for longer duration does not prevent the development of proteinuria rather it aggravated the renal dysfunction.

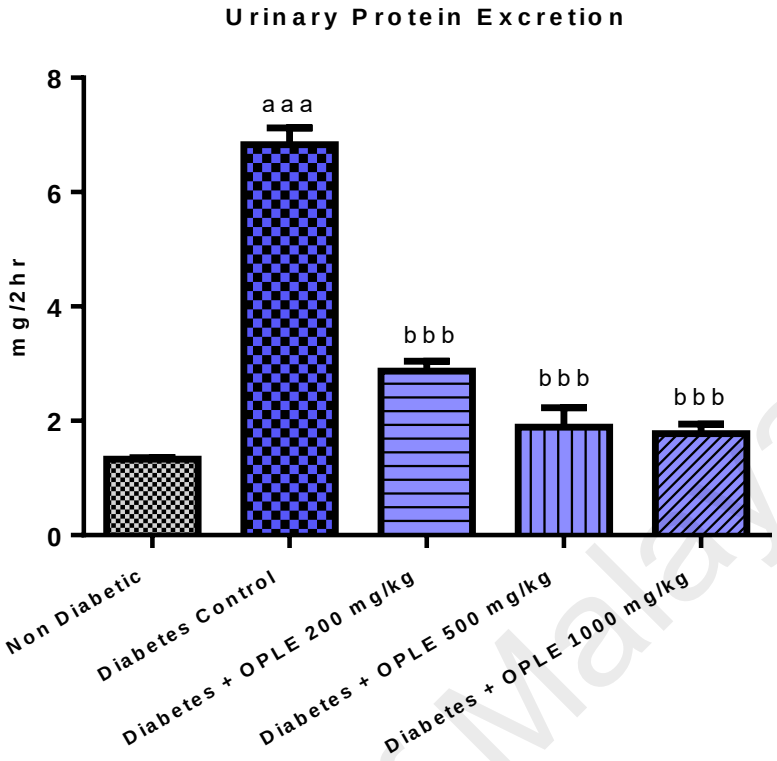


Figure 3.9(a). Effects of OPLE on urinary protein excretion in 4 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P <$ 0.001 vs. non-diabetic; $^{bbb}P <$ 0.001vs. diabetic control.

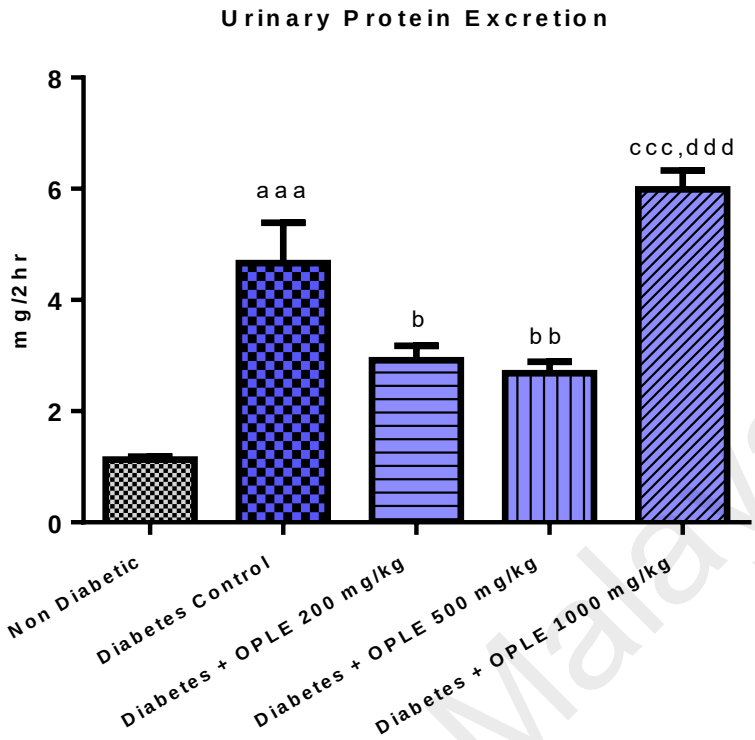


Figure 3.9(b). Effects of OPLE on urinary protein excretion in 12 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P <$ 0.001 vs. non-diabetic; $^{b}P < 0.05$; $^{bb}P < 0.01$vs. diabetic control; $^{ccc}P < 0.001$ vs diabetes + OPLE 200 mg/kg; $^{ddd}P < 0.001$ vs. diabetes + OPLE 500 mg/kg.

3.11. Inulin Clearance

Inulin clearance was used as a measure of glomerular filtration rate (GFR) in experimental rats. It is well-established that early stages of diabetic nephropathy are associated with increased glomerular filtration rate (GFR) both clinically and experimentally. Rats with 4-week diabetes exhibited elevated values for GFR when compared with those of non-diabetic rats (0.96±0.04 mL/min/gm kidney vs 0.61±0.06 mL/min/gm kidney, $P < 0.01$) assessed in terms of inulin clearance (Figure 3.10 a). Increase in GFR in diabetes may indicate hyperfiltration. Hyperfiltration was prevented by treatment with OPLE at 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day (0.67±0.07 mL/min/gm kidney, 0.63±0.09 mL/min/gm kidney and 0.63±0.06 mL/min/gm kidney, $P < 0.05$, respectively). The results of the present study indicate that the rise in RBF might be responsible for the hyperfiltration as observed in diabetic rats, although a rise in glomerular capillary pressure might have contributed. In the 12-week experimental studies (Fig.3.10 b), as compared to control animals, GFR in the diabetic group was noted to be significantly increased (0.84±0.06 mL/min/gm kidney vs 0.50±0.05 mL/min/gm kidney, $P < 0.01$). Importantly, OPLE 500 mg/kg/day treatment of diabetic animals showed significant suppression in GFR elevation (0.58±0.02 mL/min/gm kidney, $P < 0.05$). Treatment of diabetic animals with OPLE (200 mg/kg/day) reduced the GFR elevation in these animals but this reduction is not significant (0.75 ± 0.06 mL/min/gm kidney). On the other hand, in 12 weeks experimental model, OPLE treatment to diabetic rats at high dose (1000 mg/kg/day) was noted to further increase the GFR (1.07±0.10 mL/min/gm kidney, $P < 0.05$), indicating worsening of hyperfiltration.

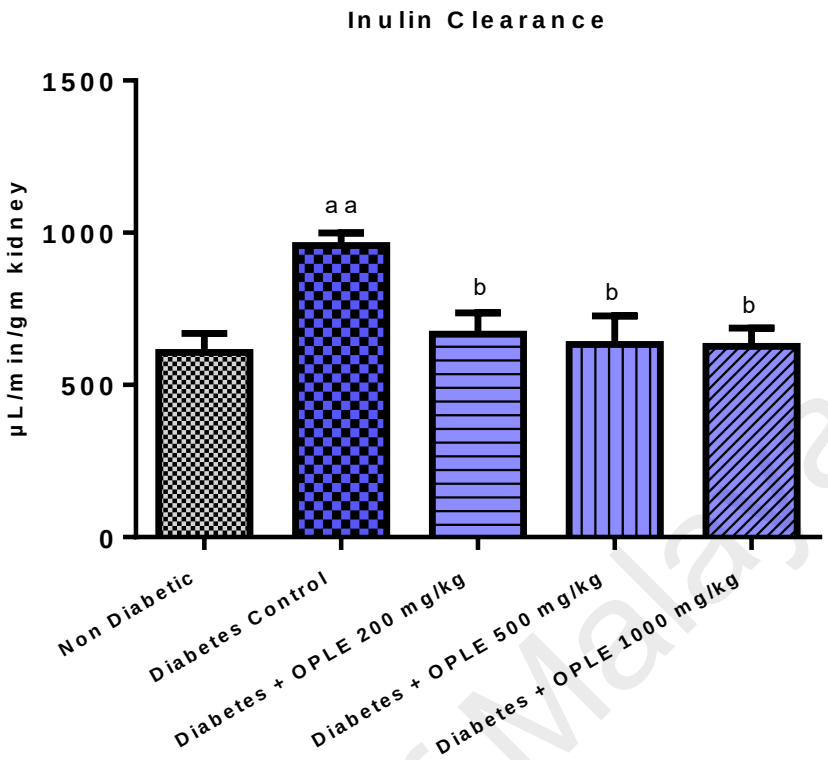


Figure 3.10(a). Effects of OPLE on GFR as assessed in terms of analyzing inulin clearance in 4 weeks experimental model. Data are expressed as mean ± SEM (*n*=6 per group). $^{aa}P < 0.01$ vs. non-diabetic; $^{b}P < 0.05$ vs. diabetic control.

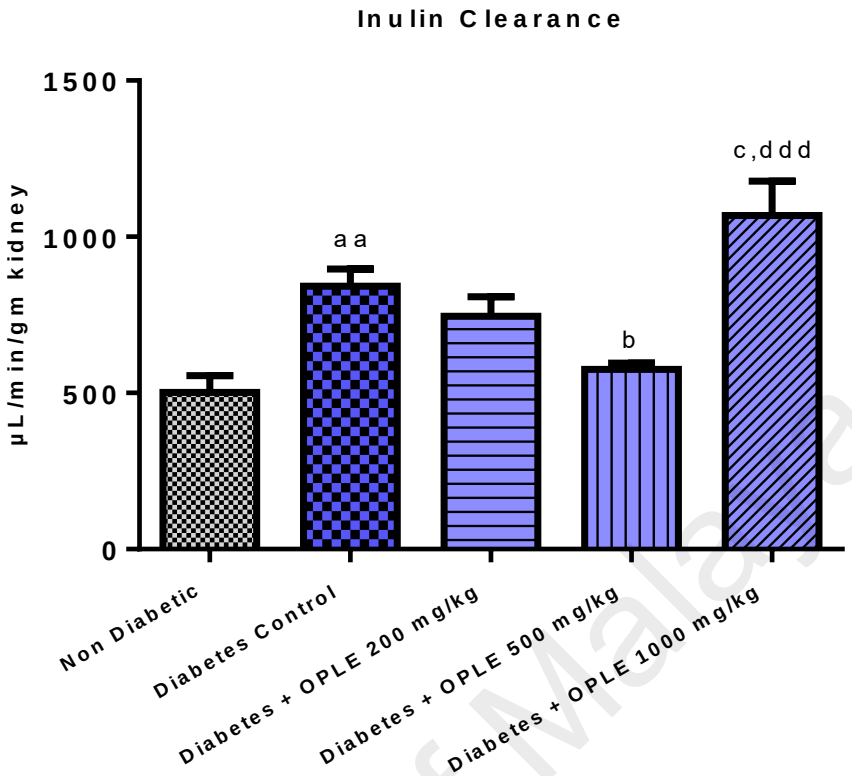


Figure 3.10(b). Effects of OPLE on GFR as assessed in terms of analyzing inulin clearance in 12 weeks experimental model. Data are expressed as mean ± SEM (*n*=6 per group). $^{aa}P < 0.01$ vs. non-diabetic; $^{b}P < 0.05$ vs. diabetic control; $^{c}P < 0.05$ vs diabetes + OPLE 200 mg/kg; $^{ddd}P < 0.001$ vs. diabetes + OPLE 500 mg/kg.

3.12. Assessment of Electrolyte Composition

Electrolyte (sodium and potassium) composition in the urine and plasma was measured by flame photometry. The electrolyte composition in all experimental animals was calculated and expressed as fractional sodium excretion and absolute potassium excretion. These data are useful to measure the efficiency of renal tubular re-absorptive function.

3.12.1. Fractional Sodium Excretion

Fractional sodium excretion was evaluated in order to obtain information on the integrity of tubular reabsorptive function. As shown in Figures 3.11 a and b, the fractional sodium was noted to be increased in diabetic rats as compared to non-diabetic control rats; the result was statistically significant in both 4 weeks and 12 weeks experimental models. The diabetic animal treatment with OPLE (200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day in 4 weeks model; 200 mg/kg/day and 500 mg/kg/day in 12 weeks model) slightly reduced the diabetes-induced increase in fractional sodium excretion, the results were not statistically significant. Moreover, the increase in fractional sodium excretion observed in diabetic rats treated with 1000 mg/kg/day in 12 weeks model was also not statistically significant when compared with diabetic control rats.

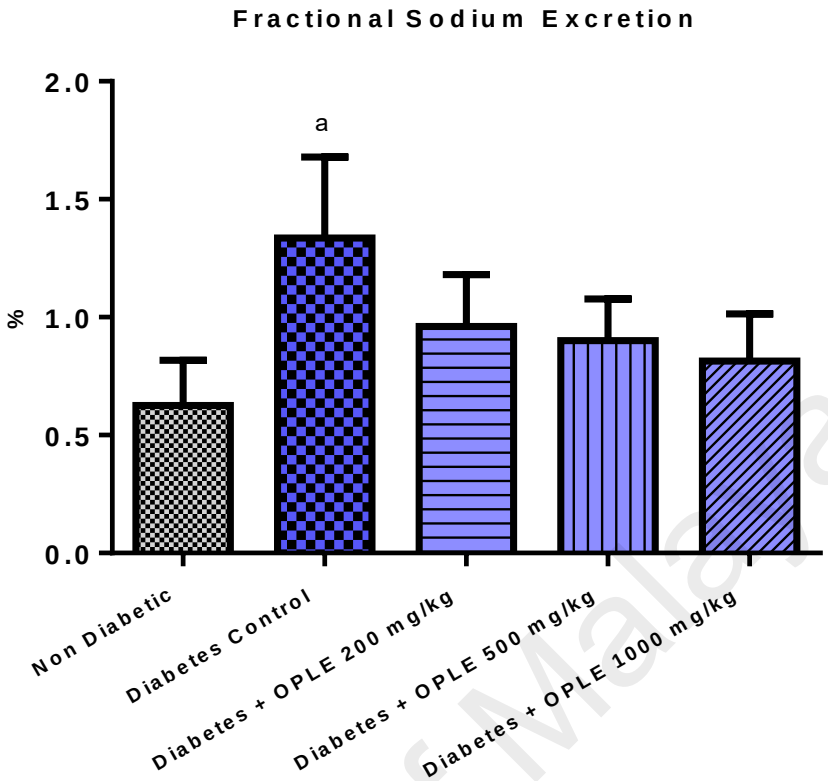


Figure 3.11(a). Effects of OPLE on fractional sodium excretion in 4 weeks experimental model. Data are expressed as mean ± SEM (*n*=6 per group). $^{a}P < 0.05$ vs. non-diabetic.

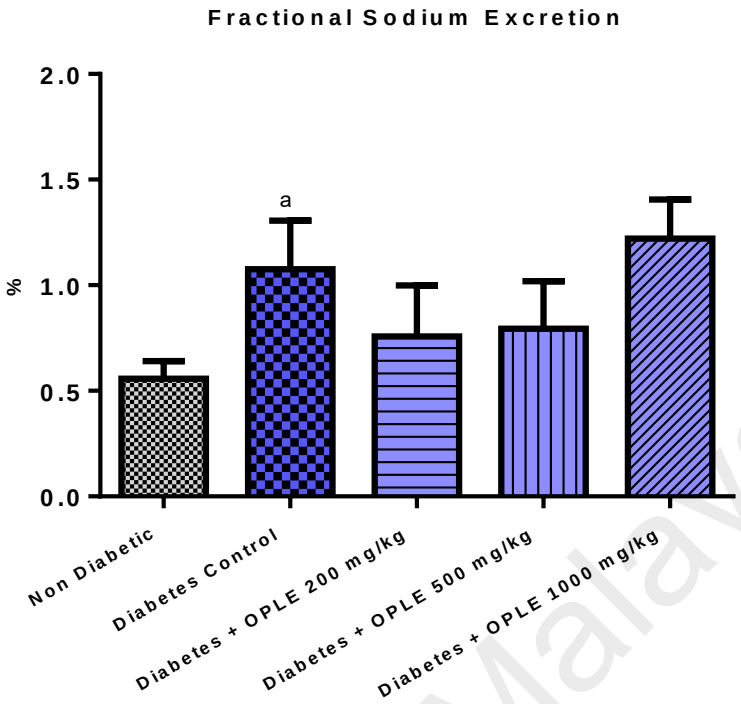


Figure 3.11(b). Effects of OPLE on fractional sodium excretion in 12 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{a}P < 0.05$ vs. non-diabetic.

3.12.2. Absolute Potassium Excretion

Absolute potassium excretion is to assess the tubular function in diabetic nephropathy. The absolute potassium excretion was measured and the results are as shown in Figures 3.12 a and b. In the 4 weeks study, diabetic animal showed significant increase in absolute potassium excretion by 2.9 fold (1374.0±208.5 µmol/min/gm kidney vs 468.9±113.1 µmol/min/gm kidney, $P < 0.01$) when compared with non-diabetic control. Treatment of diabetic animal with OPLE (200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day) showed significant reductions in absolute potassium excretion by 2, 2.3 and 2.4 fold (673.1±114.3 µmol/min/gm kidney, 580.6±167.7 µmol/min/gm kidney and 571.4±194.4 µmol/min/gm kidney, $P < 0.05$, respectively) when compared with diabetic control. In the 12 weeks study (Figure 3.12b), the diabetic animals, when compared to non-diabetic control, showed an increase in absolute potassium excretion by 2.7 fold (1141±221.2 µmol/min/gm kidney vs 424.7±60.92 µmol/min/gm kidney, $P < 0.01$). Interestingly, diabetic animal treated with OPLE (200 mg/kg/day, 500 mg/kg/day) demonstrated significant ameliorations in absolute potassium excretion by 2.2 and 2.4 fold (530.5±93.3 µmol/min/gm kidney and 468.0±86.1 µmol/min/gm kidney, $P < 0.01$ and $P < 0.05$, respectively) as compared to diabetic control animal. Diabetes animal treated with OPLE 1000 mg/kg/day for 12 weeks study did not show any improvement with regard to absolute potassium excretion (1166.0±100.3 µmol/min/gm kidney) rather it further increased potassium excretion when compared to diabetic control.

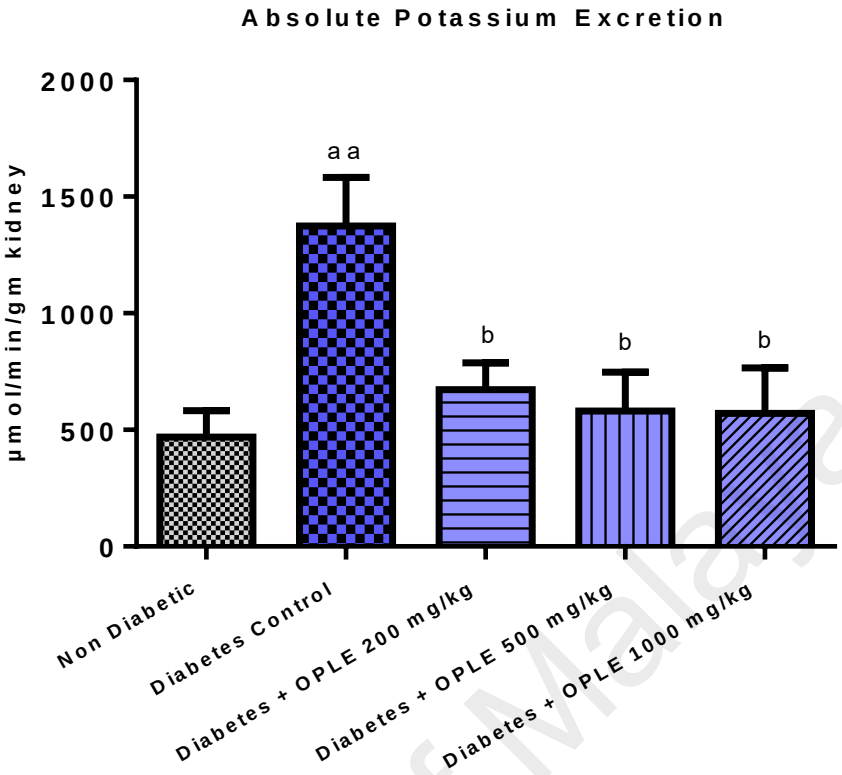


Figure 3.12(a). Effects of OPLE on absolute potassium excretion in 4 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). ^{aa}P < 0.01 vs. non-diabetic; ^{b}P < 0.05 vs. diabetic control.

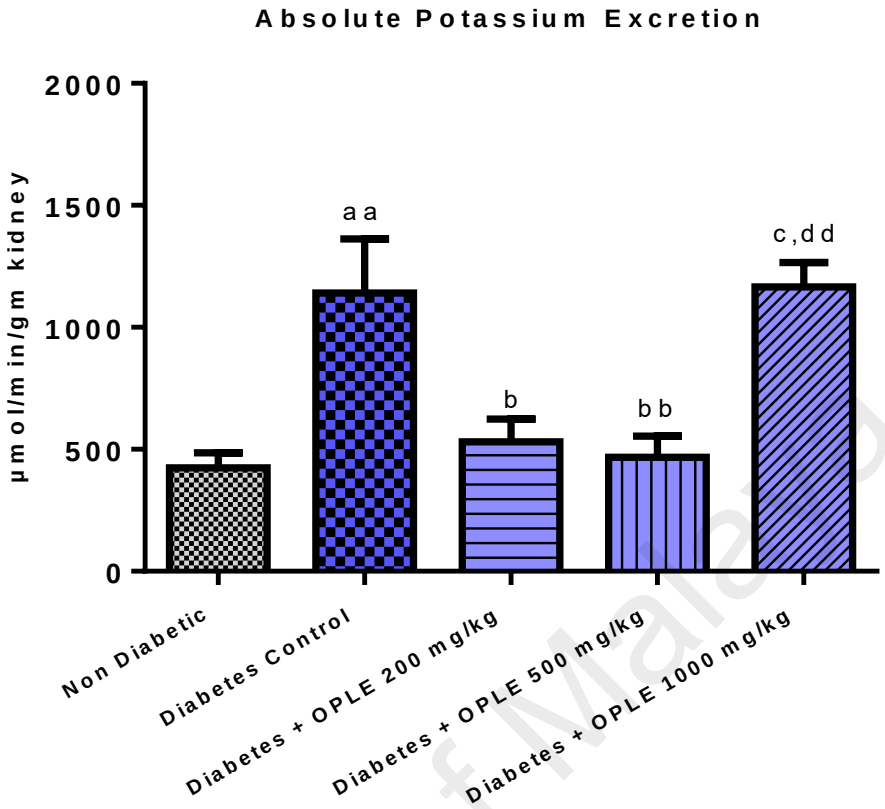


Figure 3.12(b). Effects of OPLE on absolute potassium excretion in 12 weeks experimental model. Data are expressed as mean ± SEM (*n*=6 per group). $^{aa}P <$ 0.01 vs. non-diabetic; $^{b}P < 0.05$; $^{bb}P < 0.01$ vs. diabetic control; $^{c}P < 0.05$ vs diabetes + OPLE 200 mg/kg; $^{dd}P < 0.01$ vs. diabetes + OPLE 500 mg/kg.

3.13. 8-Hydroxy-2-deoxy Guanosine (8-OHdG):

The oxidative DNA damage in kidney of diabetic rats was assessed by measuring the levels of 8-OHdG in urine samples. In 4 weeks and 12 weeks experimental models, the levels of urinary 8-OHdG (Figure. 3.13 a, b), were significantly greater in diabetic rats than in non-diabetic rats (22.56±3.51 ng/2hr vs. 7.77±0.93 ng/2hr, $P < 0.001$, 4 weeks) (25.01±3.13 ng/2hr vs. 6.45±1.09 ng/2hr, $P < 0.001$, 12 weeks). These levels were reduced significantly ($P < 0.05$) in diabetic rats treated with 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day OPLE for 4 weeks (12.62±1.31 ng/2hr, 12.40±1.93 ng/2hr and 12.23±0.75 ng/2hr, respectively).

In contrast, only OPLE 500 mg/kg/day shows significant reduction of 8-OHdG in diabetic rats when compared with untreated diabetic rats in 12 weeks experimental model (15.02±2.31 ng/2hr, $P < 0.01$). OPLE at 200mg/kg/day also reduced urinary 8-OHdG (17.98±2.43 ng/2hr) when compared with diabetic control, but the result was not statistically significant. On the other hand, in 12 weeks model, treatment with OPLE 1000 mg/kg/day further increased urinary 8-OHdG in diabetic rats (30.14±0.75 ng/2hr). Taken together, these findings indicate that treatment with OPLE (200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day) ameliorated the oxidative stress-induced DNA damage in the rat kidney of 4 weeks experimental model. In the 12 weeks study, OPLE 200 mg/kg/day and 500 mg/kg/day reduced the oxidative stress DNA damage in the kidney. However, the higher dose of OPLE 1000 mg/kg/day showed further increase in urinary 8-OHdG in diabetic rats that apparently indicates the pro-oxidant activity of the high dose of OPLE treatment for longer duration.

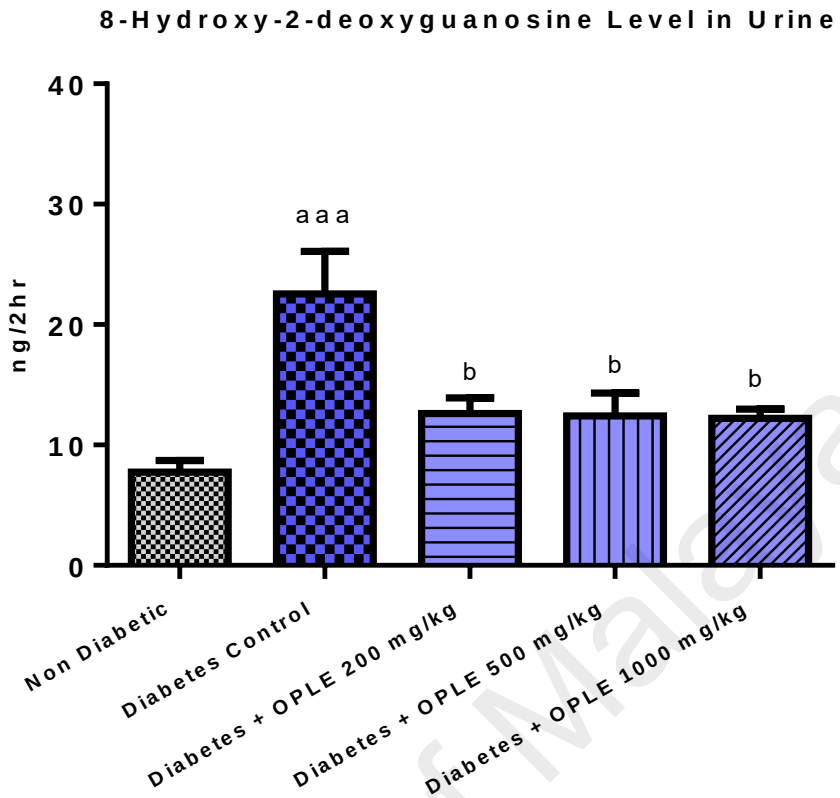


Figure 3.13(a). Effects of OPLE on urinary 8-OHdG concentration in 4 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P <$ 0.001 vs. non diabetic; $^{b}P < 0.05$ vs. diabetes control.

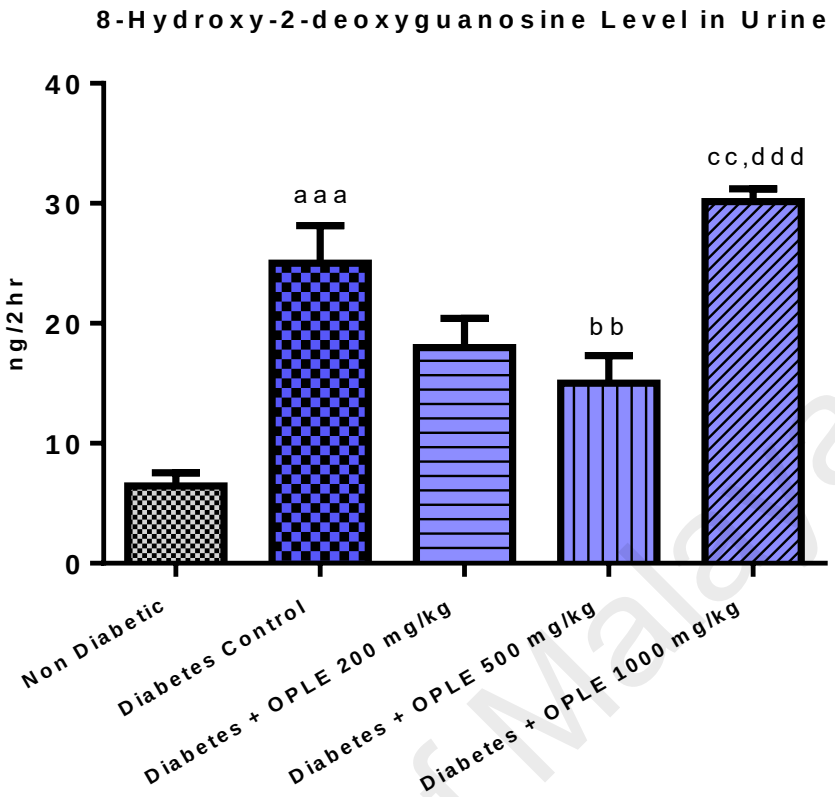


Figure 3.13(b). Effects of OPLE on urinary 8-OHdG concentration in 12 weeks experimental model. Data are expressed as mean ± SEM (*n*=6 per group). $^{aaa}P <$ 0.001 vs. non-diabetic; $^{bb}P <$ 0.01 vs. diabetes control; $^{cc}P <$ 0.01 vs diabetes + OPLE 200 mg/kg; $^{ddd}P <$ 0.001 vs. diabetes + OPLE 500 mg/kg.

3.14. Lipid Peroxides (LPO)

Renal cortical LPO were noted to be significantly higher in diabetic rats than in non-diabetic rats (Fig.3.14 a, b) at both 4 weeks (3.88±0.15 nmols/mg protein vs. 2.48±0.14 nmols/mg protein, $P < 0.001$) and 12 weeks (6.08±0.53 nmols/mg protein vs. 2.88±0.17 nmols/mg protein, $P < 0.001$) of the experimental duration.

While diabetic animals treated with 200 mg/kg/day OPLE did not affect renal cortical LPO levels in the 4 weeks model (3.45±0.10 nmols/mg protein) but in the 12 weeks model, it showed a reduction in renal cortical LPO (4.48±0.38 nmols/mg protein) but not significant when compared with diabetic control. The diabetic animal treated with OPLE 500 mg/kg/day exhibited significant reduction in the renal cortical LPO elevations in both 4 weeks (2.97±0.17 nmols/mg protein, $P < 0.001$) and 12 weeks (4.12±0.39 nmols/mg protein, $P < 0.05$) experimental models. OPLE treated with 1000 mg/kg/day reduced significantly renal cortical LPO elevations in diabetic rats in 4 weeks model (2.76±0.06 nmols/mg protein $P < 0.001$), but in the 12 weeks study, diabetic animals treated with OPLE 1000 mg/kg/day showed further increase in LPO levels (7.86±0.35 nmols/mg protein, $P < 0.001$) when compared with diabetic control group rats; once again indicating that prolonged administration of high dose of OPLE (1000 mg/kg/day) aggravated renal oxidative stress.

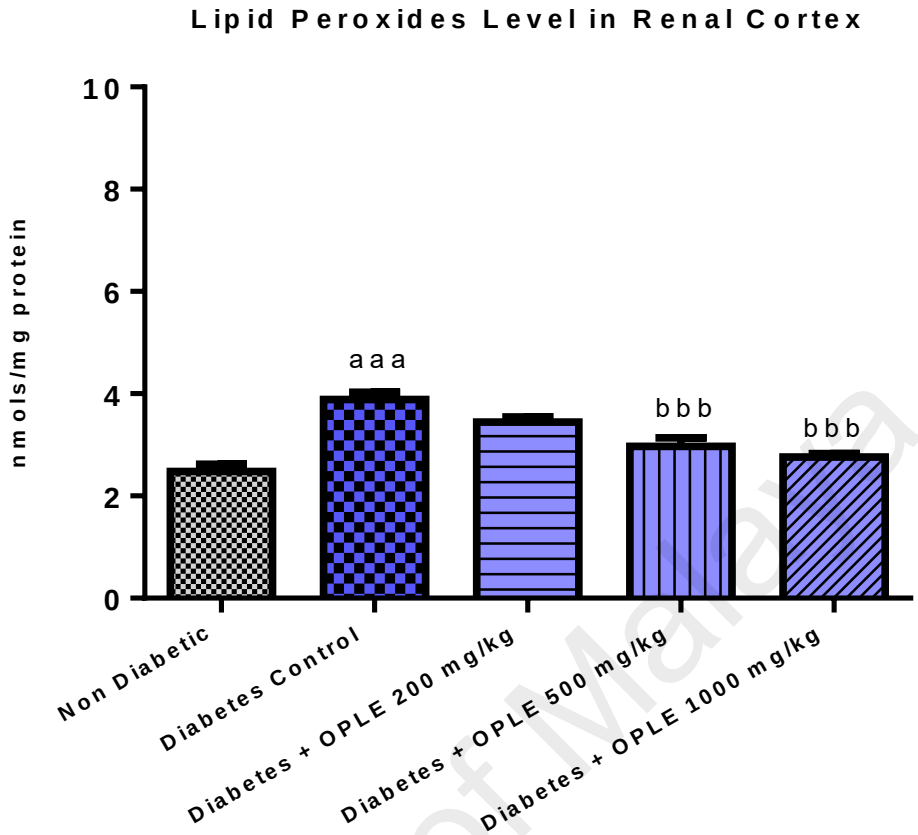


Figure 3.14(a). Effects of OPLE on renal cortical LPO concentrationin 4 weeks experimental model. Data are expressed as mean ± SEM (*n*=6 per group). $^{aaa}P <$ 0.001 vs. non-diabetic; ^{bbb}P < 0.001 vs. diabetes control.

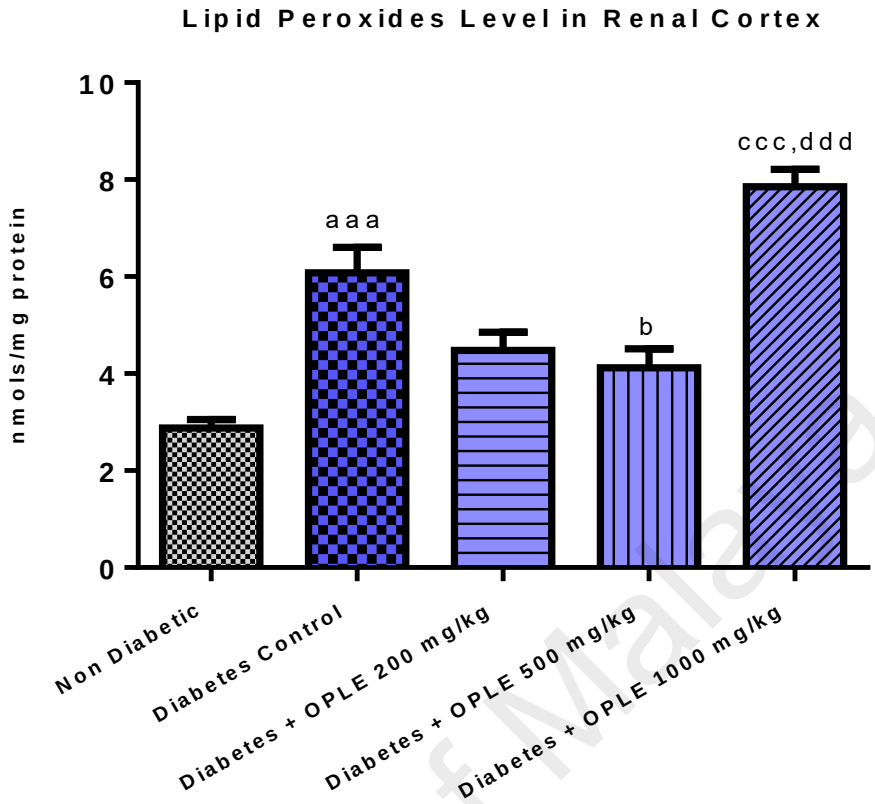


Figure 3.14(b). Effects of OPLE on renal cortical LPO concentration in 12 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). ^{aaa}P < 0.001 vs. non-diabetic; ^{b}P < 0.05 vs. diabetes Control; ^{ccc}P < 0.001 vs diabetes + OPLE 200 mg/kg; ^{ddd}P < 0.001 vs. diabetes + OPLE 500 mg/kg.

3.15. Glutathione (GSH)

GSH constitutes a part of the endogenous antioxidant defense system; the GSH redox cycle plays a major role in scavenging hydrogen peroxide (H_2O_2) under physiological conditions. To establish the effect of OPLE in modulating endogenous antioxidant defense system in diabetes, the renal GSH content was measured. As demonstrated in (Figure. 3.15 a, b), a reduction in renal cortical GSH content was noted in diabetic rats that was improved by 500 mg/kg/day OPLE and 1000 mg/kg/day OPLE in diabetic rats of 4 weeks model (4.02±0.16 nmols/mg protein and 4.08±0.22 nmols/mg protein vs. 2.98 ± 0.13 nmols/mg protein in untreated diabetic rats, $P < 0.05$). Diabetic animals treated with OPLE 200 mg/kg/day in 4 weeks study showed improvement in the GSH content (3.84±0.16 nmols/mg protein) but this improvement was not statistically significant when compared with diabetic control animals.

Similarly in the 12 weeks study, diabetic animals treated with OPLE 200 mg/kg/day also showed improvement in GSH content (3.42±0.23 nmols/mg protein) but this is also not statistically significant when compared with diabetic control. The diabetic animal treated with OPLE 500 mg/kg/day showed significant difference by 1.5 fold increases (4.13±0.19 nmols/mg protein vs 2.65±0.36 nmols/mg protein in untreated diabetic rats, $P < 0.05$). However, for 1000 mg/kg/day OPLE treatment to diabetic rats in 12 weeks model, there was a further reduction, albeit not significant, of renal GSH (2.31 ± 0.13nmols/mg protein vs. 2.65 ± 0.36 nmols/mg protein of diabetic control group rats).

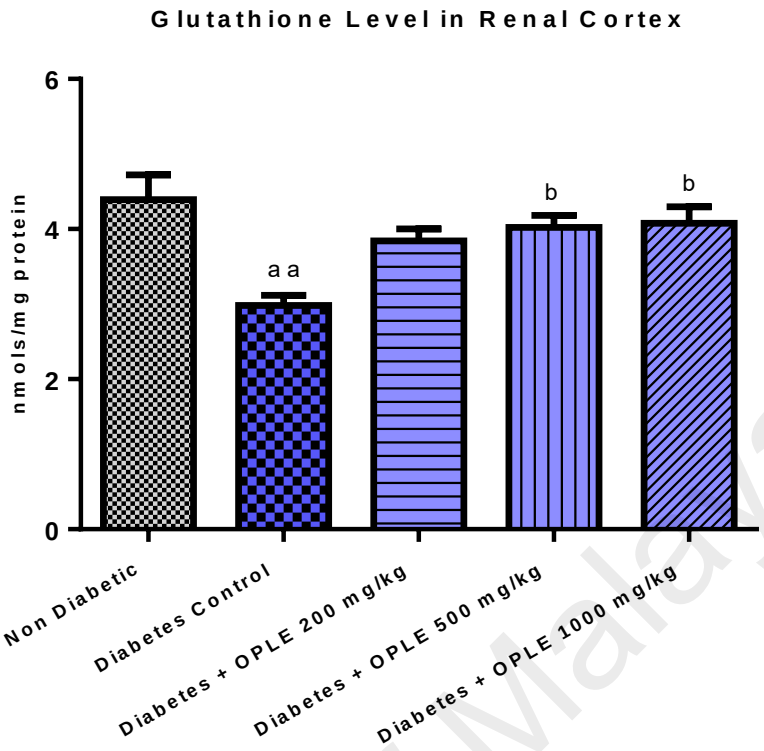


Figure 3.15(a). Effects of OPLE on kidney (renal cortex) GSH concentration in 4 weeks experimental model. Data are expressed as mean ± SEM (*n*=6 per group). $^{aa}P < 0.01$ vs. non-diabetic; $^{b}P < 0.05$ vs. diabetes control.

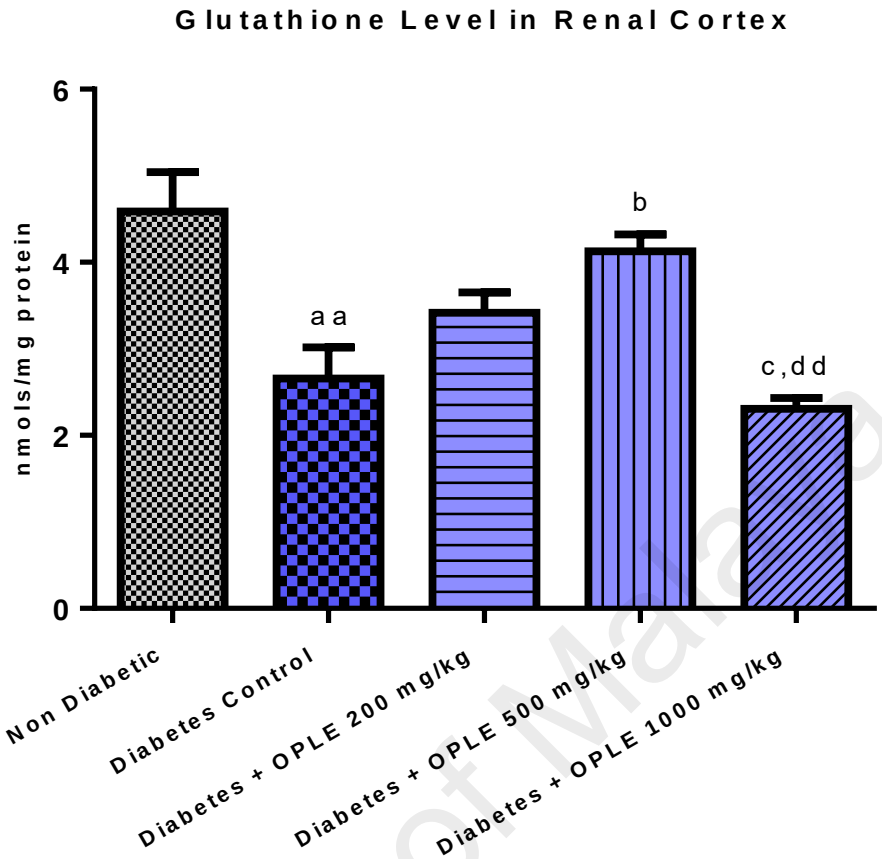


Figure 3.15(b). Effects of OPLE on kidney (renal cortex) GSH concentration in 12 weeks experimental model. Data are expressed as mean ± SEM (*n*=6 per group). $^{aa}P < 0.01$ vs. non-diabetic; $^{b}P < 0.05$ vs. diabetes control; $^{c}P < 0.05$ vs diabetes + OPLE 200 mg/kg; $^{dd}P < 0.01$ vs. diabetes + OPLE 500 mg/kg.

3.16. Transforming Growth Factor Beta-1 (TGF-β1)

TGF-β1, a fibrogenic cytokine, is purported to be a major mediator of the hypertrophic changes in diabetic kidney diseases. Thus in accord with renal dysfunction and the increased markers of oxidative stress (urinary 8-OHdG excretion and renal cortical LPO, GSH), significantly higher concentrations of TGF-β1 were detected in plasma of diabetic rats than in non-diabetic rats (Figure. 3.16 a, b), at both 4 weeks (17.09±1.10 pg/mL vs. 12.09±0.75 pg/mL, $P < 0.01$) and 12 weeks (21.34±1.27 pg/mL vs. 13.71±0.81 pg/mL, $P < 0.001$) model. Treatment of diabetic rats with OPLE at all concentrations in 4 weeks model normalized plasma TGF-β1 concentrations (12.65±0.56 pg/mL ($P < 0.05$), 11.90±0.56 pg/mL ($P < 0.01$) and 12.09± 0.82 pg/mL ($P < 0.01$) with 200 mg/kg/day OPLE, 500 mg/kg/day OPLE and 1000 mg/kg/day OPLE, respectively).

In the 12 week study, OPLE at 200 mg/kg/day did not have significant effect on plasma TGF-β1 concentrations (18.03±0.56 pg/mL) in diabetic rats, but OPLE at 500 mg/kg/day reduced the plasma TGF-β1 concentrations (16.01±0.33 pg/mL vs 21.34±1.27 pg/mL, $P < 0.01$). However, the diabetic animal treated with OPLE 1000 mg/kg/day in 12 weeks model showed a further increase in TGF-β1 concentrations in comparison to diabetic control group rats (26.42±1.44 pg/mL, $P < 0.01$), indicating that more fibrotic changes within the kidney might have occurred at high dose treatment with longer duration.

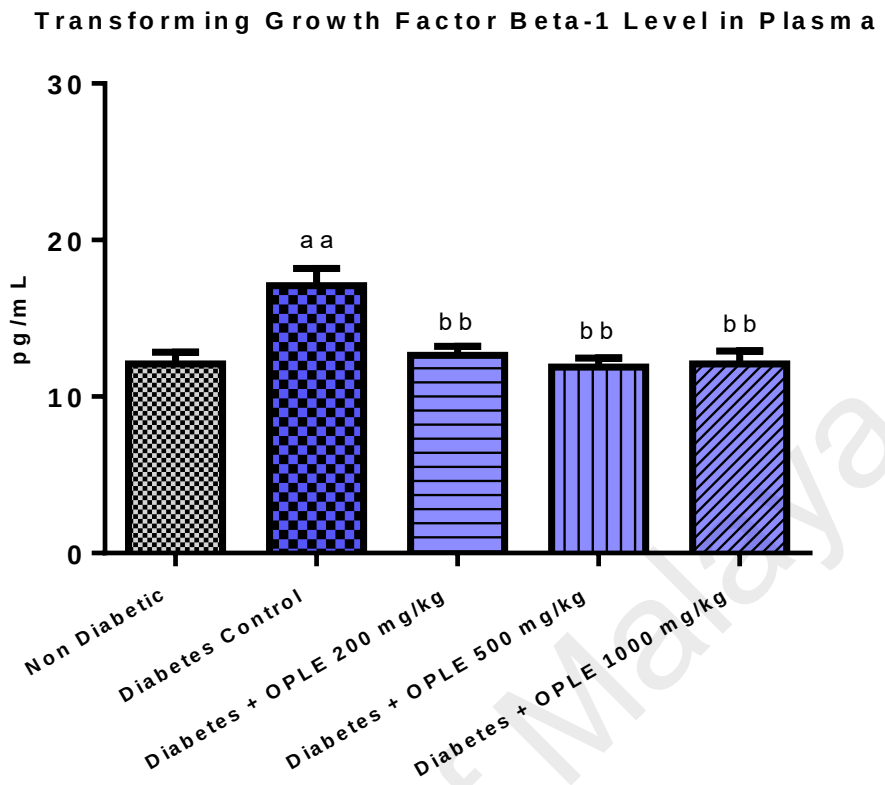


Figure 3.16(a). Plasma TGF-β1 concentration in 4 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{aa}P < 0.01$ vs. non-diabetic; $^{bb}P < 0.01$ vs. diabetes control.

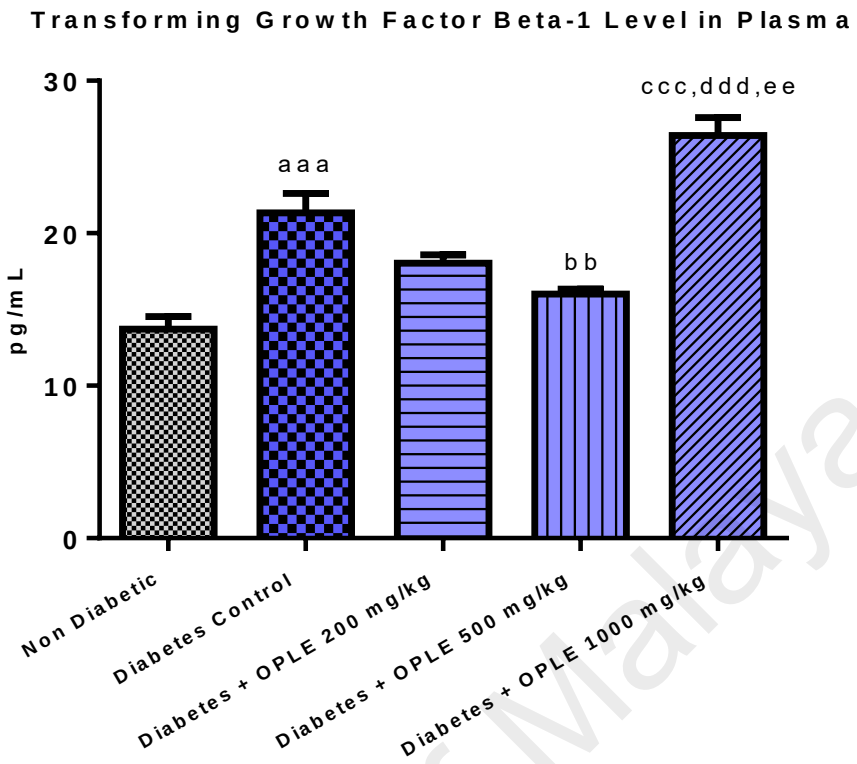


Figure 3.16(b). Plasma TGF-β1 concentration in 12 weeks experimental model. Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P < 0.001$ vs. non-diabetic; $^{bb}P < 0.01$ vs. diabetes control; $^{ccc}P < 0.001$ vs diabetes + OPLE 200 mg/kg; $^{ddd}P < 0.001$ vs. diabetes + OPLE 500 mg/kg; $^{ee}P < 0.01$ vs diabetic control.

3.17. Morphological Studies

3.17.1. Haematoxylin and Eosin (H&E)

Renal histopathological examination according to H & E staining of the normal control animals showed no pathological changes, whereas those of the diabetic control animals presented glomerular injury, tubular vacuolization-necrosis, interstitial edema and interstitial infiltration by inflammatory cells (Figure. 3.17 a). Similarly, H&E staining of diabetic kidneys in the 12 weeks study showed further increase in the glomerular injury, tubular vacuolization-necrosis, interstitial edema and interstitial infiltration by inflammatory cells. However, upon treatment of diabetic animals with OPLE 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day in the 4 weeks study, very minimal histological changes were detected in the diabetic kidneys. As for the 12 weeks study, pathological changes within the diabetic kidneys were completely prevented by OPLE 500 mg/kg/day. OPLE 200 mg/kg/day treatment showed very minimal changes in the kidneys of diabetic animals. But, the diabetic animals treated with OPLE 1000 mg/kg/day was noted to produce more severe damage than the diabetic control group rats of 12 weeks study (Figure. 3.17 b).

3.17.2. Periodic Acid-Schiff (PAS)

PAS-stained sections of the renal cortex of diabetic control rats in both the 4 and 12 weeks models (Figure. 3.18 a, b) exhibited marked glomerulosclerosis, characterized by glomerular basement membrane thickening and mesangial expansion with glomerular hypertrophy, when compared with control group rats. Remarkably, treatment with OPLE at doses 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day reduced glomerulosclerosis and attenuated the mesangial matrix

accumulation in the diabetic rats. Morphometric analysis revealed a significant decrease in the mesangial area in OPLE-treated group rats and the data clearly demonstrates that OPLE treatment alleviated the mesangial expansion (Table 3.1). But in the 12th week study, diabetic animals treated with OPLE 200 mg/kg/day and 500 mg/kg/day only show reduced glomerulosclerosis and attenuated the mesangial matrix accumulation. On the other hand, the OPLE 1000 mg/kg/day treatment did not show any improvement. Conversely, it produced an opposite effect (Figure 3.18 b).

3.17.3. Masson's Trichrome Stains

Masson's Trichrome-stained section of the diabetic rat kidney in 4 weeks model exhibited increased collagen deposition, tubular dilation, and degeneration of cortical tubules (Figure 3.19 a, b) whilst these changes were not apparent in the control group rat's kidney. Marked tubulointerstitial fibrosis characterized by accumulation of extracellular matrix (ECM) protein in cortex and medulla was observed in diabetic kidney on week 12. There were capillary occlusion, increased proliferation of interstitial fibroblasts, tubular dilatation and atrophy whereas no apparent changes were detected in kidney of non-diabetic control. Treatment with OPLE 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day in the 4 weeks study reduced tubulointerstitial fibrosis when compared to diabetic control rats (Table 3.2). But in the 12th week study diabetic animal treated with 200 mg/kg/day and 500 mg/kg/day there was reduced tubulointerstitial fibrosis, but OPLE treatment at high dose (1000 mg/kg/day) showed further increase in the tubulointerstitial fibrosis, capillary occlusion, increased proliferation of interstitial fibroblasts, tubular dilatation and atrophy when compared with diabetic control group.

Table 3.1. Glomerulosclerotic index in 4 weeks and 12 weeks experimental model

Groups/Durations	Number	Week 4	Week 12
Non Diabetic	6	0.23 ± 0.03	0.34 ± 0.03
Diabetes Control	6	1.14 ± 0.06^{aaa}	1.36 ± 0.04 ^{aaa}
Diabetes + OPLE 200mg/kg	6	0.89 ± 0.07 ^{b}	0.93 ± 0.05^{bb}
Diabetes + OPLE 500mg/kg	6	0.79 ± 0.5 ^{b}	0.88 ± 0.04^{bb}
Diabetes + OPLE 1000mg/kg	6	0.68 ± 0.06 ^{bb}	1.62 ± 0.03^{cc,dd,e}

Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P < 0.001$ vs. Non-diabetic; $^{b}P < 0.05$; $^{bb}P < 0.01$ vs. Diabetes control; $^{cc}P < 0.01$ vs Diabetes + OPLE 200 mg/kg; $^{dd}P < 0.01$ vs. Diabetes + OPLE 500 mg/kg; $^{e}P < 0.05$ vs Diabetic control.

Table 3.2. Tubulointerstitial fibrosis index in 4 weeks and 12 weeks experimental model.

Groups/Durations	Number	Week 4	Week 12
Non Diabetic	6	0.49 ± 0.05	0.61 ± 0.06
Diabetes Control	6	2.48 ± 0.18^{aaa}	2.75 ± 0.16^{aa}
Diabetes + OPLE 200mg/kg	6	1.48 ± 0.06^{bb}	1.31 ± 0.23^{b}
Diabetes + OPLE 500mg/kg	6	1.16 ± 0.03^{bb}	1.05 ± 0.14^{bb}
Diabetes + OPLE 1000mg/kg	6	1.03 ± 0.08 ^{bb}	3.05 ± 0.07^{cc,dd}

Data are expressed as mean ± SEM (n=6 per group). $^{aaa}P < 0.001$ vs. Non-diabetic; $^{b}P < 0.05$; $^{bb}P < 0.01$ vs. Diabetes control; $^{cc}P < 0.01$ vs Diabetes + OPLE 200 mg/kg; $^{dd}P < 0.01$ vs. Diabetes + OPLE 500 mg/kg.

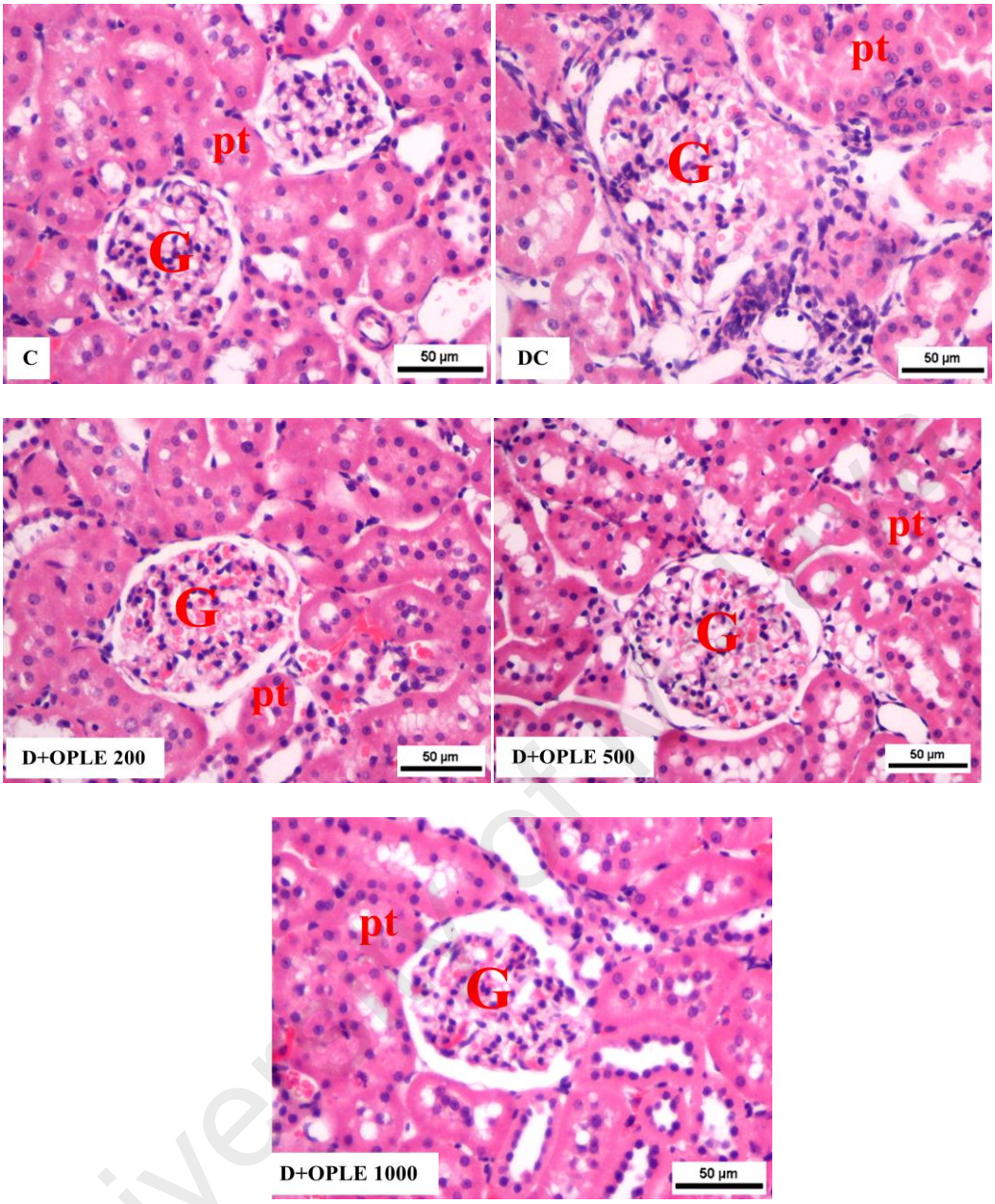


Figure 3.17(a). Histological sections of kidneys stained with Haematoxylin and Eosin in 4 weeks experimental model. Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg /day (D+OPLE 1000), Bar = 50 µm.

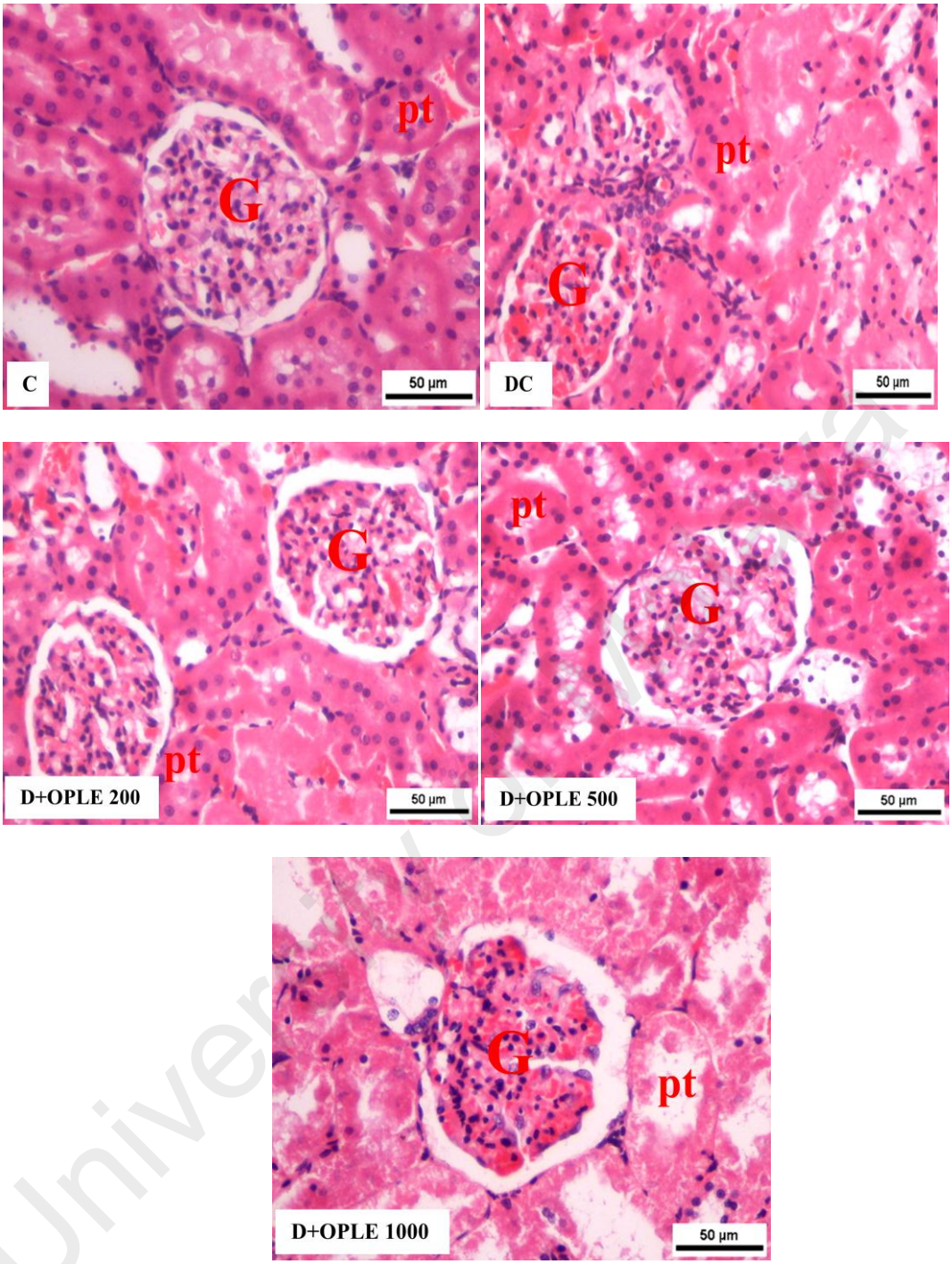


Figure 3.17(b). Histological sections of kidneys stained with haematoxylin and eosin in 12 weeks experimental model. Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000), Bar = 50 µm.

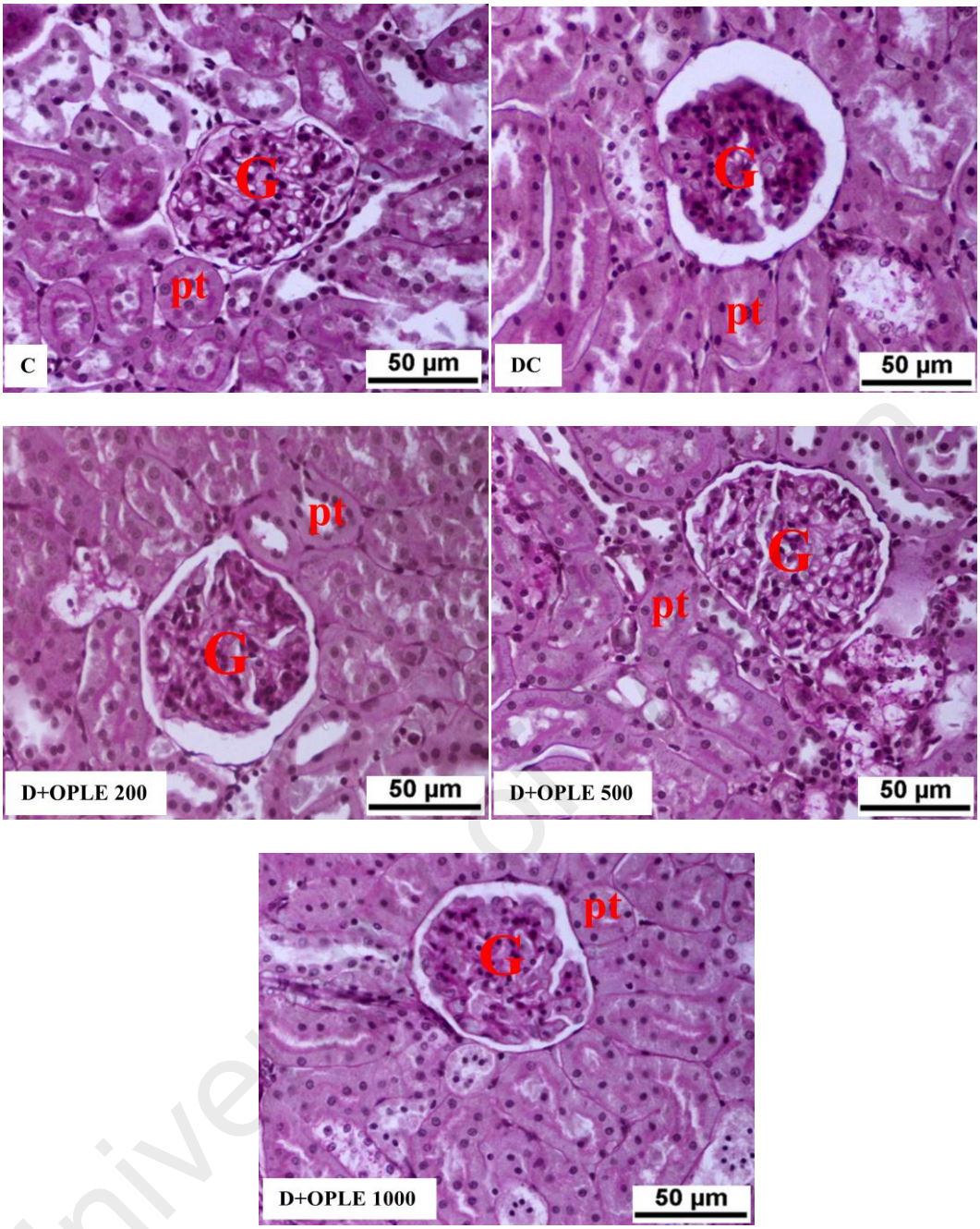


Figure 3.18(a). Histological sections of kidneys stained with periodic acid-Schiff (PAS) in 4 week experimental model. Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000), Bar = 50 µm.

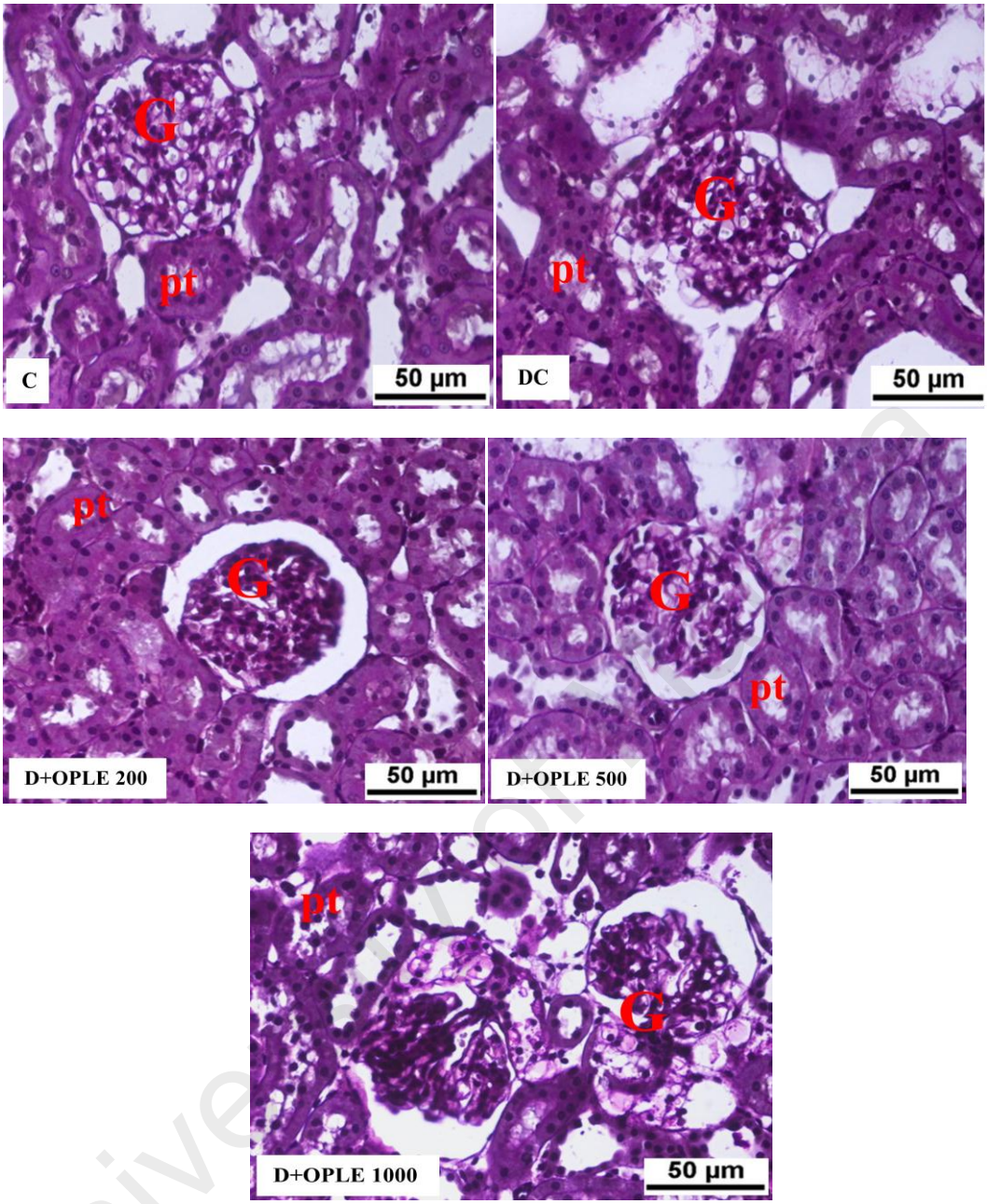


Figure 3.18(b). Histological sections of kidneys stained with periodic acid-Schiff (PAS) in 12 weeks experimental model. Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000), Bar = 50 µm.

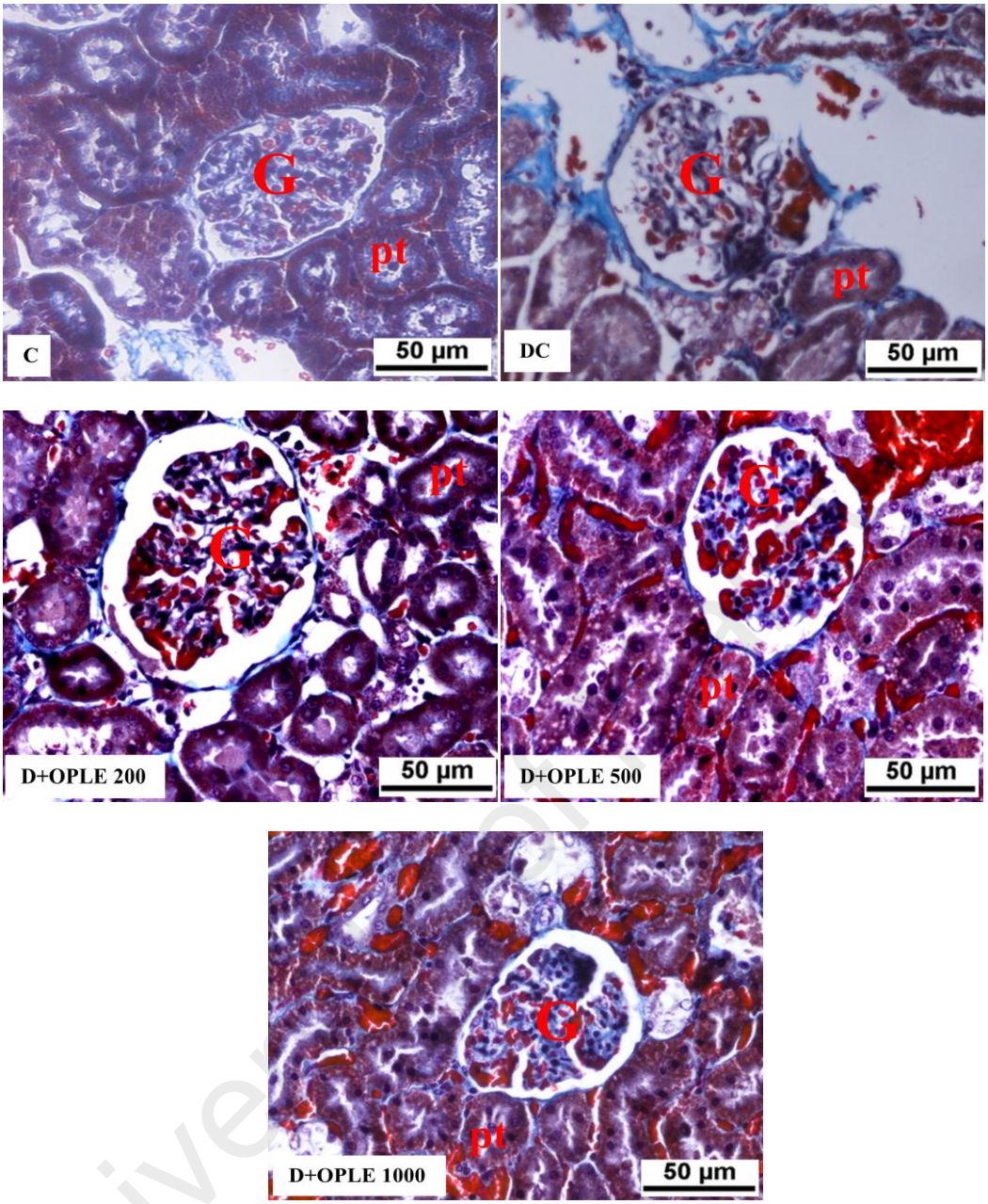


Figure 3.19(a). Histological sections of kidneys stained with Masson's trichrome in 4 weeks experimental model. Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000), Bar = 50 µm.

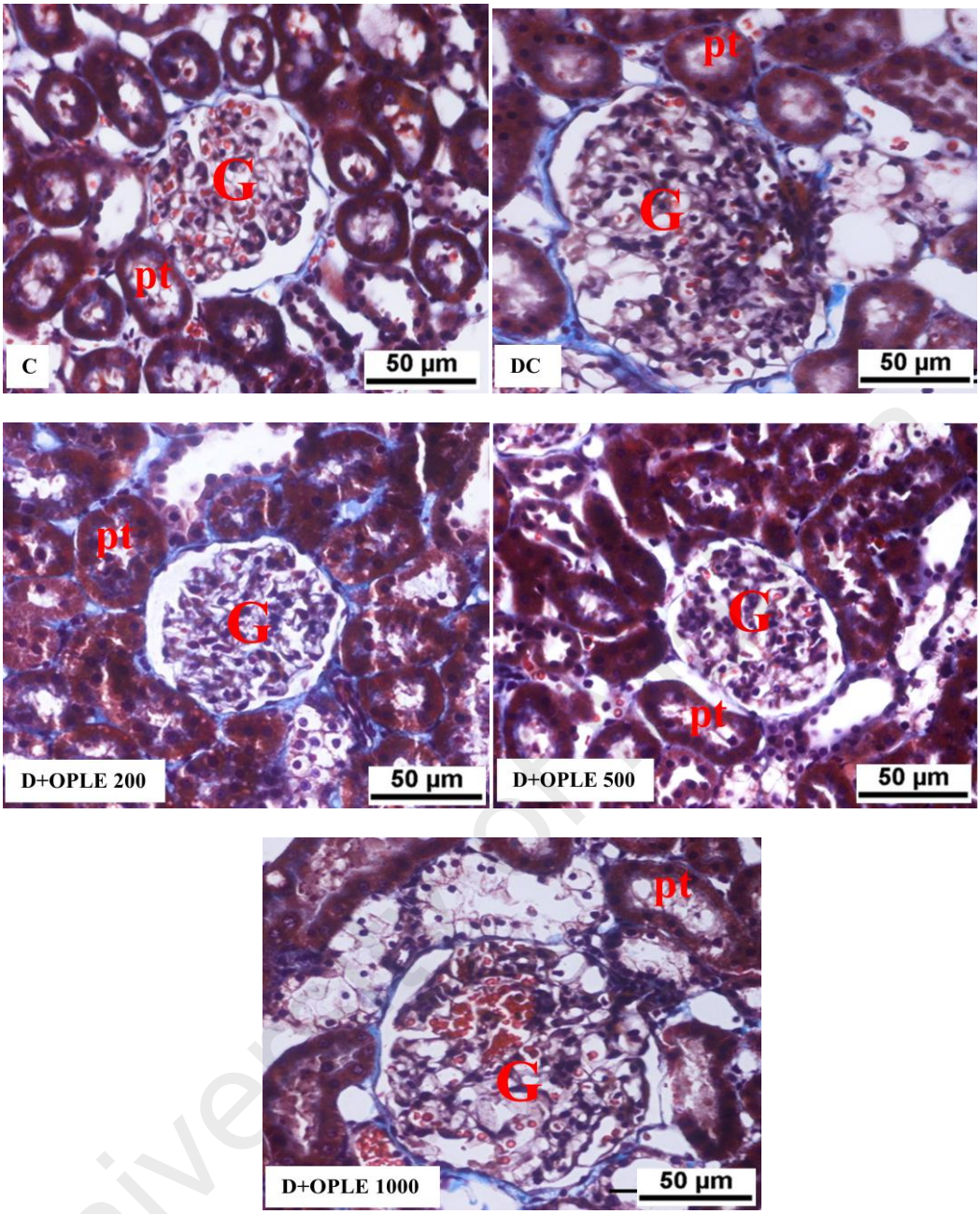


Figure 3.19(b). Histological sections of kidneys stained with Masson's trichrome in 12 weeks experimental model. Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000), Bar = 50 µm.

3.18. Immunohistochemistry

NADPH oxidase subunits (p22phox and p67phox proteins) were immunolocalized to the macula densa and distal tubule (Figure.3.20 (a, b) and Figure 3.21 (a, b)). The intensities of p22phox and p67phox immunostaining were noted to be increased in diabetic control rats, and attenuated with OPLE 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day treatments in the 4 weeks study. Comparably, in the 12 weeks study, the intensities of p22phox and p67phox immunostaining were noted to be increased in diabetic control rats when compared with non-diabetic control group rats. The diabetic animals treated with OPLE 200 mg/kg/day and 500 mg/kg/day over 12 weeks showed attenuated renal intensities of p22phox and p67phox immunostaining. Diabetic animals treated with OPLE 1000 mg/kg/day in the 12 weeks model showed further increase in the intensities of p22phox and p67phox immunostaining as compared to diabetic control rats. This indicates the possibility of a pro-oxidant property of prolonged (i.e. 12 weeks) OPLE treatment at high dose (1000 mg/kg/day).

3.19. Western Blotting

Western blot analysis confirmed the immunohistochemical observation of p22phox and p67phox proteins expression up-regulation in the kidney of diabetic control rats, and this NADPH oxidase subunits level elevation was attenuated by OPLE 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/dayin the 4 weeks study (Figure 3.22 (a), Figure 3.23 (a)).

In the 12 weeks study, diabetic animals treated with only the lower doses of OPLE at 200 mg/kg/day and 500 mg/kg/day showed attenuated upregulation of

protein expression of NADPH oxidase subunits. On the other hand, diabetic animals treated with OPLE 1000 mg/kg/day showed further increase in the protein expression of NADPH oxidase subunits such as p22phox and p67phox as compared to diabetic control rats. This results support the pro-oxidant action of prolonged OPLE treatment at high dose (1000 mg/kg/day) (Figure 3.22 (b), Figure 3.23 (b)).

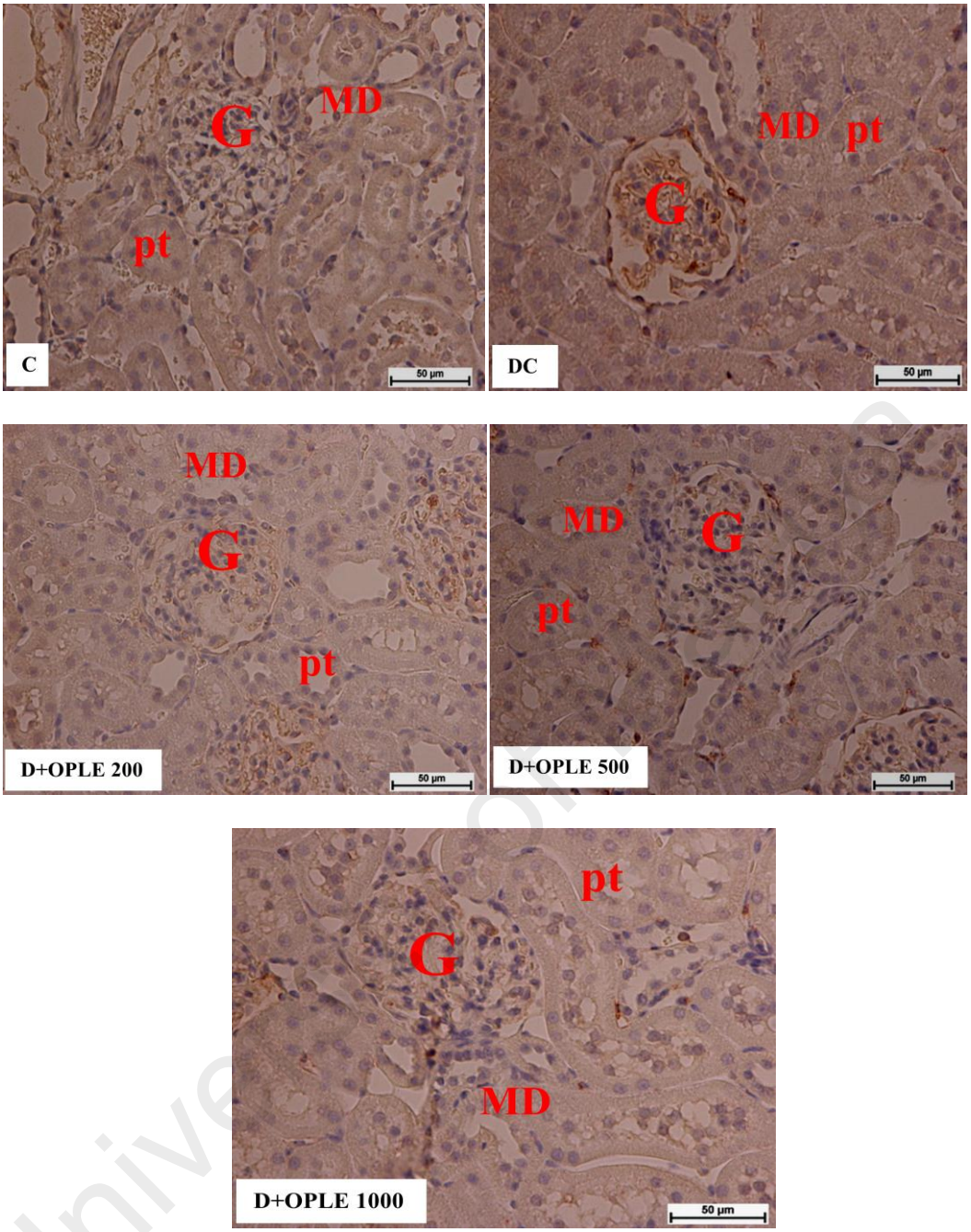


Figure 3.20(a). Immunohistochemical staining of kidney section showing localization of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits of p22phox in 4 weeks experimental model. Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000), MD-macula densa; Bar = 50 µm.

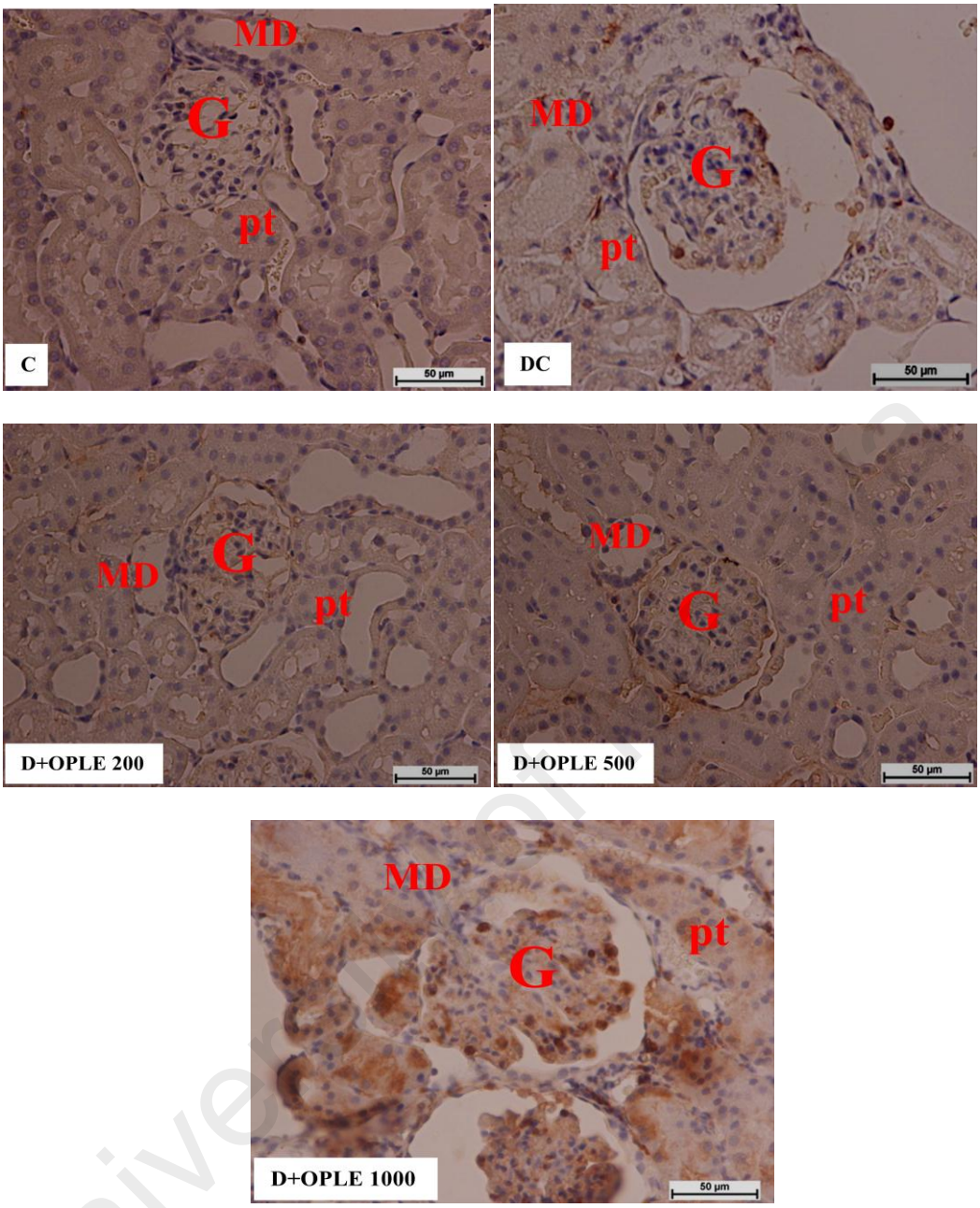


Figure 3.20(b). Immunohistochemical staining of kidney section showing localization of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits of p22phox in 12 weeks experimental model. Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000), MD-macula densa; Bar = 50 µm.

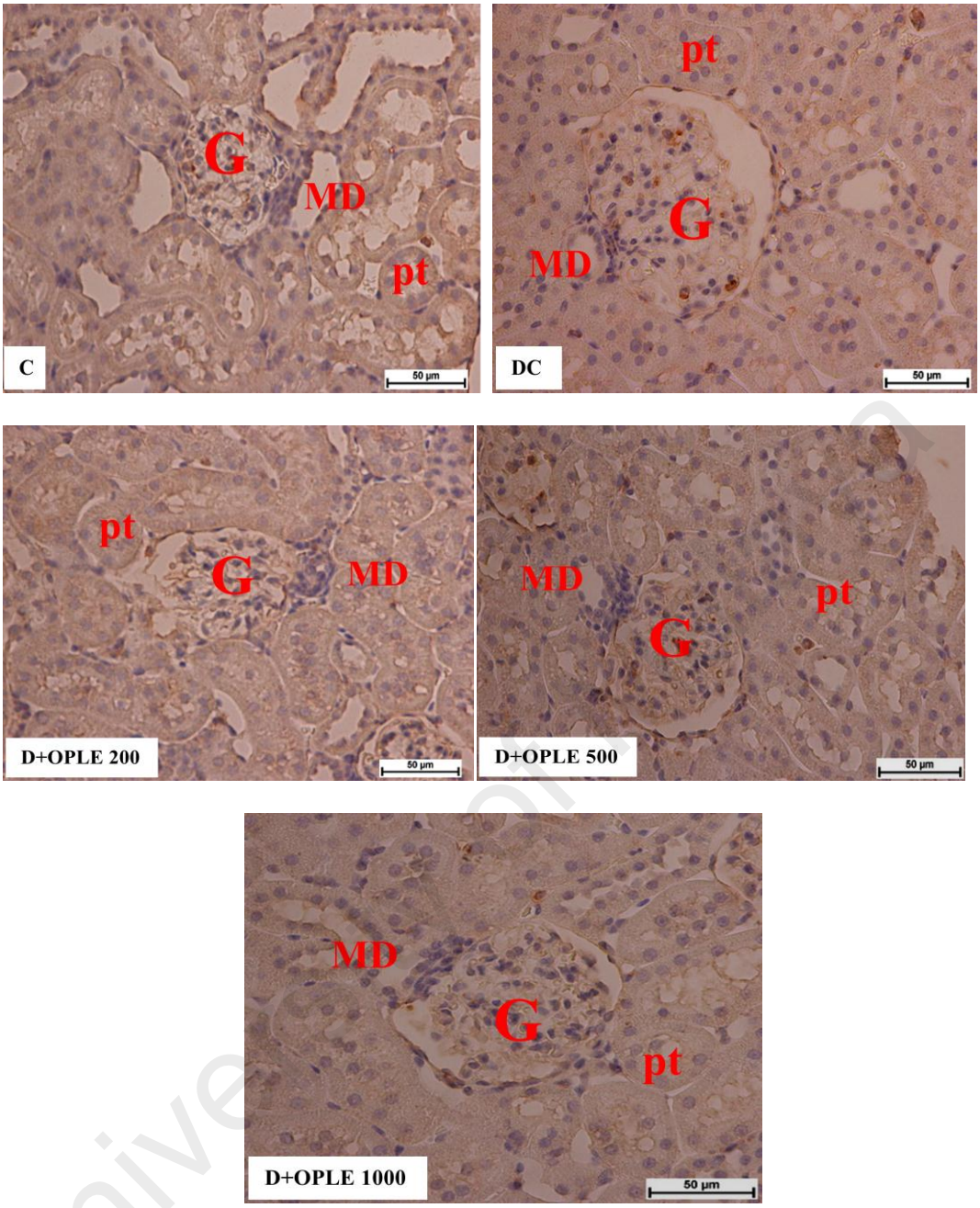


Figure 3.21(a). Immunohistochemical staining of kidney section showing localization of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits of p67phox in 4 weeks experimental model. Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000), MD-macula densa; Bar = 50 µm.

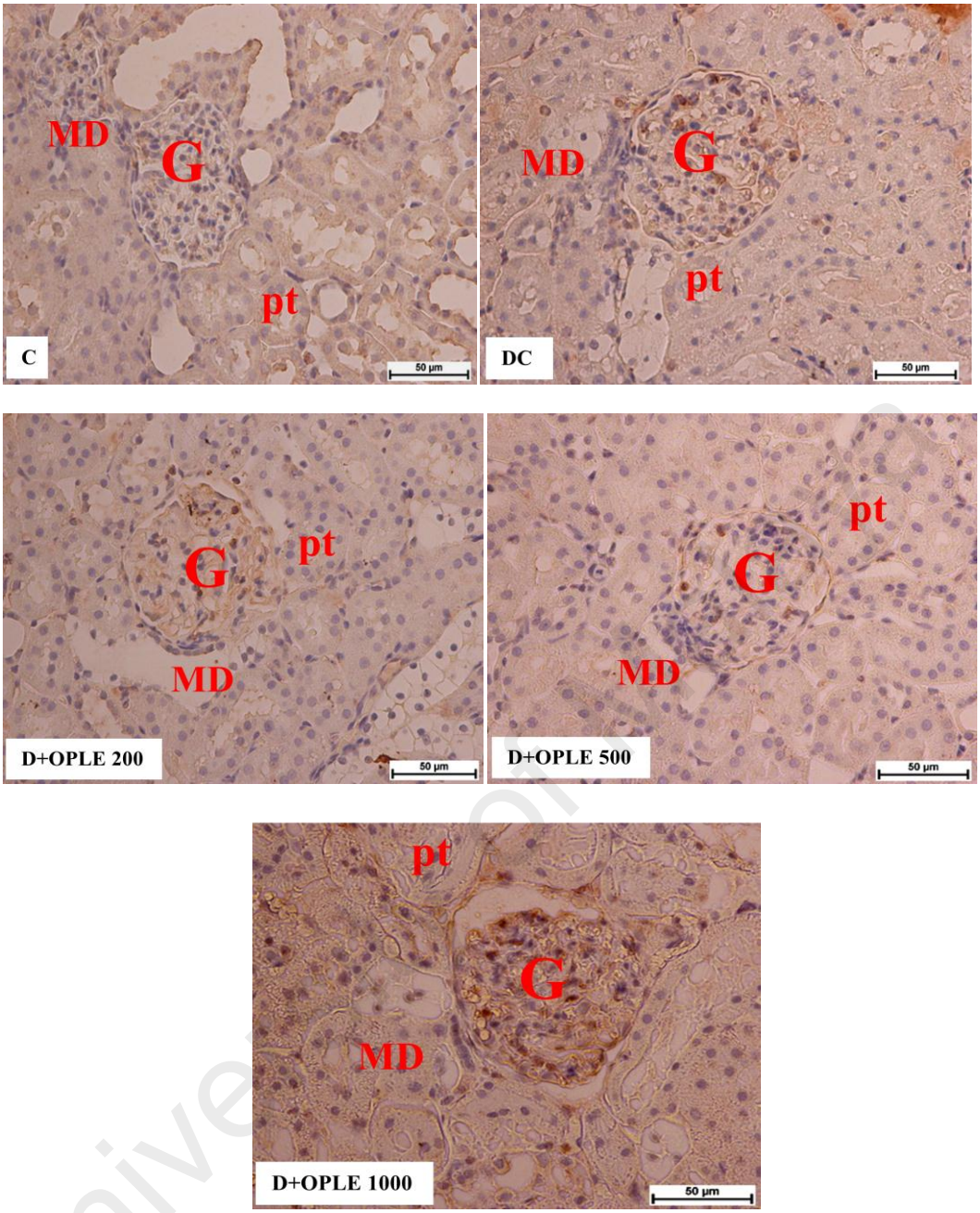


Figure 3.21(b). Immunohistochemical staining of kidney section showing localization of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits of p67phox in 12 weeks experimental model. Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000), MD-macula densa; Bar = 50 µm.

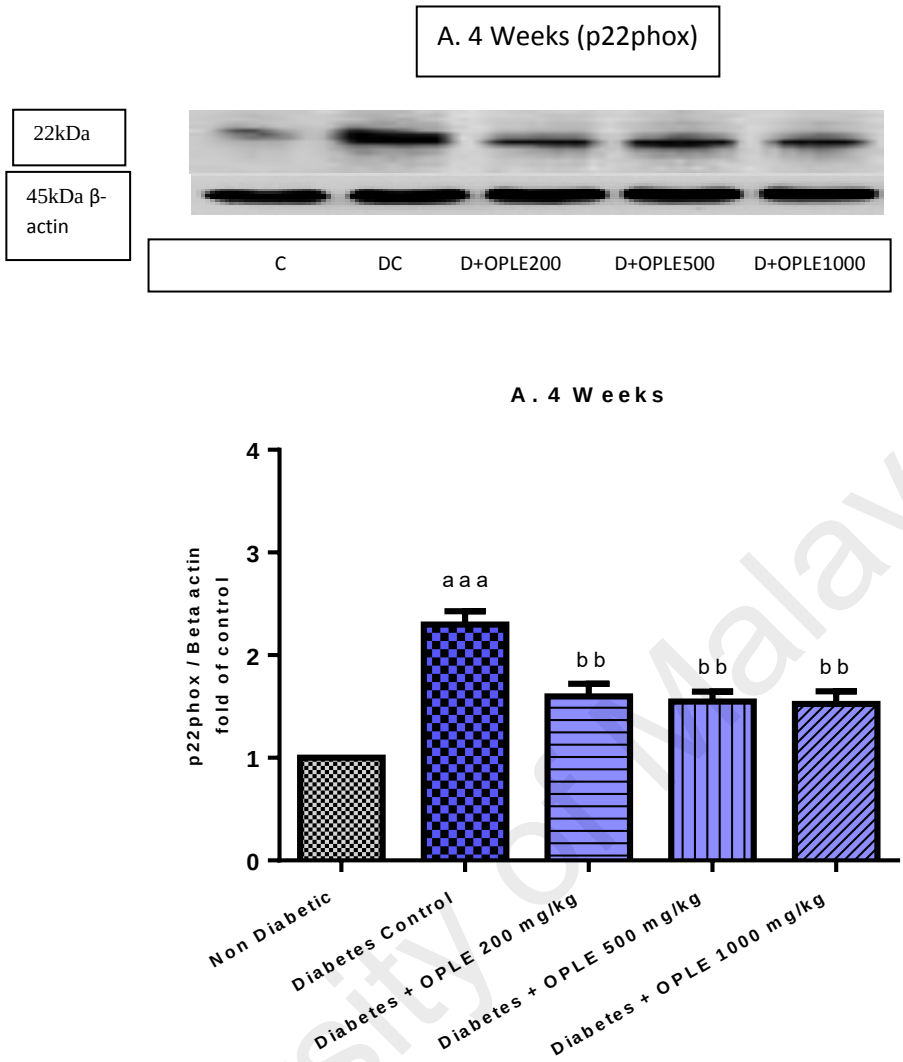


Figure 3.22(a). Renal expression of p22phox NADPH oxidase subunits in 4weeks experimental model. Data are expressed as mean ± SEM of four experiments for each group (n = 4 per group). Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000). ^{aaa}P < 0.001 vs. non-diabetic; ^{bb}P < 0.01 vs. diabetes control.

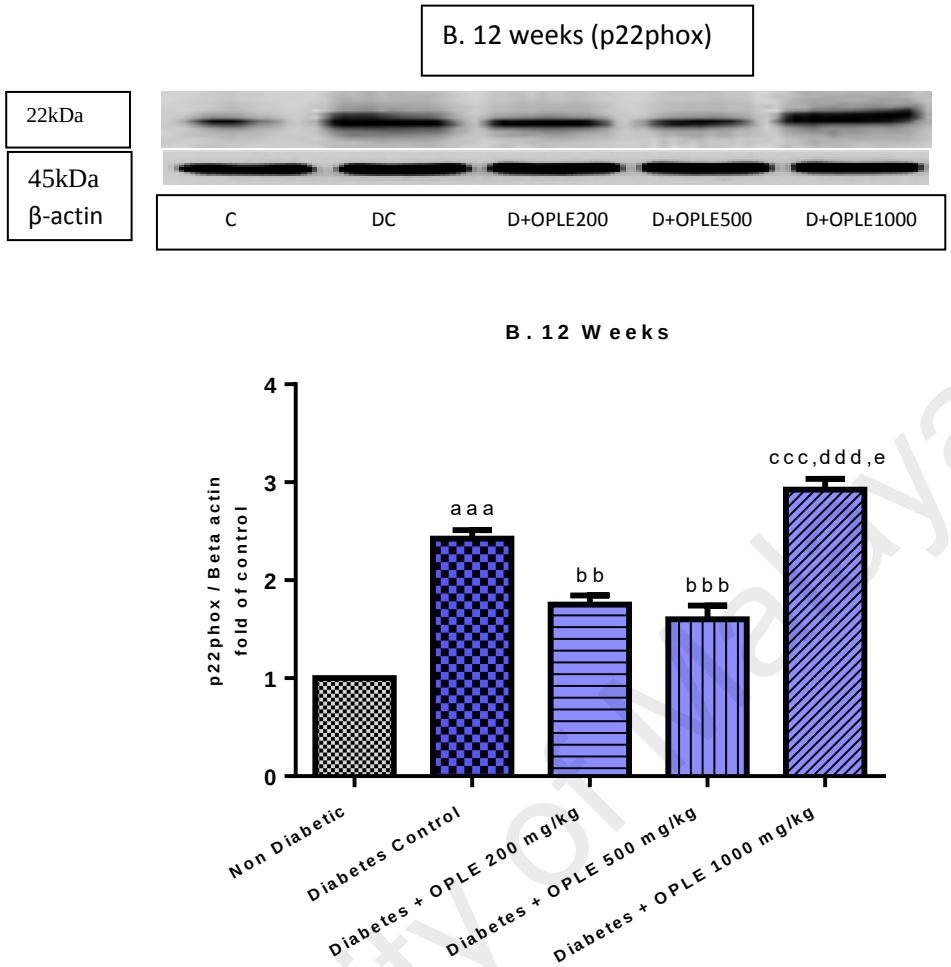


Figure 3.22(b). Renal expression of p22phox NADPH oxidase subunits in 12 weeks experimental model. Data are expressed as mean ± SEM of four experiments for each group (n = 4 per group). Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000). $^{aaa}P < 0.001$ vs. non-diabetic; $^{bb}P < 0.01$ $^{bbb}P < 0.001$ vs. diabetes control; $^{ccc}P < 0.001$ vs diabetes + OPLE 200 mg/kg; $^{ddd}P < 0.001$ vs. diabetes + OPLE 500 mg/kg; $^{e}P < 0.05$ vs diabetic control.

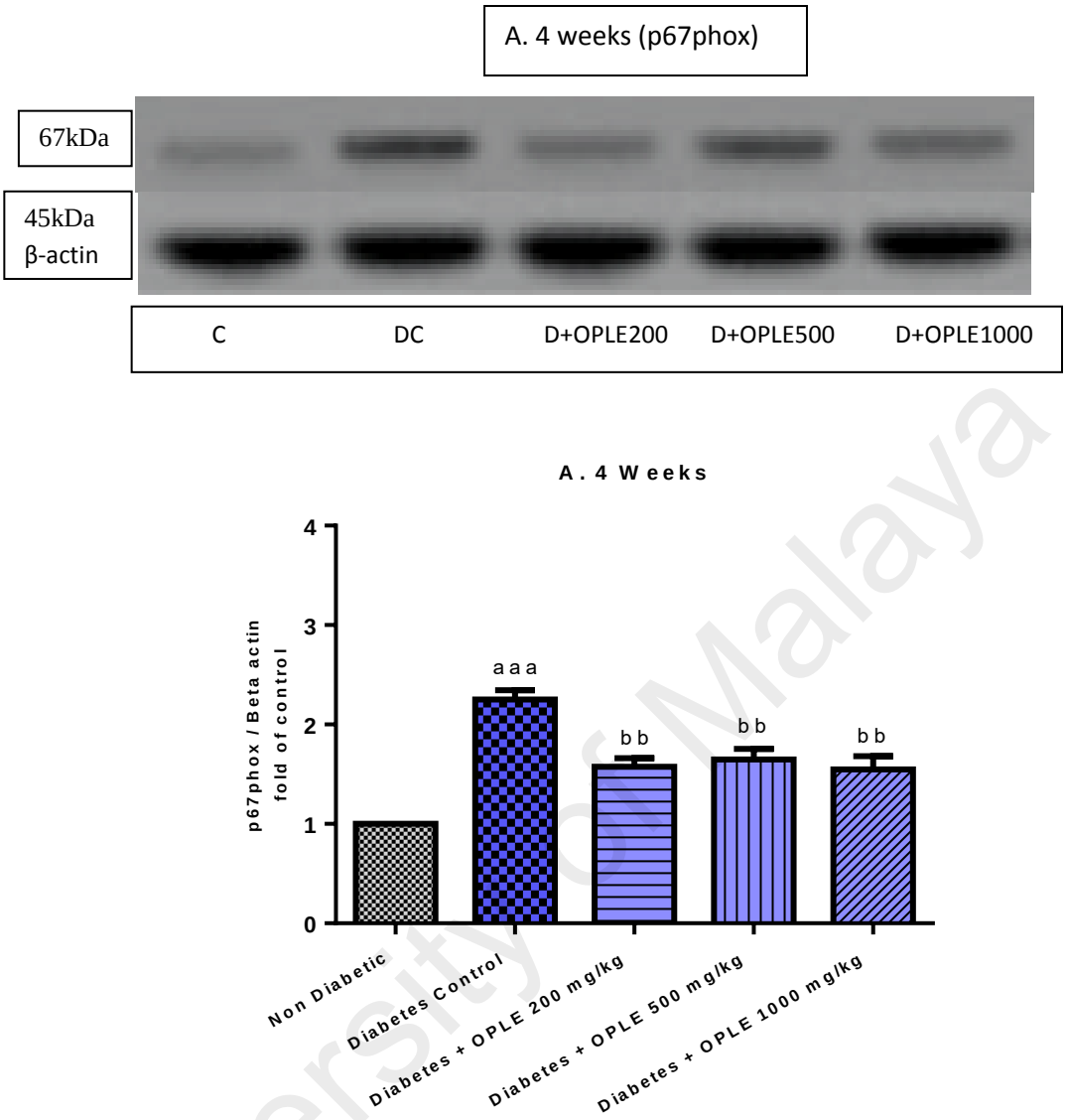
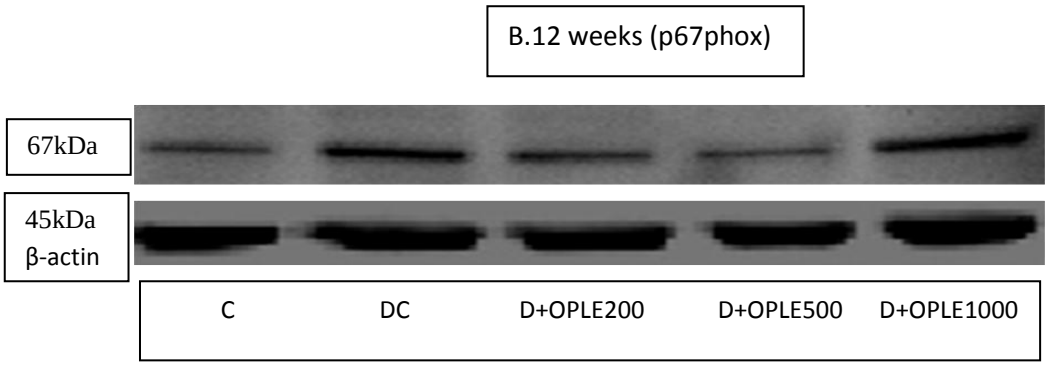


Figure 3.23(a). Renal expression of p67phox NADPH oxidase subunits in 4 weeks experimental model. Data are expressed as mean ± SEM of four experiments for each group (n = 4 per group). Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000). $^{aaa}P < 0.001$ vs. non-diabetic; $^{bb}P < 0.01$ vs. diabetes control.



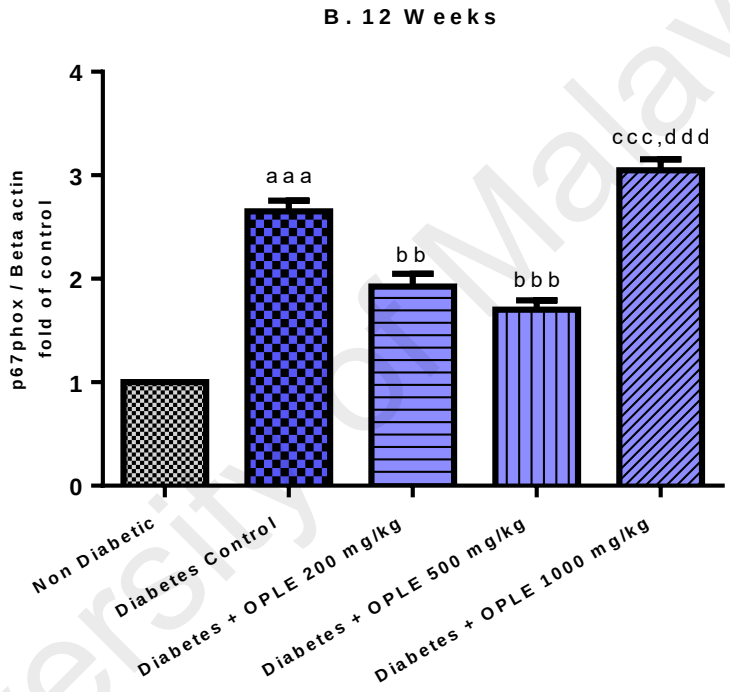


Figure 3.23(b). Renal expression of p67phox NADPH oxidase subunits in 12 weeks experimental model. Data are expressed as mean ± SEM of four experiments for each group (n = 4 per group). Control (C), diabetes control (DC), diabetes + OPLE 200 mg/kg/day (D+OPLE 200), diabetes + OPLE 500 mg/kg/day (D+OPLE 500) and diabetes + OPLE 1000 mg/kg/day (D+OPLE 1000). $^{aaa}P < 0.001$ vs. Non-diabetic; $^{bb}P < 0.01$ $^{bbb}P < 0.001$ vs. diabetes control; $^{ccc}P < 0.001$ vs diabetes + OPLE 200 mg/kg; $^{ddd}P < 0.001$ vs. diabetes + OPLE 500 mg/kg.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1. Discussion

Diabetic nephropathy is one of the major microvascular complications of diabetes mellitus where renal oxidative stress could play a key role (Singh et al., 2011; Ankita et al., 2014). Thus, diabetes mellitus-induced renal oxidative stress could cause renal structural and functional abnormalities. This study was therefore designed to investigate the effects of an antioxidant herbal agent, OPLE, in diabetes mellitus-induced nephropathy in rats. The primary objective of this study is to investigate the effect of OPLE on renal histopathological and functional abnormalities associated with nephropathy in STZ-induced diabetic rats.

Streptozotocin was used to induce diabetes mellitus in Sprague-Dawley rats. Indeed, STZ-induced diabetic rat model has been well reported to develop complications like diabetic nephropathy. The administration of a single dose of STZ (60 mg/kg) was sufficient to induce diabetes mellitus in rats (Giribabu et al., 2014; Altinoz et al., 2014; Erturkuner et al., 2014; Gomathi et al., 2014). Upon diabetic induction by a single dose of STZ administration, the blood glucose level of the diabetic animals remained high throughout the experimental period and this was also observed in other studies (Erbas et al., 2014; Zhang et al., 2012; Kuloglu and Aydin 2014). In this study, rats were kept for a period of 4 weeks and 12 weeks after STZ administration. Both 4 weeks and 12 weeks renal effects of OPLE were investigated in diabetic rats.

4.1.1. Study outcomes

OPLE treatment at various doses (200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day) for 4 weeks exhibited a potent anti-oxidant action and provided renoprotection in diabetic rats. On the other hand, OPLE treatment at these doses for 12 weeks exhibited mixed results. Both 200 mg/kg/day and 500 mg/kg/day of OPLE treatments for 12 weeks afforded an anti-oxidant and renoprotective action in diabetic rats whereas 1000 mg/kg/day of OPLE treatment for 12 weeks showed a pro-oxidant action and aggravation of renal structural and functional abnormalities in diabetic rats.

4.1.2. Renoprotective effect of OPLE in experimental diabetic nephropathy

It is well-established that chronic hyperglycaemia is the main determinant in the development and progression of diabetic nephropathy, and enhanced oxidative stress has been considered to contribute to the pathological processes of diabetic renal complication (Forbes et al., 2008). In the diabetic kidney, ROS is generated by several pathways that include glycolysis, polyol pathway flux, uncoupling of nitric oxide synthase (NOS), xanthine oxidase, NAD(P)H oxidase and advanced glycation. Clinical studies have demonstrated that strict control of hyperglycaemia can reduce the occurrence or progression of diabetic nephropathy; however this is extremely difficult to maintain (Dronavalli et al., 2008; Shamoon et al., 1993; Turner 1998; Ohkubo et al., 1995). Therefore the use of alternate additional therapies that specifically target oxidative stress implicated in diabetic microvascular complication may be advantageous in addition to strict glucose control.

The present study initially investigated the renoprotective effect of OPLE in experimental diabetes. OPLE is rich in catechins (Runnie et al., 2003; Jaffri et al., 2011b), and these polyphenolic compounds are considered to have antioxidant capacity that is several folds higher than that of vitamins C and E (Jaffri et al., 2011a). We tested three different doses of OPLE (200 mg/kg, 500 mg/kg and 1000 mg/kg) to study the effectiveness of this extract in abrogating diabetic nephropathy in short term (4 weeks) and relatively longer term (12 weeks) diabetes. The results of this study provide evidence that OPLE (200 mg/kg, 500 mg/kg) treatment introduced 72 h after diabetes induction and maintained for 4 and 12 weeks, prevented the diabetes-induced renal dysfunction as well as kidney structural injury in diabetic rat. Oral administration of the highest dose of OPLE (1000 mg/kg) for 4 weeks similarly attenuated renal dysfunction and renal pathology in the STZ-induced diabetic rat. We hypothesised that OPLE may retard renal dysfunction and renal pathology associated with early diabetic nephropathy in the STZ-induced diabetic rat, in part, through attenuation of oxidative stress in the kidney, in view of the fact that OPLE suppressed the elevation of oxidative stress markers (8-OHdG, LPO), and improved antioxidant defences as evidenced by increased levels of GSH. In contrast to other study (Rosalina Tan et al., 2011), these beneficial effects of OPLE could not be ascribed to improvement of the diabetic condition because blood glucose levels were unaffected.

The STZ-diabetic rats in our study displayed increased renal perfusion and hyperfiltration which are features of early diabetes in both humans and animal models of diabetes. On the single-nephron level, diabetic hyperfiltration is the

result of renal vasodilation predominantly of the pre-glomerular or afferent resistance vessels (Hostetter et al., 1981). This haemodynamic abnormality was suggested to be mediated by an increase in nitric oxide (NO) due to increased glomerular expression of endothelial constitutive nitric oxide synthase (Veelken et al., 2000; Komers et al., 1994). On the contrary, altered arachidonic acid metabolism has been linked to diabetic hyperfiltration; it was proven that cyclooxygenase (COX)-2 expression was increased in STZ-diabetic rats and inhibition of this enzyme reversed the hyperfiltration in diabetic rats without altering GFR in normal rats (Komers et al., 2001). Later studies implicated oxidant peroxynitrite ($ONOO^-$) as a stimulus for up-regulation of COX-2 in diabetes (Li et al., 2005; Chen et al., 2006). Consistent with earlier interventional studies that used antioxidant vitamins in STZ-diabetic rat (Koya et al., 1997; Melhem et al., 2001); the current results demonstrated that hyperfiltration was blunted to control values by renoprotective doses of OPLE when given for the appropriate length of time. The mechanism by which OPLE improved hyperfiltration however remains to be researched. A natural antioxidant was reported to reduce 3-nitrotyrosine proteins, a marker of $ONOO^-$ production, in renal tissue of STZ-diabetic rat (Su et al., 2010). While this is conjectural, we proposed that OPLE ameliorated hyperfiltration probably due to its antioxidant property that limits the generation or activity of $ONOO^-$ and in consequence prevents up-regulation of COX-2 and reduces formation of vasodilatory prostanoids.

Proteinuria, a marker of diabetic nephropathy is an important risk factor for progressive renal impairment. We therefore assessed the effect of OPLE on

urinary protein excretion to further evaluate the renal protective effect of this extract. Hyperfiltration and alteration in glomerular filtration barrier (alterations in endothelium with its glycocalyx, glomerular basement membrane and podocyte function) contribute to proteinuria in diabetes mellitus. ROS is believed to be one of the key players involved in the pathogenic pathways that lead to glomerular filtration barrier damage. Glomerular ROS production was increased in experimental diabetes (Satoh et al., 2005) and podocytes produced ROS in response to high glucose (Susztak et al., 2006). ROS has been shown to directly decrease heparan sulphate proteoglycans production within the glycocalyx (Kashihara et al., 1992), disrupt the endothelial glycocalyx (Vink and Duling, 1996) and was implicated in early podocyte damage and apoptosis (Susztak et al., 2006). On the contrary, transgenic overexpression of superoxide dismutase attenuated renal injury, including increases in albumin excretion rate (DeRubertis et al., 2004) and antioxidant therapies potentially prevented podocyte damage in early diabetic nephropathy (Spurney and Coffman, 2008; Zheng et al., 2008). In our present study, diabetic rats developed proteinuria by 4 weeks, and renoprotective doses of OPLE administered for the appropriate length of time had a positive effect on this parameter. These results may indicate that OPLE can attenuate renal damage in diabetic rats. Moreover, known antioxidants such as polyphenols and catechins are biological active components of OPLE and these compounds were found to improve proteinuria in diabetes-induced oxidative stress (Rosalina Tan et al., 2011; Yamabe et al., 2006).

STZ-diabetic rat manifested renal hypertrophy in the present study and OPLE has the tendency to reduce the renal enlargement although this effect was not

significant. Experimental studies suggested that amelioration of oxidative stress by catechin and other antioxidants abrogated renal enlargement in diabetic nephropathy (DeRubertis et al., 2004; Bhatti et al., 2005; Kataya and Hamza 2008). Pathologic hallmarks of early diabetic nephropathy include increased glomerular basement membrane thickness and mesangial expansion and these features are in concordant with our present results. In conjunction with these ultrastructural changes, our present study demonstrated elevation in plasma TGF-β1 levels in STZ-diabetic rats. Expansion of the mesangial matrix and thickening of the glomerular basement membrane in diabetic nephropathy as a result of excessive deposition of ECM proteins may be due to upregulation of TGF-β1 and other growth factors (Mason and Wahab, 2003). It is well-established that hyperglycaemia-induced ROS activate signal transduction mechanisms and transcription factors and upregulate TGF-β1 and ECM genes and proteins (Kanwar et al., 2008). Treatment of diabetic rats with renoprotective doses of OPLE for the appropriate length of time preserved renal architecture and this was conceivably reflected in suppression of the increases in plasma TGF-β1 levels. In corroboration with our findings, antioxidants significantly inhibit high glucose- and H_2O_2-induced TGF-β1 and fibronectin (glomerular matrix protein) upregulation (Craven et al., 1997; Ha and Lee, 2000; Iglesias-De La Cruz et al., 2001).

Quantification of ROS would provide direct evidence of the involvement of oxidative stress in diabetic kidney (Bhatti et al., 2005; Peixoto et al., 2009; Li et al., 2011). Although we did not measure ROS directly, several line of evidence suggested that lipid peroxidation and formation of 8-OHdG constituted a

condition of increased oxidative stress (Ha and Kim, 1999; Obrosova et al., 2003; Sugimoto et al., 2001). Our present findings of increased LPO, 8-OHdG in parallel with compromised concentration of the non-enzymatic antioxidant GSH in diabetic kidney clearly suggested that enhanced oxidative stress was present at an early stage of diabetes. The suggestion that renal oxidative stress in STZ-diabetic rats was a consequence of hyperglycaemia rather than well-established pro-oxidant effect of STZ itself was supported by findings which demonstrated correction of impaired antioxidative defense and DNA oxidative damage in the diabetic kidney after insulin administration to animals with established STZ-induced diabetes (Van Dam et al., 1996; Kakimoto et al., 2002). The diabetes-induced changes in oxidative markers (LPO, 8-OHdG, GSH) in our present study were partially or completely prevented by OPLE and this is quite consistent with the effect of this compound in other tissues of diabetic animals (Rosalina Tan et al., 2011) as well as in other, non-diabetic models of oxidative stress (Runnie et al., 2003; Jaffri et al., 2011a). These results indicate that increased oxidative stress which was present in diabetic kidney may be counteracted by OPLE.

This study indicates that catechins-rich OPLE can modulate oxidative stress caused by hyperglycaemic-induced generation of free radicals in diabetic kidney as well as preventing renal dysfunction and structural injury. Diabetic nephropathy is a major cause of end-stage renal disease and it is initially characterised by glomerular hemodynamic abnormalities that result in glomerular hyperfiltration, leading to glomerular damage as evidenced by microalbuminuria. The main advantage of OPLE was that it could ameliorate both hyperfiltration and proteinuria unlike other antioxidant vitamins. Furthermore, renal protection by

OPLE in diabetic nephropathy depends on the dose of the extract relative to body weight as well as progression of the disease implying that the dose given should correspond to the degree of *in vivo* oxidative stress within the kidney.

4.1.3. Aggravation of renal injury by OPLE in experimental diabetic nephropathy

In contrast to the above renoprotective effects of OPLE, and most interestingly, our study revealed that when oral administration of 1000 mg/ kg OPLE was extended for 12 weeks, no renoprotection was detected in the diabetic animals. In fact, worsening of renal dysfunction as evidenced by further increase in hyperfiltration and proteinuria were observed in these diabetic rats. Moreover, oxidative stress markers (LPO) and TGF-β1 were further elevated in diabetic rats treated with this dose of OPLE for 12 weeks as compared to the untreated diabetic rats possibly indicating pro-oxidant effect of OPLE. In corroboration, structural damage was amplified, although not significantly.

4.1.4. NADPH Activity as a Possible Target of OPLE

In the present study, to explore the underlying molecular mechanisms with the aim of elucidating at least part of the anti-oxidant property of OPLE, this study examined the effects of the extract on the renal expression of NADPH oxidase subunits, p22phox and p67phox. It is well established that oxidative stress has been implicated in the pathogenesis of renal injury in diabetes mellitus and the NADPH oxidase is an important source of ROS production (Forbes et al., 2008; Satoh et al., 2005; Palsamy et al., 2011). The NADPH oxidase consists of membrane-bound subunits (p22phox and Nox4, a renal homologue of gp91phox)

and cytosolic subunits (p47phox, p40phox, p67phox, and Rac) (Babior, 2004; Griendling et al., 2000). Previous studies suggested that one of the mechanisms contributing to increased oxidative stress in the diabetic kidney is increased expression of NADPH subunits, namely p22phox, p47phox, p67phox and Nox4 (Bhatti et al., 2005; Etoh et al., 2003; Kitada et al., 2003). In consistent with these reports, this study demonstrated through western blotting and immunohistochemistry that the renal expression of the membrane-bound subunit p22phox and the cytosolic subunit p67phox were enhanced in rats with 4 and 12 weeks diabetes respectively. A significant finding in this study was that administration of OPLE at 200 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day for 4 weeks reduced the diabetes-associated up-regulation of both subunits and this effect was independent of changes in blood sugar. Similar observations were made with administration of 200 mg/kg and 500 mg/kg OPLE for 12 weeks in diabetic kidney. It may be suggested that inhibition of NADPH oxidase, the enzyme that is involved in formation of O_2.-, may in part contribute to the antioxidant effect of OPLE. Studies have adduced evidence that catechins and their metabolites were capable of protecting vascular endothelial cells against $O_2^{\cdot}$-. through inhibition of endothelial NADPH oxidase activity (Steffen et al., 2008). OPLE is rich in catechins such as epigallocatechin, catechin, epicatechin, epigallocatechin gallate and epicatechin gallate (Jaffri et al., 2011a), and inhibition of NADPH oxidase in the present study in all probability could be due to the actions of these catechins, although other compounds such as ferulic acid which is also a component of OPLE, may also play a antioxidant role. Indeed, ferulic acid has been shown to have higher NADPH oxidase-inhibitory potency than apocynin (Steffen et al., 2008).

4.1.5. Pro-oxidant Property of OPLE

This study clearly suggests that in addition to inhibition of NADPH oxidase, increase in endogenous antioxidant enzyme (GSH) could be beneficial in abrogating indices of diabetic nephropathy. The beneficial effects of antioxidants mainly spotlight on their defensive functions against undue oxidative damage induced by ROS. However, from a health perspective one must be conscious that a powerful antioxidant could also exhibit pro-oxidant performance, leading to oxidative damage of cellular mechanism (Galati et al., 2006; Heim et al., 2002). Indeed, what is intriguing in the present study was the unmasking of the pro-oxidant effect of OPLE when 1000 mg/kg/day of the extract was given to diabetic rats for an extended period i.e. 12 weeks as opposed to 4 weeks. We are not certain of the mechanisms that trigger the transition of OPLE antioxidant effect to the pro-oxidant effect when administered for a longer duration; however our findings showed increase expression of the NADPH subunits, p22phox (significant) and p67phox (non-significant) when 1000 mg/kg/day OPLE was administered to diabetic animals for 12 weeks. Aggravation of renal dysfunction and structural injury by the high dose of OPLE administered to diabetic animals for 12 weeks in the present study is purported to be due to the pro-oxidant effect of OPLE but we would not exclude some other unknown ways. Szeto and Benzie (2002) reported that 200 μM epigallocatechin and epigallocatechin gallate induced oxidative damage in human DNA due to the production of hydrogen peroxide. Green tea extract (10-200 μg/ml) which contain catechins similar to OPLE, and epigallocatechin gallate (20-200 μM) have also been shown to exacerbate oxidant activity, oxidative stress, genotoxicity and cytotoxicity induced by hydrogen peroxide in RAW 264.7 macrophages (Elbling et al., 2005).

4.2. Conclusion

In view of these findings and the supported data, it is evident that the development of oxidative stress in renal tissues in diabetes may in part have contributed to the pathological changes leading to diabetic nephropathy in STZ-induced diabetic rat model. The differences observed in the diabetic control and the OPLE receiving diabetic animal as regard to renal function and structure demonstrated the importance of oxidative stress in the pathogenesis of renal failure in diabetes.

To the best of our knowledge, for the first time we have shown that OPLE (500 mg/kg) successfully reduced pathological changes within the kidney, proteinuria and improved other renal dysfunction in diabetes even in the presence of hyperglycaemia. OPLE treatment although in part was effective in significantly reducing the detrimental effects of diabetes on the kidneys; these reductions were not an absolute restoration of the normal functions. Thus, we propose that there could be other confounding factors that could be important in the pathogenesis of diabetic nephropathy that is triggered by the hyperglycaemic status.

Although this study has proven the beneficial effect of OPLE in experimental diabetic nephropathy, it is imperative that we acknowledge the pro-oxidant nature of this extract when used at a high dose (1000 mg/kg) and for an extended length of time. Indeed, the antioxidant/pro-oxidant properties of OPLE could be important in determining the functional outcome of a cell, and the biological response could either be beneficial or harmful, depending on the oxidative condition existing within a cell. Furthermore, this study provides some mechanistic insight into the antioxidant and pro-oxidant effects of OPLE.

This study suggests that with subsequent sequence-specific toxicity studies, OPLE (500 mg/kg) can be a potential therapeutic tool in delaying nephropathy in diabetes. The therapeutic potential of OPLE can also be explored in other fibrotic diseases such as fibrotic liver and lung diseases where oxidative stress could be responsible for the pathogenesis of these diseases. Ultimately, our findings stress the importance of conducting a careful dose–response and treatment duration studies for OPLE before intake of the product can be recommended to diseased individuals where oxidative stress plays a major role.

Limitations

The results obtained in our study regarding the use of OPLE for treatment of diabetic nephropathy are based on animal studies. Therefore caution is needed when extrapolating our findings to humans as statistics show irrefutably that animal-based methods used in preclinical testing to select drugs for human use are sometimes unreliable.

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