Abstract

Leucaena leucocephala (Lam.) deWit (Leguminosae) also known as 'Petai Belalang' is a fast growing shrub or tree found in tropical and subtropical areas. Its fruit has been consumed as food by people in Malaysia, Thailand, Phillipines, Indonesia and Mexico. In Indonesia and Mexico the fruits have been used to treat diabetes. The aim of this study was to assess *in vivo* antioxidant and anti-diabetic potential of *L. leucocephala.* Preliminary screening was done using *in vitro* antioxidant (data not shown), complemented by food nutrition analyses. The anti-diabetic *in vitro* study was conducted using adipocyte cellular model and RT-PCR method, and the *in vivo* efficacy of the extract was assessed using OGTT method as well as oral feeding in Streptozocin-induced diabetic rats. The finding exhibited that *L. leucocephala* dry fruit water extract posessed high phenolic content and rich in vitamin B_2, calcium and magnesium. In preliminary adipocyte cellular study, *L. leucocephala* up-regulated GLUT-4 gene expression twofold which implied the potential to facilitate glucose uptake. In OGTT, *L. leucocephala* fruit showed significant reduction in glucose level at the 60^{th} minute ($p < 0.05$) in normal rats and exhibited glucose lowering effect comparable to Glibenclamide (no significant difference at the 150^{th} minute) in diabetic rat. Thus, *L. leucocephala* posess hypoglycemic activity *in vitro* and *in vivo* which was further investigated in chronic study (30 days). *L. leucocephala* dry fruit water extract were partially purified to separate bioactive compounds using solvent extraction (hexane and ethyl acetate) and the bioactivity was confirmed using sub-chronic study (7 days) in Streptozocin-induced diabetic rats. Both chronic and sub-chronic study showed that *L. leucocephala* water extract prevented worsening of hyperglycemia and its partially purified extract exerted hypoglycemic effects ($p < 0.001$), respectively. Both studies also showed stimulation in secretion of insulin possibly by exerting protection against pancreatic damage. In

chronic study (30 days), *L. leucocephala* dry fruit water extract restored body weight and stimulated adiponectin levels. Lipid and protein damages in the brain and kidney were significantly reduced in diabetic rats treated with *L. leucocephala* extract. In *L. leucocephala* water-hexane partially purified extract treated rats, a high total non-enzymic antioxidant level and high glutathione peroxidase activity were observed. This extract was subjected to further identification of bioactives compounds using HPLC and LC-MS. The results revealed that the active compound is a polar compound with a molecular weight of 217.0937 g/mol and molecular formula of $C_7H_{13}N_4O_4$. However the exact structure could not be identified as it required more extensive investigation and is beyond the scope of this study. In conclusion, *Leucaena leucocephala* has a potential to be developed as an anti-diabetic agent.

Abstrak

Leucaena leucocephala (Lam.) deWit (Leguminosae) atau telah dikenali sebagai Petai Belalang adalah tumbuhan renek atau pokok yang mempunyai tumbesaran yang cepat dan ditemui di kawasan-kawasan tropika dan sub-tropika. Buahnya dimakan sebagai makanan oleh orang di Malaysia, Thailand, Filipina, Indonesia and Mexico. Di Indonesia dan Mexico buah tersebut telah digunakan untuk merawat penyakit diabetes. Tujuan kajian ini adalah untuk menilai potensi antioksidan dan anti-diabetik *L. leucocephala.* Saringan awal telah dilakukan menggunakan ujian antioksida *in vitro* (data tidak ditunjukkan), dilengkapkan dengan analisis nutrisi pemakanan. Kajian anti-diabetik *in vitro* telah dijalankan menggunakan model sel adipos dan kaedah RT-PCR, dan keberkesanan ekstrak secara *in vivo* telah dinilai menggunakan kaedah OGTT serta secara oral ke atas Streptozocin-induksi tikus diabetik. Hasil telah menunjukkan bahawa ekstrak air buah kering *L. leucocephala* memiliki kandungan fenol yang tinggi dan kaya dengan vitamin B_2, kalsium dan magnesium. Pada kajian awal sel adipos, *L. leucocephala* telah meningkatkan ekspresi gen GLUT-4 sebanyak dua kali ganda yang menunjukkan potensi untuk memudahkan pengambilan glukosa. Pada kajian OGTT, buah *L. leucocephala* telah menunjukkan penurunan signifikan dalam tahap glukosa pada minit ke 60 ($p < 0.05$) dalam tikus normal dan mempamerkan kesan penurunan glukosa setanding dengan Glibenclamide (tidak menunjukkan perbezaan signifikan pada minit ke 150) dalam tikus diabetik. Dengan ini, *L. leucocephala* memiliki aktiviti hipoglisemik secara *'in vitro'* dan '*in vivo'* yang mana penyelidikan telah dilanjutkan dalam kajian kronik (30 hari). Ekstrak air buah kering *L. leucocephala* telah disepara tulenkan untuk mengasingkan kandungan bioaktif menggunakan pengekstrakan pelarut (heksana dan etil asetat) dan bioaktiviti telah disahkan menggunakan kajian separa kronik (7 hari) keatas Streptozocin-induksi tikus diabetik. Kedua-dua kajian kronik dan

sub-kronik telah menunjukkan bahawa ekstrak air buah kering *L. leucocephala* telah mencegah pemburukan hiperglisemia dan ekstrak separa tulenannya telah menghasilkan kesan hipoglisemia ($p < 0.001$). Kedua-dua kajian juga telah menunjukkan stimulasi terhadap perembesan insulin yang mungkin diberikan oleh perlindungan daripada kerosakan pankreas. Dalam kajian sub-kronik (30 hari), ekstrak air buah kering *L. leucocephala* telah memelihara berat badan dan merangsang paras adiponektin. Kerosakan lipid dan protein di dalam otak dan buah pinggang ketara berkurangan dalam tikus-tikus diabetik yang telah dirawat dengan ekstrak *L. leucocephala.* Dalam kumpulan tikus yang telah dirawat dengan ekstrak separa penulenan air-heksana *L. leucocephala* telah menunjukkan jumlah antioksidan bukan enzim dan aktiviti glutatione peroksida yang tinggi. Ekstrak ini telah dipilih untuk identifikasi komposisi-komposisi bioaktif menggunakan HPLC dan LC-MS. Keputusan mendedahkan bahawa komposisi bioaktif tersebut adalah sebatian polar dengan berat jisim molekul 217.0937 g/mol dan formula molekul $C_7H_{13}N_4O_4$. Walaubagaimanapun, struktur sebenar tidak dapat dikenalpasti kerana ia memerlukan penyelidikan yang lebih luas dan ia di luar skop kajian ini. Kesimpulannya, *Leucaena leucocephala* mempunyai potensi untuk dibangunkan sebagai agen anti-diabetik.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol/Abbreviations	Description
+	Plus
–	Minus
-	Negative
±	Plus Minus
=	Equal
<	Less
≥	Equal or more
%	Percent
ºC	Degree Celcius
α	Alpha
β	Beta
δ	Gamma
ΔC_T	Delta Ct
$\Delta\Delta C_T$	Delta-delta-Ct
$2^{-}\Delta\Delta CT$	Log scale, base 2.Delta-delta-Ct
CT	Cycle threshold
µ	Micro
µl	Microlitre
µm	Micrometer
µg/ml	Microgram per millilitre
µM	Micromolar
µg/ml	Microgram per millilitre
IV	Four
xg	Times gravity
3T3-L1	Original Cell line derived from 3T3-Swiss albino rats
A	Absorbance
AACC 32-10	American Association of Cereal Chemists Method 32-10
Acrp30	Adipocyte Complement Related Protein of 30 kD
ACTH	Adrenocorticotropic hormone
ADD1	Adipocytes Determination and Differentiation Factor 1
AdipoR2	Adiponectin receptor
ADP	Adenosine diphosphate
apM1	Adipose most abundant gene transcript 1
AGE	Advanced Glycated Endproduct
AKT	Protein Kinase B
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
AOPP	Advanced Oxidation Protein Product
AOX	Antioxidant
Aq	Aqueous
ATP	Adenosine triphospate
BHA	Butylated hydroxyanisole
BSA	Bovine Serum Albumin
$C_8H_{15}N_3O_7$	2-deoxy-2-(3-(methyl-3-nitrosoureido)-D glucopyranose
CAT	Catalase
CAM	Complimentary and Alternative Medicine

Symbol/Abbreviations	Description
CBBG	Coomassie brilliant blue G-250
CDL	Clinical Diagnostic Laboratory
cDNA	Complimentary DNA
c GMP	Cyclic Guanosine Monophosphate
CVs	Coefficient of Variances
D	Diabetic rats
Da	Dalton
DAG	Diacylglycerol
DM	Diabetes Mellitus
DM1	Diabetes Mellitus type 1
DMEM I	Dulbecco's Modified Eagle's Medium I
DMEM II	Dulbecco's Modified Eagle's Medium II
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPX	Distyrene, plasticizer,xylene
EAR	Estimated Average Requirement
ELISA	Enzyme Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
EPR	Electron Paramgnetic Resonance
Et	Ethyl acetate
FAS	Fatty Acid Synthase
FBG	Fasting Blood Glucose
FBS	Fetal Bovine Serum
Fe(II)	Ferrous ion
$FeCL_3.6H_2O$	Ferric chloride
FRAP	Ferric Reducing Antioxidant Power
$FeSO_4.7H2O$	Ferrous sulfate heptahydrate
g	Gram
g/kg	Gram per kilogram
g/100g	Gram per hundred gram
g/day	Gram per day
G	Glibenclamide
GAEs	Gallic Acid Equivalents
GC	Gas Chromatography
G 1.25 mg/kg	Glibenclamide at dose of 1.25 milligram per kilogram
G6Pase	Glucose-6-Phosphatase
GlcNAc	N-acetylglucosamine
GLUT-2	Glucose Transporter 2
GLUT-4	Glucose Transporter 4
GPx	Glutathione Peroxidase
GSH	Glutathione (reduced)
GSSG	Glutathione Disulphide (oxidized)
GSSG-R	Glutathione Reductase
H_2O_2	Hydrogen Peroxide
Hb	Haemoglobin
HDL	High Density Lipoprotein
He	Hexane
His	Histidine
HM	Hydrophobic Motif

Symbol/Abbreviations	Description
HOCl	Hypochlorous Acid
HPLC	High Performance Liquid Chromatography
HRP	Horse Reddish Peroxidase
HSL	Hormone Sensitive Lipase
H + E	Heamatoxylin and Eosin
ICP-OES	Inductively coupled plasma atomic emission spectroscopy
ID	Identify
IDDM	Insulin Dependent Diabetes Mellitus
IgG	Immunoglobulin G
IL-6	Interleukin 6
IRS-1	Insulin Receptor Substrate-1
K	Pottassium
K ATP	ATP-sensitive potassium
KK/Ay	KK mice with Ay alelle congenic strains
LD_{50}	Median Lethal Dose
LDL	Low Density Lipoprotein
LIPE	Lipase
LL	*Leucaena leucocephala*
LY294002	2-morpholin-4-yl-8-phenylchromen-4-one
Lys	Lysine
M	Molar
MCP-1	Monocyte chemotactic protein-1
MDA	Malodialdehyde
mg/100g	Milligram per hundred gram
mg kg^{-1}	Milligram per kilogram
mRNA	Messenger Ribonucleic Acid
ml	Millilitre
mg/dL	Milligram per decilitre
mg/mL	Milligram per mililitre
mM	Millimolar
mins	Minutes
min/mg	Minute per milligram
ml/kg	Mililitre per kilogram
mmol/L	Milimolar per Litre
ng/ml	Nanogram per mililitre
nmol	Nanomolar
N	Normal rats
Na_2CO_3	Disodium carbonate
NAD^+	Nicotinamide Adenine Dinucleotide
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate(reduced)
NIDDM	Non- Insulin Dependent Diabetes Mellitus
nm	Nanometer
$O_2^{\bullet-}$	Superoxide anion species
•OH	Hydroxyl radical
ONOO-	Peroxynitrite
NO	Nitric oxide
NOS	Nitric Oxide Synthase

Symbol/Abbreviations	Description
O-GlcNAc	O-linked N-acetylglucosamine
O-GlcNAcase	Enzyme of O-linked N-acetylglucosamine
OGTT	Oral Glucose Tolerance Test
P	Probability
p110	Catalytic subunit
p85	Regulatory subunit
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDK 1	Phosphoinositide-dependent kinase-1
PDE3B	Phosphodiesterases
PEPCK	Phosphoenolpyruvate carboxykinase
pH	Power of Hydrogen
PH	Pleckstrin Homology
Phe	Phenylalanine
PKB	Protein Kinase B
PKC	Protein Kinase C
PPAR ∝	Peroxisome Proliferator-Activated Receptor Gamma
PUFA	Polyunsaturated Fatty Acids
PI3K	Phosphatidylinositol 3-kinases
PIP	Porcine Insulin Precursor
PtdIns(4,5)	Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5) P_3	Phosphatidylinositol 3,4,5-trisphosphate
PtdIns(4,5) P_2	Phosphatidylserine
qRT-PCR	Quantitative Real-Time PCR
r	Correlation coefficient
®	Registered trademark
R^2	Coefficient of determination
RBC	Red Blood Cells
RDA	Recommended Daily Allowance
RDV	Recommended Daily Value
Rn	*Rattus norvegicus*
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
RO•	Alkoxy
ROO•	Peroxy radicals
ROOH	Organic hydroperoxide
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal Ribonucleic Acid
RT	Reverse transcription
RT-PCR	Reverse transcription Polymerase Chain Reaction
SD	Standard Deviation
sdH_2O	Sterile distilled water
SEM	Standard Error Mean
sec	Second
SLC2A4	Gene encoded GLUT 4
SOD	Superoxide Dismutase
SPSS	Statistical Package for Social Sciences

Symbol/Abbreviations	Description
SREBP	Sterol Regulatory Element Binding Proteins
STZ	Streptozotocin
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Reactive Substances
TCA	Trichloroacetic Acid
TG	Triacylglycerol
TGFβ	Transforming Growth Factor Beta
™	Trade mark
TNF	Tumor Necrosis Factor
TPTZ	Tripyridyltriazine @ 2,4,6-tripyridyl-s-triazine
Tyr	Tyrosine
UDP	Uridine diphosphate
UMMC	University Malaya Medical Centre
UK	United Kingdom
USA	United State America
USRDA	Universal Standard Recommended Daily Allowance
VEGF	Vascular Endothelial Growth Factor
Vers	Version
VIC/MGB	Probe labeled with VIC™ dye – MGB
v/v	Volume per Volume
WHO	World Health Organization
w/v	Weight per Volume
X	Times
XOD	Xanthine Oxidase

CHAPTER ONE: INTRODUCTION

1.1 Research background

1.1.1 Medicinal plants

Since ancient times, plants had been used to preserve and improve human body wellness. Traditionally, regular consumption of plants was believed to enhance health status probably by increasing energy, reduce disease symptoms or cure a disease. Therefore, the beneficial plant was referred to as 'medicinal plant'. Providentially, the valuable information regarding medicinal plants such as their common name, medicinal use and method of preparation was documented by the Indians in Ayuverdic text, Egyptian with Greek Epidemic Book III by Hippocrates, Chinese with Chinese Nei Ching (Vuksan & Sievenpiper, 2005). At the same time, the knowledge about medicinal plants was also practiced by old folks and passed down to the next generation.

Today, the traditional medical practice is also known as Complimentary and Alternative Medicine (CAM). CAM is defined as a group of medical and health-care systems, practices and products that are not presently considered part of conventional medicine (Kumar, Bajaj, & Mehrotra, 2006). To be more specific, complementary medicine is used together with conventional medicine or therapy meanwhile alternative medicine is used alone without conventional medicine.

1.1.2 Why medicinal plants?

Malaysia has tropical rain forest which is being preserved.

Before the existence of modern medicine, ancestors used medicinal plants to treat minor ailments. The knowledge and practices based on belief and observations of traditional medicine which had been used for more than hundreds of years should not be wasted. Today, it has been proven that some medicinal plants that had been used in ancient times are used as modern day drugs (Vuksan & Sievenpiper, 2005). A good example is metformin an anti-diabetic drug which is a bioactive compound extracted from French lilac (*Galega officinalis*) (Marles & Farnsworth, 1995).

Interestingly, the use of herbal medicine was double to triple fold more than conventional drugs and 80% of the world population especially in developing countries still depend on plant-derived medicines for maintenance of health and treatment of disease (World Health Organization, 2002). The high demand for medicinal plants is due to the long history of usage and local beliefs of its curative capability. However, the medicinal plants 'healing properties' must be identified, purified and proven using scientific methods.

1.1.3 Problems faced with medicinal plant

Scientific evidence on the benefits of herbal medicine in this country are at an infancy stage. Therefore, there is a need to develop a basis for standardization that ties the composition of medicinal plant to efficacy and understand the mechanism involved, side-effect and safety.

1.2 Previous findings and potential of *Leucaena leucocephala*

To date, very limited studies have been carried out on *Leucaena leucocephala* with regard to its medicinal properties and bio-activity. Most of the available information about *L. leucocephala* was its use as a crop food for goats. Its anti-diabetic properties on alloxan induced diabetic rats was reported recently (Syamsudin, Ros Sumarny, & Partomuan Simanjuntak, 2010).

Previous *in vitro* research in our laboratory showed that *L. leucocephala* exhibited mild lipogenesis and high lipolysis activity in adipocyte cell culture (Lim, 2007), this implied the potential use of this plant as anti-obesity and anti-diabetic agent for the treatment of Type II Diabetes.

1.3 Objectives of this study

1. To assess anti-diabetic potential of *L. leucocephala* using adipocyte cellular model.
2. To investigate the anti-diabetic effects of *L. leucocephala* on Streptozocin induced diabetic rats.
3. To assess the *in vivo* antioxidant effects of *L. leucocephala* in Streptozocin induced diabetic rats.
4. To perform partial purification of *L. leucocephala* extract.

1.4 Research outline and approach

1.4.1 Overview of research methods

An overview of research and methods used in this study is as listed in *Table 1.1* and *Fig. 1.1*.

Table 1.1: Overview of research outline

Research aspects	Method	Reference
Preparation of *L. leucocephala* extract	Extraction Liquid-liquid partitioning	(Adrian, Freitag, & Maurer, 2000)
Food nutrition analyses	Carbohydrate:by calculation Protein: Kjeldahl method Total fat: Solvent extraction Fiber: AACC 32-10 Trans fatty acid Vitamin A: HPLC Vitamin B_1: HPLC Vitamin B_2: HPLC Vitamin B_3: HPLC Vitamin C: HPLC Vitamin E: HPLC Sodium : ICP-OES Calcium: ICP-OES Magnesium: ICP-OES Zinc: ICP-OES Potassium: ICP-OES Manganese: ICP-OES Selenium: ICP-OES	(Pither, 2005) (Eitenmiller & Ye, 2003)
In vitro evaluation for anti-diabetic potential of *L. leucocephala* using Gene expression in adipocytes	Adipocyte cell culture RNA extraction Real time-reverse transcription polymerase chain reaction (TaqMan®)	(Bjorntorp et al., 1978) (Madsen et al., 2003)
Screening for glucose lowering effect *in vivo*	OGTT in normal and STZ-induced diabetic rats	(Meenakshi et al., 2010) (Ortiz-Andrade et al., 2008)
Induction of diabetes mellitus	Chemical induction using Streptozocin	(Pari, 2007) (Achyut, Rajesh, Santosh, Sandhya, & Geeta, 2006)
Anti-diabetic activity	Fasting blood glucose Body weight changes Serum insulin level Adiponectin level	(Ortiz-Andrade, et al., 2008)

Research aspects	Method	Reference
Antioxidant activity	*In vivo*: using tissue homogenates from chronic study and sub-chronic study. FRAP assay AOPP assay MDA assay GPx assay	 (Benzie & Strain, 1996) (Witko-Sarsat et al., 1996)
Histology	Histology using Hematoxylin and Eosin staining on rats liver and kidney tissues	(Wills & Asha, 2006) (Wang, Hsu, Liu, Kao, & Chen, 2008)
Identification of *L. leucocephala* bioactive compounds	HPLC LC-MS	(Oleszek & Bialy, 2006)

1.4.2 Schematic overview of this study

The schematic overview to illustrate the flow of the study is as shown in *Figure 1.1*.

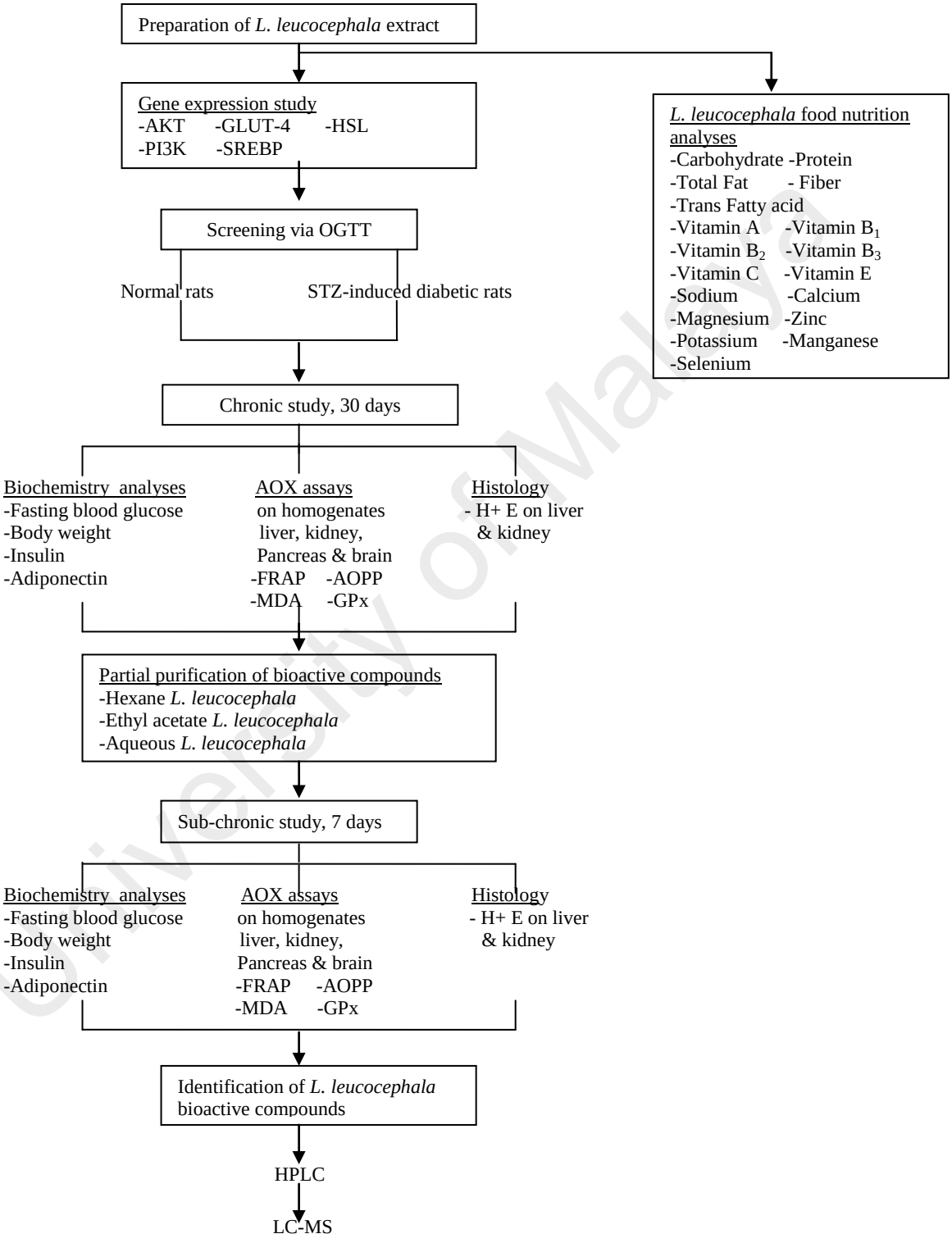


Figure 1.1 : Schematic overview of overall study.

CHAPTER TWO: LITERATURE REVIEW

2.1 *Leucaena leucocephala*

2.1.1 Habitat and Morphology

Leucaena leucocephala (Lam. de Wit) is classified into the family Leguminosae and subfamily Mimosoideae with three subspecies glabrata, ixtahuacana and leucocephala. The species may be distinguished on the basis of their tree size, flower colour, leaflet size and pod size. The trees could be found in tropical and subtropical areas around the world (Acamovic, D'Mello, & Fraser, 1982; Devendra, 1993). The tree is native to southern Mexico and northern Central America. In 16th century, the tree was introduced in Philipines as a feed for ruminant livestock and subsequently spread throughout Asia-Pacific region. In Malaysia, the local name is 'Petai Belalang' and other common names are White Leadtree, Jumbay, and White Popinac. The "white head" refers to its flowers.

L. leucocephala is a fast growing shrub or tree and could tolerate dry season because of its deep roots (Adejumo & Ademosun, 1991). The young trees could reach a height of more than 20 feet in 2-3 years. The bark is greyish and has prominent lenticels. Leaves bipinnate with 4-9 pairs of pinnae, variable in length up to 35 cm, with a large gland (up to 5 mm) at the base of the petiole, leaflets 11-22 pairs/pinna, 8-16 mm x 1-2 mm, acute. Flowers numerous, in globose heads with a diameter of 2-5 cm, stamens (10 per flower) and pistil 10 mm long, anthers pilose, dehiscing at dawn. Pod 14-26 cm x 1.5-2 cm, pendant, brown at maturity. Seeds 18-22 per pod, 6-10 mm long, brown. The flowering and fruiting occur throughout the year as long as moisture permits; fruiting is associated with suppression of vegetative growth. Fruits ripen in 10-15 weeks.

Figure 2.1 : Morphology of *Leucaena leucocephala* fruits.

Figure cited from (syifa-kesihatandalaman.blogspot.com, 2013)

2.1.2 Ethnopharmacology

During the 1970s and 1980s *L. leucocephala* was known as a "miracle tree" because of its multiple uses. Generally, the tree was used for firewood, timber, green manure, shade and erosion control and the leaves of *L. leucocephala* are highly nutritious for ruminants (Yami et al., 2000) and human (Ekpenyong, 1986).

In Malaysia, the fruit is eaten as raw vegetable called '*ulam*' the same way as *Parkia speciosa Hassk* also known as 'Petai' and classified under the same family. It is considered as an edible fruit in Malaysia, Thailand, Indonesia and Mexico. Since ancient times, the unripe pods and seeds had been used by the native inhabitants of Mexico and Central America as a food or medicine meanwhile Indonesians used fermented leucaena seeds to prepare 'tempe lamtoro', which is a delicacy. Thais used very young shoots as food. *L. leucocephala* had traditionally been used to treat diabetes especially by Indonesians and Mexicans (Andrade-Cetto & Heinrich, 2005). Indonesians used mature or fried dry *L. leucocephala* seeds, crushed into powder and stirred with boiled water to treat diabetes.

2.1.3 Composition and reported activities

L. leucocephala has been shown to have great potential as a source of high quality feed for ruminants. *L. leucocephala* is very rich in protein (Adeneye, 1979; Akingbade, Nsahlai, Bonsi, Morris, & du Toit, 2001). Higher protein content was reported in younger than mature leaves, seed meal than husks (Adeneye, 1979). The seed legumes are potential substitute for soybean meal because of similarity in their amino acid composition and energy profile (Wiryawan, 1997). *L. leucocephala* seeds are rich in isoleucine and have a greater content of lysine, leucine, proline and serine than soya bean proteins (Ekpenyong, 1986). *L. leucocephala* leaves were found to be v-

ery rich in minerals such as calcium, phosphorus, potassium, magnesium and iron (Adeneye, 1979), trace elements as well as vitamin A.

L. leucocephala species contains secondary compounds such as mimosine and tannins which function to protect against defoliation and facilitate continuous existence (Akingbade, et al., 2001). The seeds contain mimosine, an amino acid known to be toxic to non-ruminant vertebrates and it is known to inhibit appetite. Certain breeds of animals are capable of ingesting greater amounts (100 %) of *L. leucocephala* compared to others. However, it had been proposed that *L. leucocephala* should not comprise more than 40 %-60 % of the diet or less than 1.0 g mimosine per kg (Adejumo & Ademosun, 1991). The mimosine content depends on the part and age of the plant. A research found that young seeds and mature leaves posess about half mimosine content of mature seeds or young leaves (Adeneye, 1979). Meanwhile, less or no mimosine exist in the seed coats or empty brown pods. Whole green pods with seeds are as rich in mimosine as mature leaves. The mimosine levels may range from 6 to 12 % in growing tips while young pods, seeds and green stems contain 3-5 %, 4-5 % and 2 %, respectively (J. Jones, 1979).

L. leucocephala toxicosis in ruminants has been reported in Australia, Papua New Guinea, Africa, Malaysia and Florida (Anbarasu, Dutta, Sharma, & Rawat, 2004). However, the mimosine metabolites, dihydroxy pyridone (DHP) could be degraded by *Synergistes jonesii*, which exist in certain species of goats in Hawaii and Indonesia (Allison, Mayberry, McSweeney, & Stahl, 1992). Generally, the strain had been tested using anaerobic media culture (Domínguez-Bello, Lovera, & Rincón, 1997) and was distributed to tropical areas to help those animals to acquire and transmit the ability to detoxify DHP.

2.2 DIABETES MELLITUS

2.2.1 Definition

'Diabetes' was derived from the Greek word meaning to pass through, followed by the Latin word 'mellitus' which means honeyed or sweet that was added after physician realized the urine of a diabetic patient was loaded with sugar. Before the term was established, this disease was known by the symptoms. Patients appeared thirsty, with sweet urine, tremendous reduced body weight that led to short-life span (Ahmed, 2002).

2.2.2 Classification of diabetes mellitus

World Health Organization recognizes three main types of diabetes mellitus: *type I, type II*, and *gestational diabetes* (occurring during pregnancy) (World Health Organization, 1999). Type I diabetes mellitus occurs when the body makes little or no insulin due to autoimmune or non-immune destruction of the insulin-producing β-cells of the islets of Langerhans (Hettiarachchi, Zimmet, & Myers, 2004). Type II diabetes mellitus is due to the inability of available insulin to work properly.It involves multiple organ systems and include abnormal insulin secretion and peripheral (muscle, adipose tissue and hepatic) insulin resistance (World Health Organization, 1999).

Type I diabetes was formerly known as childhood-onset diabetes, juvenile diabetes, or insulin-dependent diabetes (IDDM) whereas Type II diabetes was known as non-insulin dependent diabetes (NIDDM) (World Health Organization, 1999). Type I diabetes mellitus is usually diagnosed during childhood, Type II in adulthood and gestational diabetes throughout pregnancy (World Health Organization, 1999).

2.2.3 Pathophysiology of diabetes mellitus

In type I diabetes mellitus, pancreatic β-cells loss is due to T-cell mediated autoimmune attack (Rother, 2007). Type II diabetes correlates with central obesity and preexisting insulin resistance. Central obesity altered secretion level of adipokines that induce insulin resistance (Antuna-Puente, Feve, Fellahi, & Bastard, 2008). Thus, obesity is found in approximately 55% of patients diagnosed with type II diabetes (Centers for Disease Control and Prevention, 2004). Other factors that contribute to diabetes mellitus is ageing (impairment/dysfunction of mitochondrial function) (Petersen et al., 2003).

There are four major pathomechanism that may cause the development of complications in diabetes (Stirban, Rosen, & Tschoepe, 2008);

(1) The polyol pathway flux

(2) Increased hexosamine pathway throughput and subsequent overmodification of proteins by *N*-acetylglucosamine

(3) Hyperglycemia induced activation of protein kinase C (PKC) isoforms and

(4) Increased formation of advanced glycation end products (AGEs).

The pathomechanisms are interrelated and share common hyperglycemia-dependent cause: the overproduction of superoxide by the mitochondrial electron transport chain resulting in oxidative stress (Brownlee, 2005) . Oxidative stress affected diabetes starting from the induction of insulin resistance, destruction of β-cells leading to diabetes and generation of complications of diabetes (Ceriello, 2003; Rosen et al., 2001).

In polyol pathway, the enzyme aldose reductase will reduce toxic aldehydes to inactive alcohols in the cells. During hyperglycemia , aldose reductase will reduce

glucose to sorbitol, followed by oxidation to fructose in a process utilizing reduced NADPH, an important cofactor for regenerating reduced glutathione. When the amount of reduced glutathione decreased, the polyol pathway has increased susceptibility to intracellular oxidative stress (Brownlee, 2005).

Under normoglycemic conditions, glucose is metabolized *via* glycolysis and the initial products are glucose-6-phosphate and fructose-6-phosphate. But during intracellular hyperglycemia, some of fructose-6-phosphate is switched into glucosamine-6 phosphate and eventually to uridine diphosphate *N*-acetylglucosamine (Brownlee, 2005) by glutamine fructose-6-phosphate amidotransferase in another signaling pathway. Uridine diphosphate *N*-acetylglucosamine (UDP *N*-acetylglucosamine) is a substrate for the glycosylation of intracellular factors including transcription factors. Therefore *N*-acetylglucosamine subsequently will react with serine and threonine, resulting in changes in gene expression (Rolo & Palmeira, 2006).

Intracellular hyperglycemia induces the synthesis of diacylglycerol (DAG). Diacylglycerol is a critical activating cofactor for the three isoforms of Protein Kinase C: PKC-α, PKC-β and PKC-δ (Ishii, Koya, & King, 1998). Specifically, PKC-β isoform is strongly involved in the pathogenic processes of diabetic microangiopathy such as vascular leakage and abnormal vasodilator function (Ishii, et al., 1998).

Production of AGE precursors seem to damage cells by four mechanisms; modification of intracellular proteins, extracellular matrix molecules in the vicinity, circulating proteins in the bloodstream and circulating protein (Kalousova, Skrha, & Zima, 2002). The changes occur includes proteins involved in the regulation of gene transcription, signalling between matrix and the cells thus causing cellular dysfunction, and production of inflammatory cytokines and growth factors, which in turn are responsible for cellular and tissue damage (Kasper & Funk, 2001). . The AGE receptors

that exist on the surface of different cells are responsible for the elimination of AGEs however, it still appears to mediate most of the biological effects of AGEs (Singh, Barden, Mori, & Beilin, 2001).

2.2.4 Complications of diabetes mellitus

The 'response-to injury' theory states that endothelial dysfunction is a way toward atherosclerosis (Cai et al., 2002), diabetic retinopathy and nephropathy in diabetes (Schalkwijk & Stehouwer, 2005). The factors that contribute to the development of endothelial dysfunction are hyperglycemia, hyperinsulinemia and accelerated aging. Endothelial dysfunction occurs when there is diminished production of endothelial NO. In diabetes, the increased generation of ROS can directly react with NO to form peroxynitrite and this results in diminished concentration of bioactive NO, reduction in reactivity of eNOS, and production of another highly ROS (peroxynitrite). This will increase lipid peroxidation and nitrative stress, encourage vasoconstriction and initiate inflammatory responses (Tan, 2004).

The LDL present in the subendothelial matrix will trigger events of atherosclerosis. This will lead to increased permeability and leakage of the endothelial cell layer. Diabetes is related to increased oxidized LDL (Schalkwijk & Stehouwer, 2005). Furthermore, LDL particles in diabetic subjects are more exposed to oxidation (Bowie, Owens, Collins, Johnson, & Tomkin, 1993) and glycation of LDL particles increases in parallel with hyperglycemia (Wu, Yeh, & Yen, 2010). The LDL subclass phenotype B (small, dense, triglyceride-rich), oxidized LDL and glycated LDL are correlated with Type II diabetes (Feingold, Grunfeld, Pang, Doerrler, & Krauss, 1992). The glycated LDL increased the non-receptor-mediated uptake of particles and oxidized

LDL carrying cholesterol to the artherosclerotic plaque by macrophage uptake (Lyons, 1991).

In contrast, high-density lipoprotein (HDL) is a protective factor against artherosclerosis and was found markedly decreased in Type II diabetes patients (Feingold, et al., 1992). HDL has antioxidative and anti-inflammatory properties that could reduce the LDL peroxidation and combat ROS constantly.

In long-term, microvascular complications will affect small blood vessels throughout the body which include heart, blood vessels, eyes, kidneys, and nerves (Brownlee, 2005).

Blindness (diabetic retinopathy) occurs as a result of long-term accumulated damage to the small blood vessels in the retina (Ogata et al., 2005). Kidney failure (diabetic nephropathy) frequently occurs in diabetic patients (House et al., 2010). Foot ulcers and limb amputation (diabetic neuropathy) occur as a consequence of reduced blood flow in diabetic patient feet (Edwards, Vincent, Cheng, & Feldman, 2008).

On the whole, the risk of mortality among diabetes sufferers is at least double the risk of their peers without diabetes. After 15 years of diabetes, approximately 10-20 % of diabetes patients die of kidney failure, 10 % will develop severe visual impairment and 2 % will become blind (WHO, 2006).

2.2.5 Current prevalence of diabetes mellitus

Latest report by WHO (2006), estimated that over 220 million people worldwide have diabetes. In 2005, mortality rate of diabetics attained 1.1 million and 80 % of these were from low- and middle-income countries. More specifically, 55 % of mortality rate are women and about half of deaths occurred at the age of < 70 years. Based on past

WHO records, deaths due to diabetes is expected to increase twofold between 2005-2030.

2.2.6 Treatments available for diabetes mellitus patients

Prevention or delaying the onset of diabetes could be achieved by having healthy low-calorie diet, regular physical activity, maintaining normal body weight and avoiding tobacco use. However, most people only realise that they are diabetic once the symptoms appeared.

Controlling the diet is essential to reduce hyperglycemia (Israili, 2009). Anti-diabetic drugs could also help to decrease hyperglycemia. Most anti-diabetic drugs widely used today are 'secretagogues' that trigger insulin release by direct action on the K^+-ATP channel of the pancreatic beta cells or 'sensitizers' that reduce liver glucose output and increase uptake of glucose by the peripheral tissue or enhance production of mRNAs of insulin dependent enzyme (Kapinya, Nijjar, Stanek, & Amanullah, 2008). For example; Glibenclamide is a secretagogue which decreases blood glucose by stimulating the existing β-cells to secrete more insulin.

Metformin, an insulin-sensitizer drug acts by overcoming insulin resistance in Type II diabetes (Dunn & Peters, 1995). When a single therapy of sulfonylurea failed to control blood glucose, a combination of sufonylureas or with insulin-sensitizers/ insulin will be recommended (Turner, Cull, Frighi, Holman, & Group, 1999). Insulin therapy is usually given to Type I diabetes because functional β-cells are not available for insulin production.

However, all type of drugs has its side-effects such as sulfonylureas cause weight gain and hypoglycemia (Van Staa, Abenhaim, & Monette, 1997).

2.3 OXIDATIVE STRESS

2.3.1 Definition of oxidative stress

Oxidative stress is an imbalance between the production of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage (Mena, Ortega, & Estrela, 2009).

2.3.2 Free radical induces oxidative stress

Free radicals are defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell, 1999).

Synopsis of free radical theory:

1954: Gershman's free radical theory of oxygen toxicity

Toxicity of oxygen is due to partially reduced forms of oxygen

1954: Commoner, Townsend, and Pake

Observations of a weak electron paramagnetic resonance (EPR) signal attributable to the presence of free radicals in a variety of lyophilised biological materials.

1956: Denham Harman

Proposed the concept of free radicals playing a role in the ageing process

1969: Mc Cord and Fridovich

Discovered the enzyme superoxide dismutase (SOD)

1977: Mittal and Murad

Provided evidence that the hydroxyl radical stimulates activation of guanylate cyclase and formation of the "second messenger" cyclic guanosine monophosphate (cGMP)

Free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are small molecules / ions, reactive with small activation energies, have short halflife and are products of cellular metabolism. Free radicals have beneficial and deleterious effects (Marian Valko & Milan Mazura, 2007). Examples of beneficial effects are defence against infectious agents and cellular signalling systems. This take place at low or moderate concentrations of free radicals. The harmful effects of free radicals include oxidative stress and nitrosative stress (Droge, 2002). However, endogenous antioxidants that act as the defense system will suppress the deleterious reactions (Svend, 2003). This "Redox regulation" protects against a variety of oxidative stresses and maintains "redox homeostasis" (Droge, 2002).

Oxygen radicals is the prominent radical species generated in a living system (Miller, Buettner, & Aust, 1990). An unpaired electron gives a certain level of reactivity to the oxygen radical that leaks out during energy transduction (Marian Valko & Milan Mazura, 2007). Oxygen molecule is itself a radical and an addition of an electron to it forms the superoxide anion radical ($O_2^{\bullet -}$) (Miller, et al., 1990). ROS is classified into primary or secondary ROS. The "primary" ROS is superoxide anion, arising either through metabolic processes or oxygen "activation" by physical irradiation. Meanwhile "secondary" ROS are derived from interaction of "primary" ROS with other molecules, either directly or prevalently through enzyme- or metal-catalysed processes (Marian Valko & Milan Mazura, 2007).

2.3.3 Reaction of free radicals

Hydroxyl radical has a high reactivity, making it very harmful to near location of its formation (Marian Valko & Milan Mazura, 2007).

2.3.4 Oxidative stress and diabetes

Chronic hyperglycemia is known to trigger oxidative stress induced-diabetic complications (Choi, Benzie, Ma, Strain, & Hannigan, 2008). Hyperglycemia stimulates ROS formation from a variety of sources which includes oxidative phosphorylation, glucose autooxidation, NADPH oxidase, lipooxygenase, cytochrome P450 monooxygenases, and nitric oxide synthase (NOS) (Marian Valko & Milan Mazura, 2007).

Overproduction of ROS, arising from mitochondrial electron-transport chain and excessive stimulation of NADPH results in oxidative stress. In oxidative stress, excess of free radicals will cause damage to cellular macromolecules including lipids, proteins and DNA.

2.4 ANTIOXIDANT

2.4.1 What is an antioxidant?

An antioxidant may be defined as 'any substance that when present at low concentrations, compared with those of the oxidizable substrate significantly delays or inhibits oxidation of that substrate (Halliwell & Gutreridge, 1989).

Defence mechanisms against free radical –induced oxidative stress include:

(1) Preventive mechanisms

(2) Repair mechanisms

(3) Physical defences, and

(4) Antioxidant defences (Marian Valko & Milan Mazura, 2007; Sies, 1997)

Table 2.1 : Reaction of free radicals

Oxidant	Description
•O_2-, superoxide anion	One-electron reduction state of O_2, formed in many autoxidation reactions and by the electron transport chain. Relatively unreactive, but can release Fe^{2+} from iron-sulfur proteins and ferritin. Undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis and is a precursor for metal-catalyzed •OH formation.
H_2O_2, hydrogen peroxide	Two-electron reduction state, formed by dis-mutation of •O_2- or by direct reduction of O_2. Lipid soluble therefore able to diffuse across membranes.
•OH, Hydroxyl radical	Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. Very reactive, will attack most cellular components.
ROOH, organic hydroperoxide	Formed by radical reactions with cellular components such as lipids and nucleobases.
RO•, alkoxy and ROO•, peroxy radicals	Oxygen centred organic radicals. Lipid forms participate in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.
HOCl, hypochlorous acid	Formed from H_2O_2 by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents, including thiol groups, amino groups and methionine.
ONOO-, peroxynitrite	Formed in a rapid reaction between •O_2- and NO•. Lipid soluble and similar in reactivity to hypochlorous acid. Protonation forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen dioxide.

(Aruoma, 1998)

2.4.2 Classification of antioxidant

Generally antioxidants are classified into two categories; enzymatic antioxidant and non-enzymatic antioxidant. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants derived from nutritional intake (Sies, 1997). Metal binding proteins such as ferritin and ceruloplasmin are also known as non-enzymic antioxidants.

2.4.3 Pro-oxidant

There is no specific definition for pro-oxidant. Basically, from its term pro-oxidant has a contrasting effect to oxidant or antioxidant. This might occur due to several factors such as, different condition e.g., high dose for Vitamin C (Park & Lee, 2008) and structure-activity relationships of flavonoids (Cao, Sofic, & Prior, 1997).

2.4.4 Antioxidant and diabetes

Antioxidants are found abundantly in plants and the long history of traditional medicine supports and validates its use to prevent the complications of diabetes. The low levels of plasma antioxidants has been implicated as a risk factor for the development of the disease (McCune & Johns, 2002). Thus, antioxidants are important for inhibiting complications of diabetes. Examples of natural plants with antioxidant and anti-diabetic effect are bitter gourd (*Momordica charantia*) (Sathishsekar & Subramanian, 2005), sugar apple (*Annona squamosa*) (Kaleem, Asif, Ahmed, & Bano, 2006), cactus pear (*Opuntia ficus indica*) (Fatma, El-Metwally, Shehab, Hassan, & Gomaa, 2012) and summer-grass mycelia (*Cordyceps sinensis*) (Li et al., 2006).

2.5 ADIPOCYTES

2.5 Adipocytes

The adipocyte or adipose is an endocrine organ which also stores and secretes triglycerols (energy homeostasis). Adipocytes can increase its size and form new adipocytes from existing preadipocytes (Gregoire, Smas, & Sul, 1998). Adipocytes secrete cytokines that are known to be involved in homeostasis, inflammatory response, vasoregulation, and steroid metabolism (Diamond, 2002). Among the cytokines secreted by adipocytes, adiponectin and leptin are known to have stimulatory effects on the beta oxidation of fatty acids in skeletal muscle therefore are linked with insulin sensitivity. An increase in serum leptin or plasma adiponectin concentration improve regulation in insulin sensitivity (Kim et al., 2008) *via* activation of PPARγ (Lappas, Permezel, & Rice, 2005). Meanwhile, tumor necrosis factor (TNF), interleukin 6 (IL-6), and resistin, play a role in the development of the insulin resistance in Type II Diabetes obese patients (Diamond, 2002).

2.5.1 Types of adipocytes

Adipose tissue is classified into white and brown adipose tissue. White fat cells secrete resistin, adiponectin, and leptin (Gregoire, et al., 1998).

2.5.1.1 Adiponectin

Adiponectin or Acrp30 or apM1 is a protein hormone secreted solely by the adipocytes. This hormone is involved in glucose regulation and fatty acid catabolism (Diamond, 2002). It has anti-inflammatory and anti-atherogenic properties that is protective against insulin resistance and macroangiopathy, reduces circulating free fatty acids and corrects both hyperglycemia and hyperinsulinemia. The concentration are inversely correlated with body fat percentage in adults. Thus, it is present in high levels

in normal body weight as compared to obesity, type II diabetes, coronary artery disease individuals. High concentration level correlates with insulin sensitivity and rise in response to insulin (Diamond, 2002).

2.5.1.2 Insulin

Insulin is a hormone made of 51 amino acids with a molecular weight of 5808 Da. It is synthesized within β-cells of the islets of Langerhans in the pancreas. In human, around one million to three million islets of Langerhans exist and 60–80 % of these are β-cells. Insulin regulates the expression of more than 100 genes (Lochhead, Salt, Walker, Hardie, & Sutherland, 2000). Insulin is secreted if a stimulus such as glucose in the blood produced from digested food is detected. The release is rapidly triggered in response to high blood glucose level and later slow release is sustained before it is degraded and the response is terminated. Generally, insulin promotes the synthesis and storage of carbohydrates, proteins and lipids. Therefore, effects of insulin include increasing glucose uptake from the blood, storing it as glycogen in the liver and muscle (glycogen synthesis), inhibit glycogenolysis and gluconeogenesis in liver and stimulate lipogenesis and stop lipolysis in adipose tissue (Saltiel & Kahn, 2001; White, 1994).

2.5.2 Role of Insulin in adipocytes

Insulin controls specific protein synthesis and amount, by upregulating or downregulating certain gene expression followed by mRNA translation (Ueki et al., 1998). At cellular level, the presence of insulin in adipocytes will stimulate insulin signalling pathway as described in *Section 2.5.3*.

2.5.3 Insulin Signalling Pathway

In Insulin Signalling Pathway, insulin binds to the insulin receptor. Insulin receptor is a protein consisting of two α-subunit and attached to its two β-subunit each. The α-subunit functions to inhibit the tyrosine kinase activity of β-subunit (Saltiel & Kahn, 2001). When insulin binds to the insulin receptor, the receptor kinase activity of β-subunit phosphorylates and turns on Insulin Receptor Substrate-1 (IRS-1), this changes PIP2 to PIP3. PIP3 is attached to Protein Kinase B (PKB), and signals (phosphoinositide-dependent kinase-1) PDK1 to phosphorylate PKB. PKB is major signalling arm for PI3K. P13K consists of a p110 catalytic subunit and a p85 regulatory subunit (Seki et al., 1997). Once phosphorylated, PKB phosphorylates further targets that provoke GLUT 4 to be translocated to the plasma membrane.

GLUT 4 is an insulin regulated glucose transporter that is located in the adipose tissue and striated muscle (skeletal and cardiac). In the absence of insulin, the transporter stays at its endosome. The presence of insulin stimulates GLUT 4 to be translocated from the intracellular region to the plasma membrane. At cell surface, the transporter enables glucose uptake *via* passive diffusion into muscle or fat cells. The glucose will then undergo glycolysis.

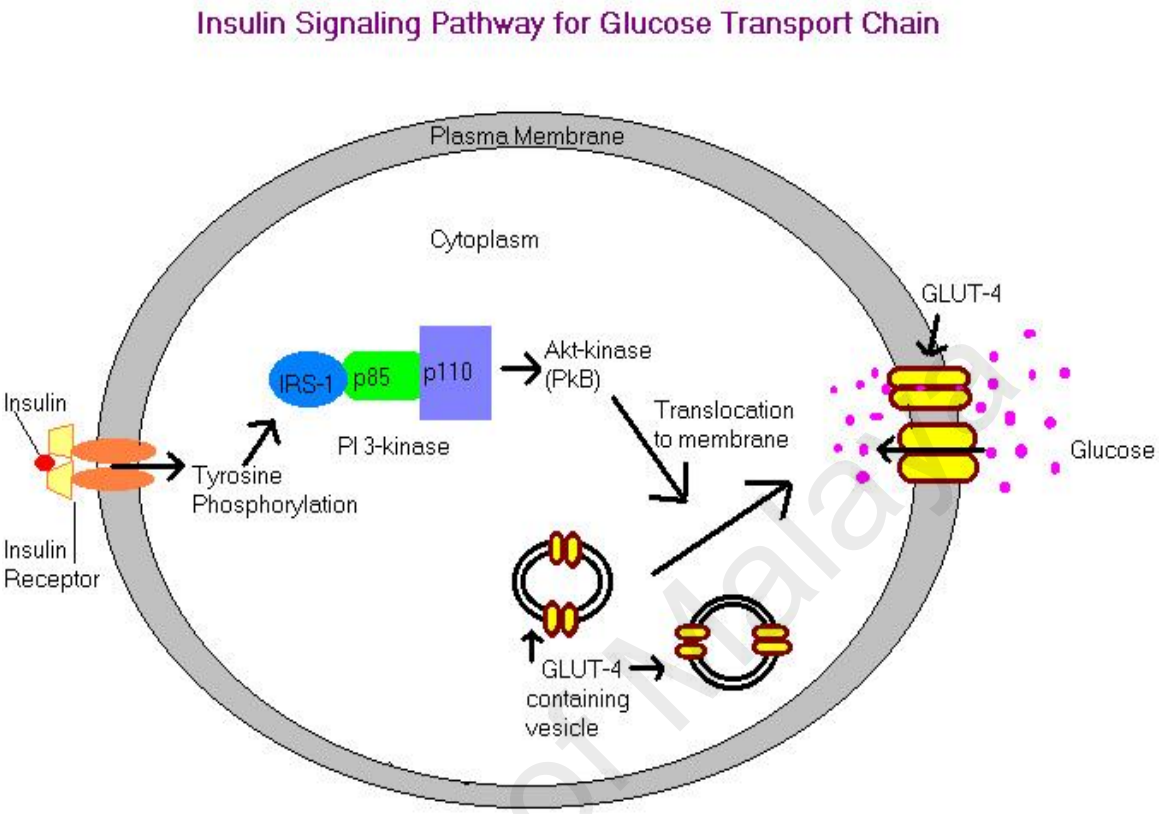


Figure 2.2 : Insulin Signalling Pathway

Figure cited from (University of Arizona, 2003)

2.5.4 Selected genes for gene expression study in rat adipocytes.

Glucose transporter 4 or GLUT-4 is encoded by SLC2A4 gene (O'Gorman et al., 2006) located on the short arm of chromosome 17 (Abel et al., 2001; Joost et al., 2002). Type II diabetes is due to insulin resistance and leading to problems in glucose transport that could be related to the down-regulation of GLUT-4 gene expression in skeletal muscle and adipose tissue (Abel, et al., 2001). In chronic exposure, rats treated with insulin showed increased GLUT-4 mRNA in adipose tissue meanwhile insulin treatment of 3T3-L1 adipocytes resulted in either no change or a reduction in GLUT-4 mRNA levels (Fernyhough, Okine, Hausman, Vierck, & Dodson, 2007). Laville and friends (1996) found that the expression of the genes coding for GLUT-4 were unaltered in obese or Type II diabetic patients.

P13K or phosphatidylinositol 3-kinases is activated by insulin receptor substrates (IRS) 1 and 2 in insulin signalling pathway. Insulin has been claimed to inhibit the transcription of genes encoding for hepatic phosphoenolpyruvate carboxykinase (PEPCK) genes, the rate limiting enzyme in gluconeogenesis, glucose-6-phosphatase (G6Pase) and regulation of gene expression of fatty acid synthase (FAS) through a PI3K pathway (Treins, Giorgetti-Peraldi, Murdaca, Semenza, & Van Obberghen, 2002). The expression of PEPCK and G6Pase genes are found abnormally regulated in type II diabetes (Lochhead, et al., 2000). PIP3 plays a role for repression of PEPCK transcription by insulin (Lochhead, et al., 2000). In diabetic patients, proinflammatory cytokines lead to serine phosphorylation of IRS-1 and IRS-2 that cause insulin resistance. Hyperglycemia, hyperinsulinemia and increased free fatty acids enhances IRS serine phosphorylation therefore reduces tyrosine phosphorylation followed by inactivation of PI3K and finally impair downstream signaling.

AKT or Protein Kinase B is activated by PI 3-kinases. The PKB subfamily comprises three mammalian isoforms, PKBα (Akt1), PKBβ (Akt2) and PKBγ (Akt3) with three functional domains; a central kinase, an N-terminal pleckstrin homology (PH) and a C-terminal regulatory with hydrophobic motif (HM) phosphorylation site (Hanada, Feng, & Hemmings, 2004; Zdychova & Komers, 2005). Akt2 has high expression level in adipocytes, skeletal muscle, heart and cerebellum (purkinje cells) (Zdychova & Komers, 2005). AKT regulations are important in regulation of metabolism, cell survival, transcription and cell-cycle progression (Elisabeth *et. al*., 2005). The serine phosphorylation of IRS-1 by PKB stabilizes the protein and improves signaling. The PKB is activated by autophosphorylation of receptor tyrosine kinases induced by insulin. PI3K generates second messenger from PtdIns $(4,5)_{P2}$ to PtdIns $(3,4,5)_{P3.}$ The plecstrin homology (PH) domain of Akt kinases has an affinity for PIP3 and the binding triggers Akt translocation to the plasma membrane. Wortmannin, rapamycin and LY294002 are PI3K inhibitors that prevent the threonine phosphorylation thus no signalling in the cascade (Hegedus et al., 2012). A research using transgenic mice expressing a kinase-dead mutant suggested that PKB was shown to be involved in the regulation of insulin secretory pathway (Bernal-Mizrachi et al., 2004). In Type II diabetes, Akt signalling was impaired due to insulin resistance (Zdychova & Komers, 2005).

Sterol Regulatory Element Binding Proteins (SREBP) are transcription factors that bind to the sterol regulatory element DNA sequence TCACNCCAC. SREBPs are attached to the nuclear membranes and endoplasmic reticulum. In a condition of low sterol concentration, SREBPs will undergo proteolytic cleavage to a water soluble N-terminal domain, which is translocated to the nucleus. These activated SREBPs then bind to specific sterol regulatory element DNA sequences which up-regulate the

synthesis of enzymes involved in sterol bio-synthesis (Ma Hongbao, 2008). SREBP that is highly found in adipose tissue and liver is SREBP-1c. SREBP-1c is involved in promoting rat adipocytes differentiation (ADD1) (Shimomura et al., 1998). Reseachers found that SREBP-1c controls the genes involved in fatty acid synthesis in adipocyte cell lines (Ma Hongbao, 2008). In diabetes, hyperglycemia up-regulated SREBP-1 as shown in renal cells cultured in a high glucose media (Sun, Halaihel, Zhang, Rogers, & Levi, 2002). High glucose also enhances expression of SREBP-1a and -1c mRNA, SREBP-1 protein, and FAS, causing increased TG. In streptozocin-induced diabetes rat, SREBP-1 and fatty acid synthase (FAS) expression were significantly high and there is stimulation of lipid synthesis in the liver (Shimomura et al., 1999). However, when the diabetic rats were treated with insulin, the renal expression of SREBP-1 and the accumulation of TG were inhibited. SREBP-1 plays an essential role in the increase of lipid synthesis, TG increase, mesangial expansion, glomerulosclerosis, and proteinuria by increasing the expression of transforming growth factor beta (TGFβ) and vascular endothelial growth factor (VEGF) (Foretz et al., 1999).

Hormone sensitive lipase or HSL regulates lipolysis of stored triglycerides in adipocytes to triacylglycerol (TG), diacylglycerol (DAG) and monoacyglycerol, cholesteryl ester and free fatty acids (Wen-Jun et al., 2007). The lipase is highly expressed in adipose tissue. The HSL gene is activated when the body has to mobilise energy stores. HSL responds positively to glucagon, catecholamines, ACTH and negatively to insulin (Muller, Jordan, Jung, Kleine, & Petry, 2003; Wen-Jun, et al., 2007). Catecholamines work by activating the different steps of the lipolytic process and cascade, resulting in the activation of HSL. It has been suggested that the presence of the truncated HSL protein is associated with an impaired adipocyte lipolysis (Ray et al., 2003).

In the present study, the gene expressions of AKT, GLUT-4, HSL, PI3K and SREBP in adipocytes treated with *L. leucocephala* was estimated as a preliminary attempt to delineate the mechanism of action of the *L. leucocephala* extract.

2.6 Rat model for anti-diabetic study

There are a few rodent model for anti-diabetic study which had been established since 1980s. Animal experimental research was well-developed as public awareness on ethical of using and handling animal during experimental increased.

Choosing the right animal model is important as each model could give certain invaluable insight of what researcher looking for. Its depend on the suitability of the animal model and aims of the research Several essential factors should also be taken into account when selecting a diabetes model. The important criteria include the ability to maintain a steady level of diabetes for the duration of the animal experiment, understanding the disease characteristics and progression of injury in the animal being used and the attainment of a pathological state which has clinical relevance (Tesch & Allen, 2007).

Small rodents such as rats are common animal model choosen by most of scientist for anti-diabetic and antioxidant study due to ease of handling especially for large group of experiment. Methods options available are surgical method e.g pancreatectomised animals and non-surgical method e.g toxin-mediated pancreatic damage, genetically altered rats/mice (Rees & Alcolado, 2004).

Alloxan and Streptozocin are popular toxin chemicals for inducing diabetic and was beneficial also for antioxidant study (Afifi, Saket, Jaghabir, & Al-Eisawi, 1997; Ahlem et al., 2009; Liu, Li, Zeng, Liu, & Wang, 2008). Streptozocin-induced diabetes mellitus are choosen in this study because of it's common way people get diabetes disease which is due to free radicals causes oxidative stress-induced diabetic complications and align with antioxidant study. This study is an first attempt for study of efficacy and safety of *Leucaena leucocephala* potential as anti- diabetic and antioxidant agent, therefore Streptozotocin-induced diabetic rats is a best option.

2.6.1 Streptozocin induced-diabetics rats

Streptozocin (STZ) selectively destroys β-cells (Szkudelski, 2001) in the pancreas leading to absence of insulin production. This method becomes an important tool for producing animal models of hyperglycemia. This type of model induce Type I diabetes, (Tesch & Allen, 2007) while injection of Streptozocin followed by nicotinamide intraperitoneally will produce Type II diabetes.

Streptozocin is a brand name for 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D glucopyranose, $C_8H_{15}N_3O_7$. (Rossini, Like, Chick, Appel, & Cahill, 1977; Szkudelski, 2001). It is a chemical derived from *Streptomyces achromogenes* (Szkudelski, 2001). It is a mimic of N-acetylglucosamine (GlcNAc) (Tesch & Allen, 2007). STZ molecules will be carried by Glucose Transporter 2 (GLUT-2) into β-cells in pancreas (Frode & Medeiros, 2008; Szkudelski, 2001). Streptozocin causes DNA alkylation, induces activation of poly adenosine diphosphate (ADP)-ribosylation and liberates nitric oxide (Frode & Medeiros, 2008). The DNA alkylation causes DNA damage. This promotes (ADP)-ribosylation that causes depletes cellular nicotinamide adenine dinucleotide (NAD^+) and adenosine triphospate (ATP). The increase in ATP dephosphorylation and subsequent formation of hypoxanthine from adenine metabolisme serves as substrate for xanthine oxidase (XOD). This results in the formation of superoxide radicals, hydrogen peroxide and hydroxyl radicals (Ruzaidi, Abbe, Amin, Nawalyah, & Muhajir, 2008). Meanwhile, the release of nitric oxide (NO) inhibits aconitase activity that also contributes in DNA damage. This will cause necrosis of β-cells (Tesch & Allen, 2007).

Streptozocin is the most commonly used drug to induce diabetes and rats were mostly used due to the availability of many different strains and transgenic or knockout breed (Frode & Medeiros, 2008; V Kecskemeti et al., 2002). Although type II diabetes is more common compared to type I, but at later stage of type II diabetes the inevitable

β-cell insufficiently / destruction could lead to insulin dependence just as in Type I diabetes.

Male rodents are generally more susceptible to the effects of STZ and tend to develop greater hyperglycemia (Tesch & Allen, 2007). An appropriate dose should be used to ensure that the number of rats that became diabetic with less complications is maximum and the rats survive until the end of the experiment. The higher Streptozocin dosage will cause greater cytotoxic and more rapid destruction of pancreatic β-cells, resulting in higher incidence and severity of diabetes. However, at higher doses, STZ has a non-specific cytotoxic effect which has been shown to cause acute kidney damage in mice and rats. Consequently, model using higher doses of STZ can develop nephropathy which results from hyperglycemia-induced injury super-imposed on acute renal STZ-cytotoxic, making it difficult to interpret findings (Tesch & Allen, 2007). Overdose STZ lead to nephropathy and other complications that shorten the survival rate although it gives higher product (Tesch & Allen, 2007).

The male rats were used in all our studies since the females are shown to be protected from lipid-induced reductions in insulin action (Hevener, Reichart, Janez, & Olefsky, 2002) and male rodents are generally more susceptible to the effects of STZ and tend to develop greater hyperglycemia (Tesch & Allen, 2007).

CHAPTER THREE: MATERIALS AND METHODS

3.1 MATERIALS

The following chemicals, consumables and kits were used in this study (*Table 3.*1).

3.2 EQUIPMENT

The following equipment were used in this study (*Table 3.*2).

3.3 PLANT COLLECTION AND PROCESSING

3.3.1 Preparation of aqueous extract

Leucaena leucocephala was obtained from Ethno Resources, Sungai Buloh, Selangor. The identity of *L. leucocephala* was confirmed by a herbal scientist from the company. *L.leucocephala* dry fruit (grinded form) was stored at Biochemistry Laboratory, Department of Biomedical Science, Faculty of Medicine, University of Malaya, Kuala Lumpur.

A litre of water was added to 50 g of the powdered extract in a 1000 ml flask and stirred for three hours at 40 °C. This mixture was filtered using filter paper, centrifuged at 3000 xg for 10 minutes, and the resulting supernatant was filtered again before freeze-drying. This extract was stored at -20 °C until further use. The procedure used for the preparation of aqueous extract is illustrated in *Figure 3.1*.

3.3.2 Solvent extraction of bio-active compounds

The aqueous extract of *L. leucocephala* was subjected to solvent extraction by using liquid-liquid partitioning method. It is a method commonly used to separate compounds based on their relative solubility in two different immiscible liquids, usually water and an organic solvent. Two grams of crude extract was dissolved in 250 ml of Millipore water. Next, equal volumes of hexane or ethyl acetate were added and mixed

well by shaking. Then, *L. leucocephala* compounds were separated based on polarity. Non-polar compounds were dissolved in hexane, while polar compounds were dissolved in water. The two fractions were well-separated after 30 minutes. The water fraction was collected from the lower end (tip) of the funnel while the solvent fraction was collected from the upper end of the funnel to prevent disturbance of the interface.

The solvent fraction was rotary evaporated while the water extract was freeze dried. The solvent product was stirred and heated at 50 °C to remove the solvent residue, until traces of solvent completely dissappeared. All the process were performed in a fume hood. Finally, it was oven-dried at 50 °C. Normal saline 0.9% was used to dissolve final dried residue from different solvent extraction.

The steps involved in the partial purification of bio-active compounds is shown in *Figure 3.2*.

Table 3.1: List of materials used in this study.

Materials	Brand	Used in
Hexane, HPLC grade	Fisher Scientific, UK	Solvent extraction
Ethyl acetate, HPLC grade	Fisher Scientific, UK	Solvent extraction
Streptozocin	Sigma-Aldrich, USA	Induction diabetes
Sodium citrate	Merck, Germany	Induction diabetes
Acid citric	Merck, Germany	Induction diabetes
Glucose	Glucolin, Malaysia	OGTT
Glibenclamide	Euglucon, Roche	OGTT, chronic and subchronic study
Collagenase	Sigma-Aldrich, USA	Tissue culture
RPMI-1640 media supplemented with 2 mM L-Glutamine, 100 units/ml Penicillin-Streptomycin-, 2 mM amphotericin B and 5 % FBS	Sigma-Aldrich, USA	Tissue culture
Dulbecco's Modified Eagle's Medium I (DMEM I) supplemented with 2 mM Penicillin-streptomycin-glutamine, 2mM amphotericin B, 10 % FBS, 33 µM biotin, 17 µM panthothenic acid, 10 µM troglitazone, 0.5 mM IBMX, 1.0 µM dexamethasone and 10 µg/ml insulin	Sigma-Aldrich, USA	Tissue culture
Dulbecco's Modified Eagle's Medium II (DMEM II) supplemented with 2 mM Penicillin-streptomycin-glutamine, 2 mM amphotericin B, 10 % FBS, 33 µM biotin, 17 µM panthothenic acid and 10 µg/ml insulin.	Sigma-Aldrich, USA	Tissue culture
Trizol® reagent (Product no.: 12183-018)	Invitrogen, USA	RNA extraction
High Capacity cDNA Reverse Transcription kit, (Product no.: 4368814)	Applied Biosystems,USA	Reverse Transcription
TaqMan® gene expression assays	Applied Biosystems,USA	Gene expression analysis
18S rRNA (*Ratus norgevicus* samples; (Product no.: 4319413E; VIC/MGB probe)	Applied Biosystems	Gene expression analysis
Rat Insulin ELISA kit	Linco Research,USA	Biochemistry assay
Rat Adiponectin ELISA assay kit	BioVision Research Products, USA.	Biochemistry assay
Sodium chloride	Sigma-Aldrich, USA	Homogenates samples preparation

Materials	**Brand**	**Used in**
PBS buffer tablet (20 mM, pH 7.4)	Sigma-Aldrich, USA	Homogenates samples preparation
2,4,6-tripyridyl-s-triazine (TPTZ)	Sigma-Aldrich, USA	FRAP assay
Ferric chloride ($FeCL_3.6H_2O$)	Sigma-Aldrich, USA	FRAP assay
Sodium acetate trihydrate	Sigma-Aldrich, USA	FRAP assay
Glacial acetic acid	Sigma-Aldrich, USA	FRAP and AOPP assay
Ferrous sulfate heptahydrate ($FeSO_4$.7H2O)	Sigma-Aldrich, USA	FRAP assay
Potassium iodide	Fisher Scientific, UK	AOPP assay
Chloramine-T	Sigma-Aldrich, USA	AOPP assay
Trichloroacetic acid (TCA)	AppliChem,Germany	MDA assay
Thiobarbituric acid (TBA)	AppliChem,Germany	MDA assay
1,1,3,3-tetraethoxypropane	Sigma-Aldrich, USA	MDA assay
Glutathione Peroxidase kit BioxyTech GPx-340,	Oxis Research product (USA).	Enzymatic antioxidant assay
Bradford reagent	Bio-Rad, USA	Bradford assay
Folin-Ciocalteu phenol reagent	Merck, Germany	TPC assay
10 % Na_2CO_3	Merck, Germany	TPC assay
Gallic acid	Sigma-Aldrich, USA	TPC assay
Diphenylpicrylhydrazyl (DPPH)	Sigma-Aldrich, USA	DPPH assay
Formalin	Scharlau Chemie, Spain	Histology
Haematoxylin and Eosin (H & E)	Scharlau Chemie, Spain	Histology
DPX Mountant	Scharlau Chemie, Spain	Histology

Table 3.2: List of equipment used in this study.

Equipment	Used in
Oven	Plant extraction
Blender	Plant extraction
Freeze-dryer	Plant extraction
Fridge	Plant extraction
Centrifuge	Plant extraction
Rotary –Evaporator BUCHI Rotavapor R-210 Buchi Waterbath B-491 Buchi Vacuum System V-700	Plant extraction
Glucometer and test strips Accu-Chek Advantage II	OGTT, chronic and subchronic study
pH meter	Induction diabetes
Weighing scale	Plant and drug preparation, induction diabetes
Intragastic tube	OGTT, chronic and sub-chronic study
Syringe	Induction diabetes
Waterbath	MDA assay
Spectrophotometer	RNA extraction
Thermalcycler	Reverse transcription
Real time PCR	Gene expression assay
StepOne software (Vers. 2.0, Applied Biosystems)	Gene expression assay
MicroAmp™; Product no.: 4358293) and capped with optical caps (Product no.: 4323032).	Gene expression assay
Vacutainer blood tubes	Blood sample preparation
Homogenizer	Homogenates tissue samples preparation
Digital weight scale	Homogenates tissue samples preparation
96 well plate	All enzymatic and non-enzymatic antioxidant assays
Microplate Reader Power Wave X 340 Software: KC Junior Programme	All enzymatic and non-enzymatic antioxidant assays
Safety cabinet Class II biohazard NU 425-400E, Nuaire ™, USA	Cell culture
CO_2 water-jacketed incubator, Nuaire ™, IR autoflow	Cell culture
Inverted microscope, CK-40 Olympus	Cell culture
Light microscope	Histology
SPSS (vers. 13)	Statistical analysis
Graph Pad Prism (vers 5.02)	Statistical analysis
Microsoft Excel	Statistical analysis

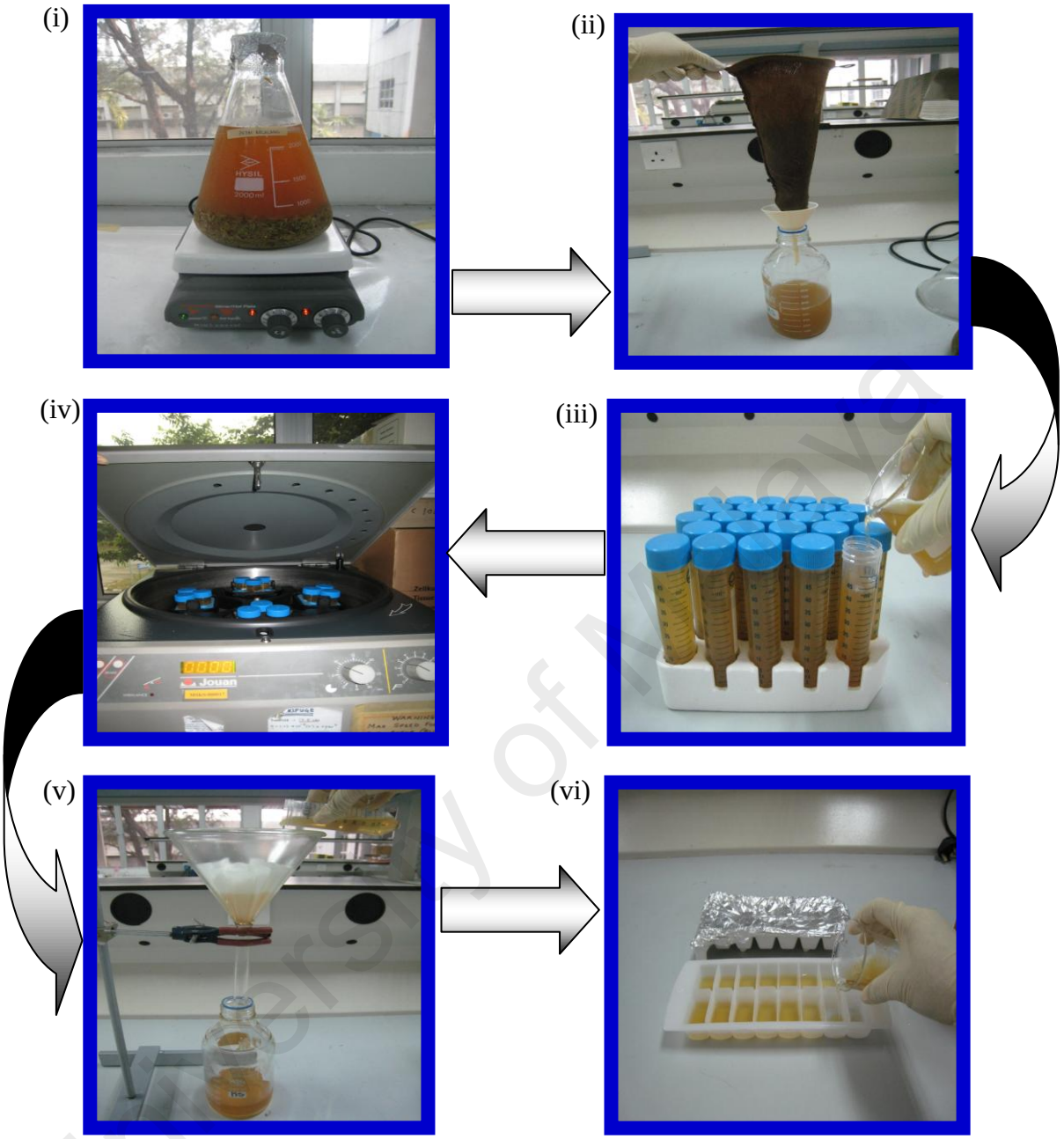


Figure 3.1: Preparation of aqueous crude extract.

(i) The extract was stirred for three hours at 40 °C. (ii) The extract mixture was filtered using tea filter. (iii) & (iv) Filtered supernatant were poured into centrifuge tubes and centrifuged at 3000 *xg* for 10 minutes. (v) Supernatant was filtered again using filter paper. (vi) Clear supernatant was made into ice cubes for freeze-drying.

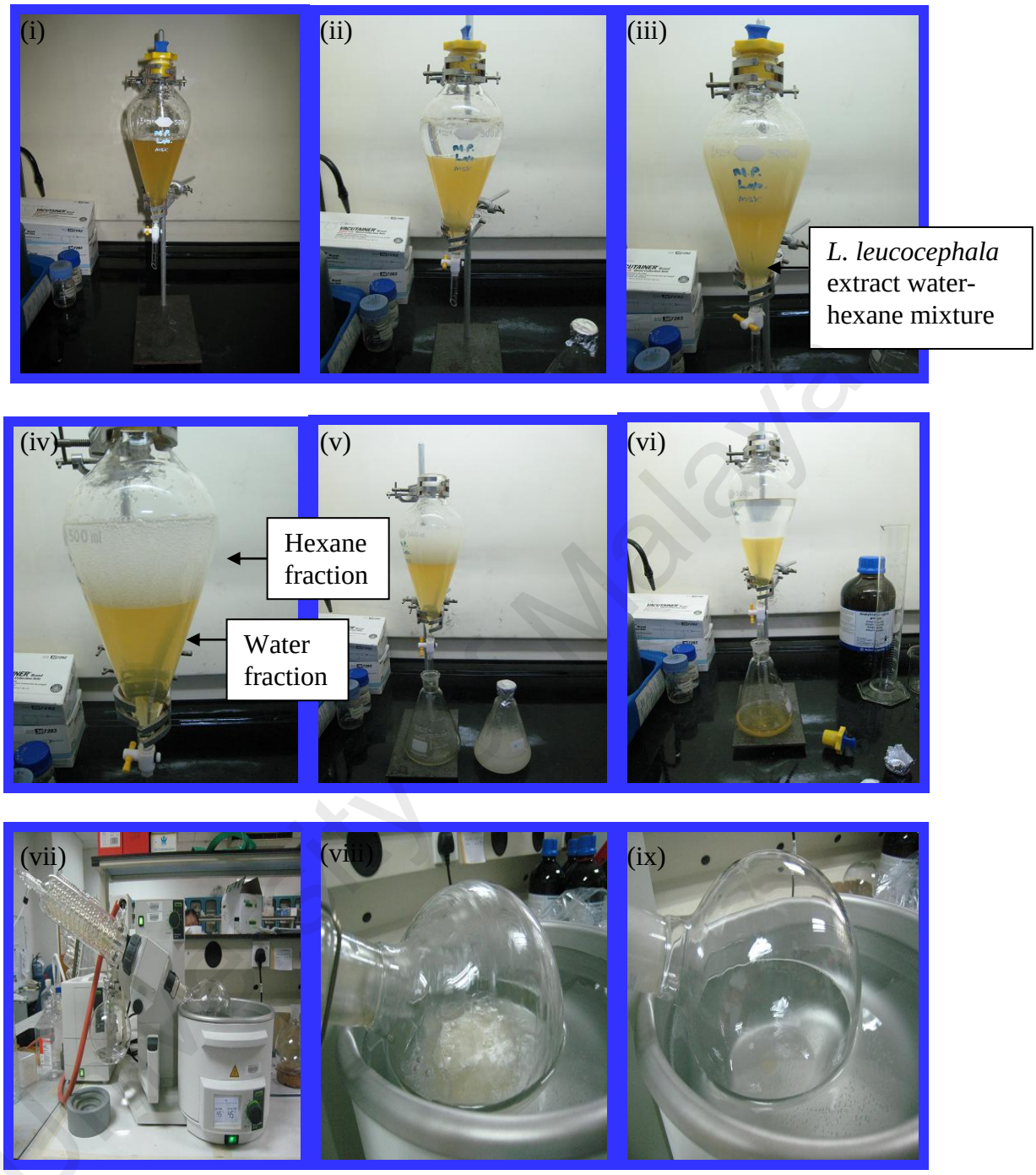


Figure 3.2: Solvent extraction of bioactive compounds.

(i) *L. leucocephala* extract dissolved in Millipore water was poured into the separator funnel. (ii) An equal volume of hexane was added. (iii) The *L. leucocephala* extract-hexane were mixed well by shaking the separator funnel several times. (iv) Two separate bands were clearly seen after 30 minutes. (v) The different solvent bands were collected in different flasks. (vi) The water band were re-poured into separator funnel and same volumes of hexane were added, then step iii-v were repeated for thrice. The same procedures were repeated with ethyl acetate solvent. (vii) The products were rotary-evaporated to remove the solvent. (viii) The hexane product in rotary–evaporator flask. (ix) The ethyl acetate product in rotary-evaporator flask.

3.4 FOOD NUTRITION ANALYSES

3.4.1 Parameters and methods

The tests were done by Consolidated Laboratory Sdn Bhd. *L. leucocephala* crude aqueous powdered extract were subjected to food nutrition analyses and the parameters measured are depicted in *Table 3.3.*

3.4.2 Evaluation

The results obtained were compared with Universal Standard Recommended Daily Allowance (USRDA). Recommended Dietary Allowances (RDA), the daily dietary intake level of a nutrient considered sufficient by the Food and Nutrition Board to meet the requirements of nearly all (97- 98 %) healthy individuals in each life-stage and gender group. It is calculated based on the Estimated Average Requirement (EAR) and is usually approximately 20 % higher than the EAR. The RDA is used to determine the Recommended Daily Value (RDV) which is printed on food labels in the U.S. and Canada. There is no RDA specifically for people with diabetes. Diabetic patient have to modify their diet.

Table 3.3: Parameters tested and methods used in food nutrition analyses.

Parameter	Unit	Method
Carbohydrate	g/100 g	By calculation
Protein	g/100 g	Kjeldahl method
Total fat	g/100 g	Solvent extraction
Dietary Fiber	g/100 g	AOAC 985.29
Trans fatty acid	g/100 g	GC
Vitamin A	mg/100 g	HPLC
Vitamin B_1	mg/100 g	HPLC
Vitamin B_2	mg/100 g	HPLC
Vitamin B_3	mg/100 g	HPLC
Vitamin C	mg/100 g	HPLC
Vitamin E	mg/100 g	HPLC
Sodium	mg/100 g	ICP-OES
Calcium	mg/100 g	ICP-OES
Magnesium	mg/100 g	ICP-OES
Zinc	mg/100 g	ICP-OES
Potassium	mg/100 g	ICP-OES
Manganese	mg/100 g	ICP-OES
Selenium	mg/kg	ICP-OES

Abbreviations:

AOAC stands for Association of Official Agricultural Chemists, where an enzymatic-gravimetric method for analyzing total dietary fibre was adopted from. GC stands for gas chromatography, HPLC stands for high performance liquid chromatography and ICP-OES stands for inductively coupled plasma atomic emission spectroscopy.

Table 3.4: Recommended Dietary Allowances (RDA).

Parameter	**Unit**	**RDA** (RDA for males and females aged 40-50 years) **Male**	**Female**
Carbohydrate	g/100 g	130 g/day	130 g/day
Protein	g/100 g	56 g/day	46 g/day
Total Fat	g/100 g	20–35 % of calories	
Fiber	g/100 g	38 g/day	25 g/day
Trans Fatty acid	g/100 g	nil	nil
Parameter	**Unit**	**RDA** (RDA for an average healthy 19-50 year old male / female) **Male**	**Female**
Vitamin A	mg/100 g	900 µg	700 µg
Vitamin B_1	mg/100 g	1.2 mg	1.1 mg
Vitamin B_2	mg/100 g	1.3 mg	1.1 mg
Vitamin B_3	mg/100 g	16 mg	14mg
Vitamin C	mg/100 g	90 mg	75 mg
Vitamin E	mg/100 g	15 mg	
Sodium	mg/100 g	1500 mg	
Calcium	mg/100 g	1000 mg	
Magnesium	mg/100 g	400- 420 mg	310- 320 mg
Zinc	mg/100 g	11 mg	8 mg
Potassium	mg/100 g	4700 mg	
Manganese	mg/100 g	2.3 mg	1.8 mg
Selenium	mg/kg	55 µg	

3.5 Adipocyte Cell Culture

3.5.1 Cell Culture, Isolation and Differentiation

Male *Sprague-dawley* rats of 6-8 weeks were sacrificed humanely by cervical dislocation. Primary preadipocytes were harvested from the intra-abdominal fat pads and epididymal fats under sterile condition (*Figure 3.3*) (Bjorntorp, et al., 1978). The fat tissues were cut into smaller fragments, followed by collagenase digestion at 37 ˚C for an hour in a water-bath. After that, it was centrifuged to separate mature adipocytes and preadipocytes. The pelleted preadipocytes were re-suspended and cultured in RPMI-1640 supplemented with 2 mM L-Glutamine, 100 units/ml Penicillin-Streptomycin-, 2 mM amphotericin B and 5 % FBS. Preadipocytes were seeded into 12-well plates and chemically-induced to mature adipocytes. The medium was changed every 2-3 days until confluent state was reached. Induction of differentiation was done on Day 0 (confluence) using DMEM I supplemented with 2 mM Penicillin-streptomycin-glutamine, 2 mM amphotericin B, 10 % FBS, 33 µM biotin, 17 µM panthothenic acid, 10 µM troglitazone, 0.5 mM IBMX, 1.0 µM dexamethasone and 10 µg/ml insulin. From Day 2 onwards, cells were cultured in similar medium, but free of troglitazone, IBMX and dexamethasone until full differentiation was obtained (Madsen, et al., 2003) as its prolonged exposure will produce cell toxicity and lipolysis. Experiments were carried out on Day 9- Day 12 where there was optimum differentiation to mature adipocytes indicated by development of large lipid globules in cytoplasmic region of the cells. The development of preadipocytes to mature adipocytes were shown in *Figure 3.4*.

3.5.2 Cell culture treatments

On day 12 after the initiation of differentiation, the spent media was aspirated and replaced with media containing treatment compounds. The cells were incubated with *L.leucocephala* dry fruit water crude extract at concentration of 10 μg/ml. Cells treated with insulin 10 μg/ml act as a positive control. The cells was incubated for 24 hours before total RNA was extracted.

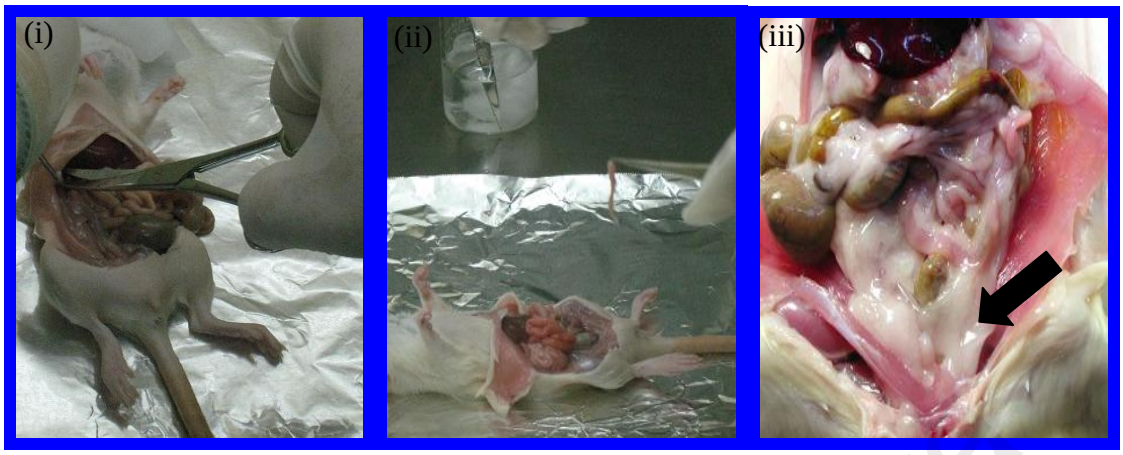


Figure 3.3: Rat dissection for collection of rat abdominal fat tissue.

Harvesting of rat abdominal fats was done under sterile condition**.**

Figure iii clearly showed the abdominal fat indicated by black arrow.

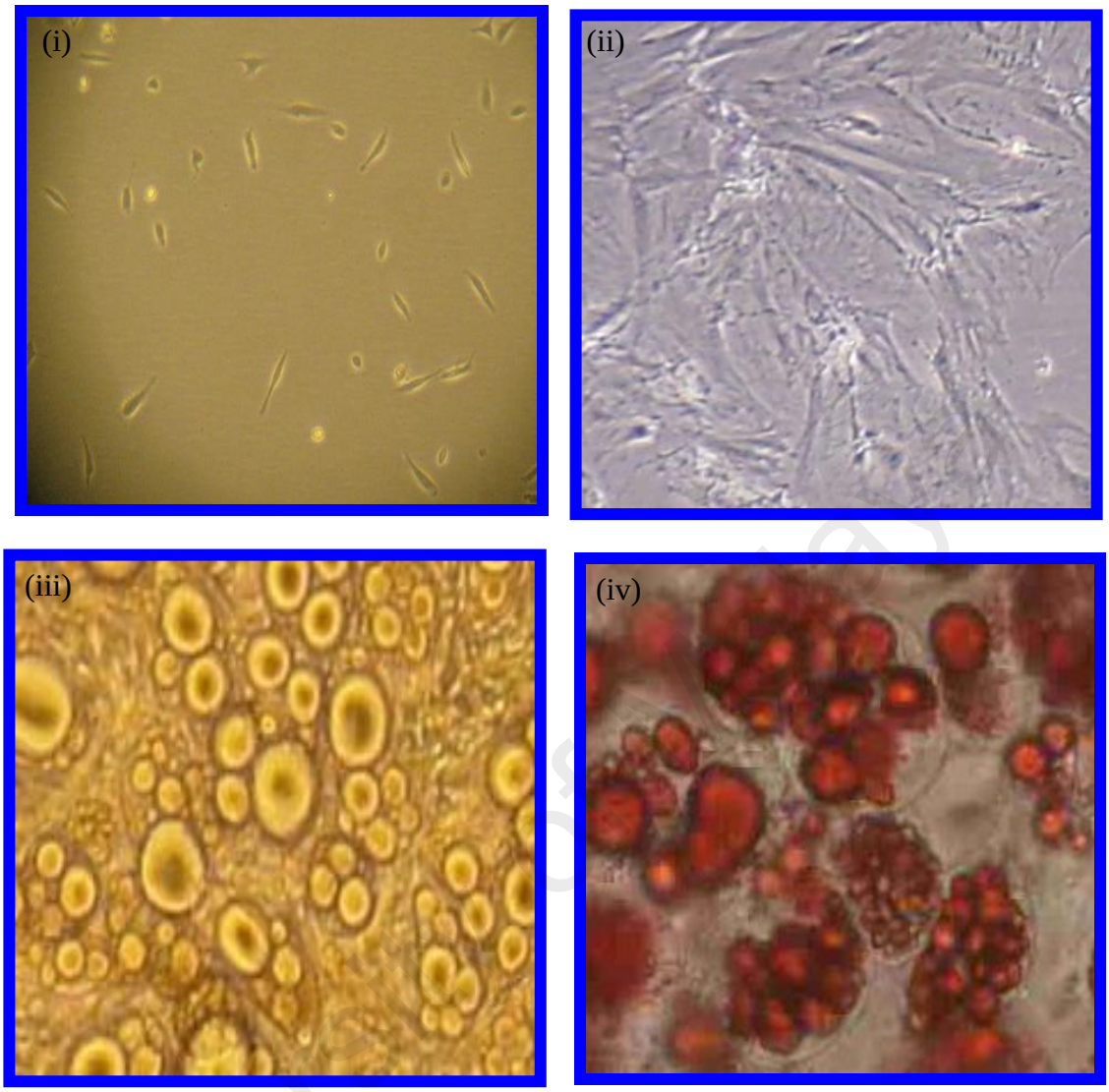


Figure 3.4: Adipocyte differentiation.

i) Preadipocytes (day 1).

ii) Confluent preadipocytes (day 5).

iii) Fully differentiated adipocytes (full of lipid) which appear yellowish (day 12).

iv) Fully differentiated adipocytes (full of lipid) which appear red with Oil Red O stain.

All photos were taken under 200 X magnification.

3.5.3 RNA extraction

3.5.3.1 Principle of the assay

RNA organic extraction is the process of purifying RNA (Ribonucleic acid) from a biological source. The method performed was Guanidinium thiocyanate-phenol-chloroform extraction using Trizol® reagent provided with the kit. The method was established by Piotr Chomczynski and Nicoletta Sacchi in 1987.

Ribonuclease enzymes present in the cells will destroy the RNA therefore, guanidium isothiocyanate was used to protect RNA from endogenous RNAses by denaturing the RNases and protein. The guanidium isothiocyanate also separates rRNA from ribosomes. This extraction using organic solvents resulted in separation into layers; aqueous layer which contains of RNA and inter-phase layer which contains of DNA and protein. Then, RNA was recovered from the aqueous phase by precipitation with 2-propanol or ethanol. The sample was then added into a spin column which binds nucleic acids. The column was washed and purified RNA material was eluted with water or buffers.

3.5.3.2 General procedures

RNA was extracted and purified by using a commercialized spin cartridge kit by Invitrogen (Product no.: 12183-018). The extraction and purification processes were performed as in the instruction protocol. Firstly, spent media was discarded and the cells were washed with PBS. The cells were detached used a cell scraper and was transferred into 50ml falcon tube and the flask was washed again with PBS to collect the remaining cells. Then, it was subjected to centrifuging at 784 xg for five minutes.

After centrifuging the supernatant was discarded. Cells were lysed and homogenized using guanidium isothiocyanate in proprietary acidic phenol reagent (lysis buffer). The homogenization was performed by draining it through a small-bore needle syringe (27G) several times. The lysate was transferred into a microcentrifuge tube and mixed with an equal volume of ethanol. The mixture was transferred into a spin column and centrifuged in a RNA spin cartridge at 12000 xg for 15 seconds. In this process, RNA binds to the silica base membrane in the cartridge meanwhile the impurities were washed out by with buffer. RNA was finally eluted out with water and collected in a recovery tube. The RNA was kept ice-cold to prevent any residual RNase activity that could degrade the RNA.The RNA purity was checked and was immediately subjected to reverse transcription.

3.5.3.3 Calculations

The purity of the RNA extracted is indicated by ($A_{260/280} \geq 1.8$) and this was checked using a spectrophotometer. The concentration of the RNA was calculated by the following formula.

Total RNA (μg) = A_{260nm} X 40 μg/ml X Dilution factor X Volume (ml)

3.5.4 Reverse transcription (RT)

3.5.4.1 Principle of the assay

Reverse transcriptase, also known as RNA-dependent DNA polymerase, is a DNA polymerase enzyme. Reverse transcription is a process of transcription of single-stranded RNA into complementary DNA (cDNA). Normal transcription involves the synthesis of RNA from DNA; hence, reverse transcription is the reverse of this.

Once the RNA has been reversed transcribed into a single strand complementary DNA (cDNA) it can help in the formation of a double helix DNA via Polymerase Chain Reaction (PCR). The process needs enzyme reverse transcriptase, known functional primers, random primers or in some cases polyT primers due to the presence of polyA tail in most transcribed RNAs.

3.5.4.2 General procedures

Reverse transcription was performed by using a commercialized kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Product no.: 4368814) (*Figure 3.5 i*). In every 20 µl reaction, two µg of total RNA was used and was mixed with an equal volume of the 2X reaction master mix as instructed in the user's protocol. The content of the master mix is depicted in *Table 3.5*. After mixing thoroughly, it was centrifuged to remove air bubbles (*Figure 3.5 ii*). The reverse transcription process were done in a thermalcycler, (*Figure 3.5 iii*) and the process was carried out at different temperatures and period as shown in *Table 3.6*. The cDNA product was stored under -20 °C until downstream analysis.

Table 3.5: Preparation and composition of 2X concentrated reaction master mix for reverse transcription.

Reaction master mix (2X)	
Component	**Volume (µl)**
10X RT buffer	2.0
25X dNTP mix (100 mM)	0.8
10X RT Random primers	1.0
Multiscribe reverse ™ transcriptase	1.0
Nuclease-free water	3.2
Total volume per reaction	10

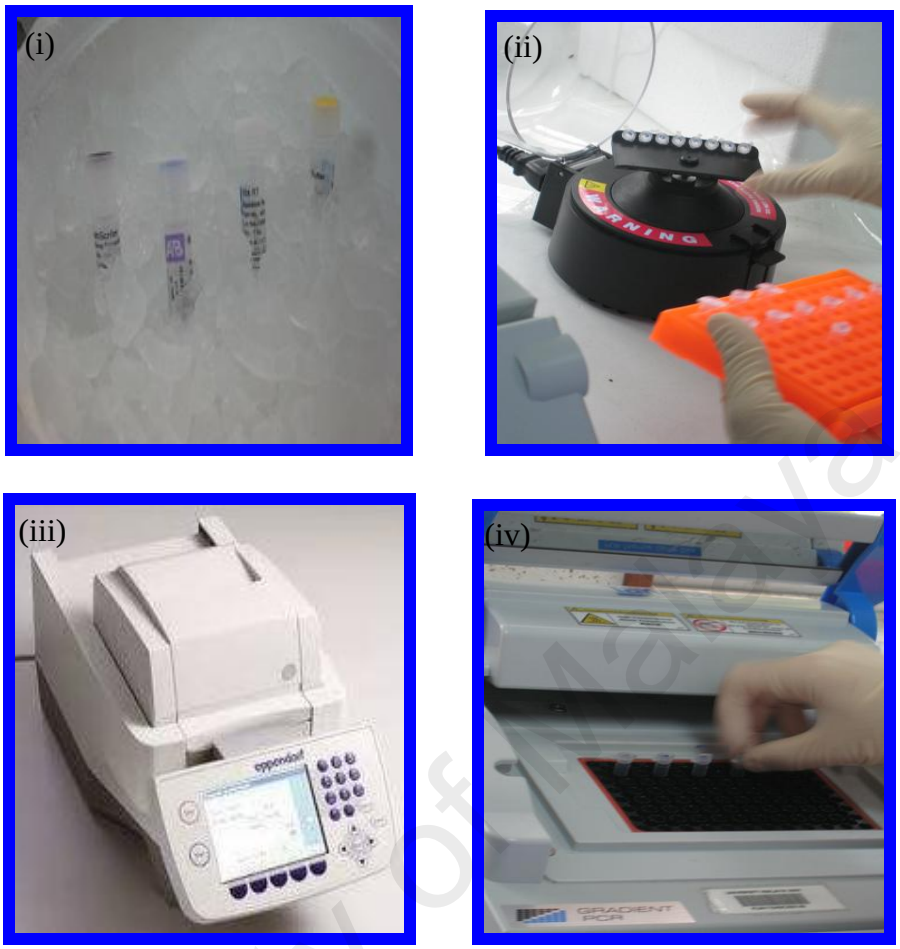


Figure 3.5: Reverse transcription process.

i) Components of master mix.

ii) The micro-centrifuge tubes containing RNA-master mix samples was centrifuged to remove air bubbles and accumulate the content at the bottom tube.

iii) Thermalcycler.

iv) Loading the micro-centrifuge tubes containing RNA-master mix samples into thermalcycler.

Table 3.6: Thermalcycler conditions for reverse transcription.

Step	**1**	**2**	**3**	**4**
Temperature (°C)	25	37	85	4
Time	10 minutes	120 minutes	5 seconds	Hold

3.5.4.3 Calculations

The quantity of the cDNA was not quantified prior to RT-PCR.

3.5.5 Gene expression assay

3.5.5.1 Principle of the assay

3.5.5.1.1 TaqMan® chemistry

Polymerase Chain Reaction (PCR) is a precious tool for measuring gene expression. PCR amplifies target DNA sequences present in small quantities therefore generating many copies of a specific DNA sequence through a series of reaction *in vitro*. Basically, PCR involves three steps; denaturing of double-stranded DNA whereby the two strands of DNA template are separated from one another at 99 °C; annealing of primers where a hybridization of the oligonucleotide primer is carried out on the single-stranded DNA template occur at 55 °C and extension of the primers by heat stable DNA polymerase; elongation until a double-stranded DNA at the optimal temperature for *Taq* polymerase which is 72 °C. Each set is referred to as a cycle. This process occurs along both strands of the single-stranded DNA template therefore DNAs is doubled in the course of one cycle. If this cycle is repeated, a fourfold quantity will be obtained. With the advanced technologies available such as Quantitative Real-Time PCR (qRT-PCR) and fluorogenic probes, highly sensitive and specific, rapid quantification of amplicon is easier.

TaqMan® or 5′nuclease assay is a heat-stable DNA polymerases from *Thermus aquaticus,* a eubacterium that grows in the high temperatures of aquatic hot springs. TaqMan® utilize the 5' to 3' exonuclease activity of Taq DNA polymerase concept. TaqMan® dye-labeled probe is designed to anneal the target sequence in forward and reverse primers. The probe contains a fluorescent dye that is attached to the probe's 5′ end therefore called 5' reporter dye and a quencher dye attached to the probe's 3′ end which absorbs the energy from the light source used to excite the reporter dye. When the reporter and the quencher are linked to each other *via* intervening probe, quencher suppresses the fluorescence of the reporter dye. During amplification, TaqMan® cleaves the probe and detaches it from template, allowing extension to continue. This cleavage separates the reporter dye and quencher dye resulting in an increase in fluorescence. The increased fluorescence only occurs if the target sequence is amplified and is complimentary to the probe, thus preventing detection of non-specific amplification. The fluorescence signal is directly proportional to the quantity of initial copy which is generated in proportion to the amount of amplicon in each cycle of amplification. Therefore, the relative amount of template can be determined. Thus, higher copy number templates will cross a fluorescence detection threshold before lower copy templates. TaqMan® probes used in this study are labeled with VIC/MGB The TaqMan® chemistry reaction is simplified in *Figure 3.6.*

Two assumptions were considered, the endogenous control used must not vary in number of copies or expression level under different experimental conditions and amplification efficiency of the genes is assumed 100 % if the $\Delta\Delta C_T$ method is used to evaluate relative expression (Livak & Schmittgen, 2001).

3.5.5.1.2 Relative expression

Relative quantification determines fold difference of a target nucleic acid in a starting material with statistical confidence. The relative expression of each gene is obtained from comparison with endogenous control or housekeeping genes which act as genetic normalizer. Genetic normalizer is used to offset variations in sample mass and/or metabolism occurs in the assay (*e.g.*, cell number, total mass DNA/RNA, volume *etc*,.).

Eukaryotic18S rRNA (for *Rattus norgevicus* samples; Product no.: 4319413E; VIC/MGB probe) was the endogenous control used in this study purchased from Applied Biosystems. 18S ribosomal RNA (rRNA), a highly abundant post-transcriptional-regulated product of the ribosomal genes, has great predictability as an internal control (Bonini & Hofmann, 1991). 18S rRNAs are better as loading controls than gene expression controls because they are products of RNA polymerase I. These RNA polymerases show varying sensitivities to different xenobiotics; it is thus possible that an experimental manipulation could cause an across-the-board arrest of RNA polymerase II-transcription, while the RNA polymerases I and III continue to transcribe at near-control levels.

In this assay system, amplification of genetic template, cycle by cycle, can be visualized in real time (*Figure 3.7*) allowing for precise and quantitative measurements during the exponential phase of PCR as it occurs. The comparative CT method, $2\Delta\Delta CT$ was used to calculate relative changes in gene expression.

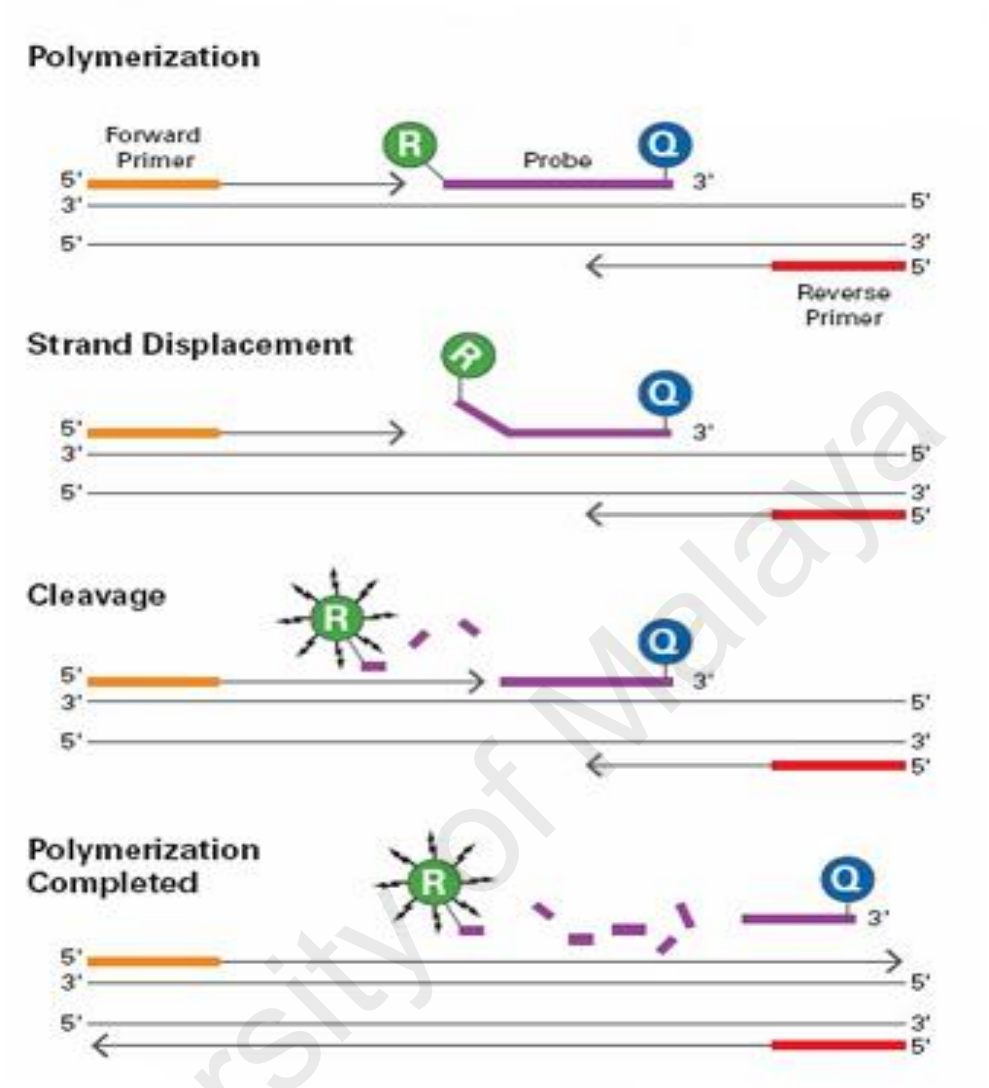


Figure 3.6: TaqMan® gene expression assay chemistry.

("AB TaqMan® Gene Expression Services,")

In polymerization, the fluorescent reporter (R) dye and a quencher (Q) of a TaqMan® probe anneal to the 5' target sequence within the forward and reverse PCR primers. In Strand displacement the reporter dye emission is quenched when the probe is intact. Cleavage occur during each extension cycle, where the Taq polymerase cleaves the reporter dye from the probe. Upon completion of polymerization, reporter dye is separated from the quencher, and this emits fluorescence signal.

3.5.5.2 General procedures

An experimental Design Wizard in StepOne software (Ver. 2.0, Applied Biosystems) was used in setting up real-time PCR experiment for the entire process, including designing the experiment and reactions mix. After all the reaction mix prepared as in *Table 3.5* and assay mix prepared, it was transferred into fluorescence-compatible fast reaction tubes / strips (MicroAmp™; Product no.: 4358293) and capped with optical caps (Product no.: 4323032). Negative control was prepared using sterile ultra-pure water to replace the assay mix. Each reaction was run in triplicate. All reagents were kept on ice, and thawed only once before use. The strips were centrifuged briefly before loading into the real time PCR thermal cycler (StepOnePlus™ Real Time PCR system) *(Figure 3.8).*

3.5.5.2.1 Genes studied

Table 3.7 shows the list of genes and its accession numbers chosen in this gene expression study.

3.5.5.3 Calculations

The comparative CT (ΔΔCT) method is used to determine the relative target quantity in samples. Comparative CT experiments are commonly used to compare expression levels of a gene in different tissues, in a treated sample vs. an untreated sample and levels of wild-type alleles vs. mutated alleles. It describes the change in expression of the target gene in a test sample relative to a reference sample (calibrator sample). StepOne software measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the

endogenous control (18S rRNA). The software determines the relative quantity of target in each sample by comparing normalized target quantity in each sample to normalize target quantity in the reference sample. This involves comparing the C_t values of the samples of interest with a control (non-treated sample). The C_t values of both the control and the samples of interest are normalized to 18S rRNA. The calculations and formulas involved were as follows;

$$C_{T\ Target} - C_{T\ Endogenous\ control} = \Delta C_T$$

$$\Delta C_{T\ Sample} - \Delta C_{T\ Calibrator} = \Delta\Delta C_T$$

$$\text{Relative fold change} = 2^{-\Delta\Delta CT}$$

Cycle threshold number (C_T) is the calculated fractional cycle number at which the PCR product crosses a threshold of detection. ΔC_t value for any sample normalized to the endogenous housekeeping gene. $\Delta C_{t,\ reference}$ is the C_t value for the calibrator also normalized to the endogenous housekeeping gene. C_T is in exponential relationship to quantity.

Calculation is valid if the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how ΔC_t varies with template dilution. If the plot of cDNA dilution versus delta C_t is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, then the standard curve method is preferred.

Results were expressed as n-fold difference over respective controls. Fold variation values less than 0 were expressed as negative values (*e.g.*, a n-fold variation of 0.50 is expressed as -2.00). For statistical analyses of real time RT-PCR experiments, gene expression results were expressed as difference from the ΔC_T value obtained between treated versus untreated.

Table 3.7: Genes investigated.

No.	Gene name (Target) and abbreviation	Assay ID	Accession number
1	Protein kinase B (Akt)	Rn00583646_m1	NM_033230.1
2	Glucose transporter 4 (GLUT4)	Rn00562597_m1	NM_012751.1
3	Hormone sensitive lipase (HSL)	Rn00689222_m1	NM_012859.1
4	Phosphatidylinositol 3-kinase (PI3K)	Rn00564547_m1	NM_013005.1
5	Sterol regulatory element binding protein 1c (SREBP1)	Rn01495772_g1	AF286470.2

General abbreviation of genes selected for this study and corresponding assay ID and accession number. All corresponding assay ID and accession number information available on Applied Biosystems website. Assay ID refers to the Applied Biosystems Gene Expression Assay kits with patented primer and TaqMan® probe mix. Assay ID with "Rn" prefix is defined as "*Rattus norvegicus*". All Gene Expression Assay kits listed here are FAM/MGB probed.

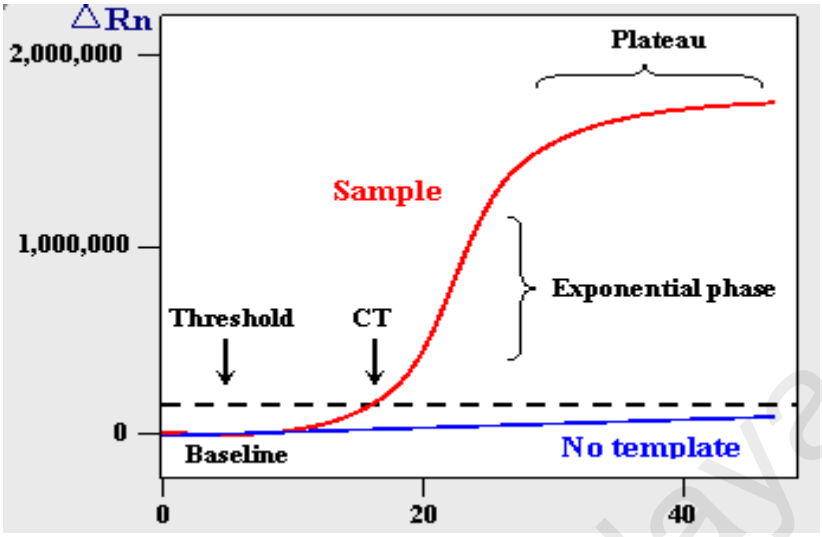


Figure 3.7: Amplification plot of real time PCR.

Figure shows three phases of PCR product growth that are exponential, linear and plateau in which each sample undergo. Baseline indicated the initial cycle of PCR where at this stage little change in fluorescence signal was observed. Threshold reflects a statistically significant point above the calculated baseline. Threshold cycle (CT) is the calculated fractional cycle number at which the PCR product crosses a threshold of detection.

Figure 3.8: StepOnePlus, a Real Time PCR machine from Applied Biosystems.

3.6 Rats model

Healthy male *Sprague-dawley* rats aged 8-10 weeks were obtained from the Animal house, Faculty of Medicine, University of Malaya . The animals were housed in cages, maintained under standard conditions (12 hour light; 12 hour dark cycle; 22 ± 27 °C; 35-60 % humidity). The animals were fed with standard rat pellet diet and water. The rats were divided randomly into several group according to *Table 3.8, Table 3.9* and *Table 3.10* for the various experiments. Each group consisted of six rats. Each rat that weighed between 225-250 g was housed separately (one rat per cage) (*Figure 3.9 i*). [Note: The ethics approval number was PM/02/01/2008/MAA (R). All animal experiments were conducted humanely according to *Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research* 2010 developed by American Psychological Association Committee.

3.6.1 Induction of diabetes mellitus with Streptozocin

Diabetes were induced in an overnight fasted rats by a single intraperitoneal injection of 50 mg/kg Streptozocin (STZ). Streptozocin was freshly prepared by dissolving STZ powder in 0.1M Sodium Citrate Buffer; pH 4.5 (Brosky & Logothetopoulos, 1969; R. K. Gupta et al., 2005; Jeong-Sook, 2005) (*Figure 3.9 ii*). The STZ solution (volume according to body weight) was injected within 10 minutes (*Figure 3.9 iii*). Hyperglycemia was confirmed by the elevated glucose level in the blood, determined at 72 hours after injection (Pari, 2007; Prasad, Alka Kulshreshtha, & Qureshi, 2009) and then on day seven after injection (Pari, 2007). The rats with fasting blood glucose of between 6 and 18 mmol/L were used for anti-diabetic studies. The diabetic rats were subdivided into mild diabetic rats that had glucose range between 6 mmol/L-13.88 mmol/L and severely diabetic rats that had a range of more than 13.88 mmol/L. (Achyut, et al., 2006; R. K. Gupta, et al., 2005).

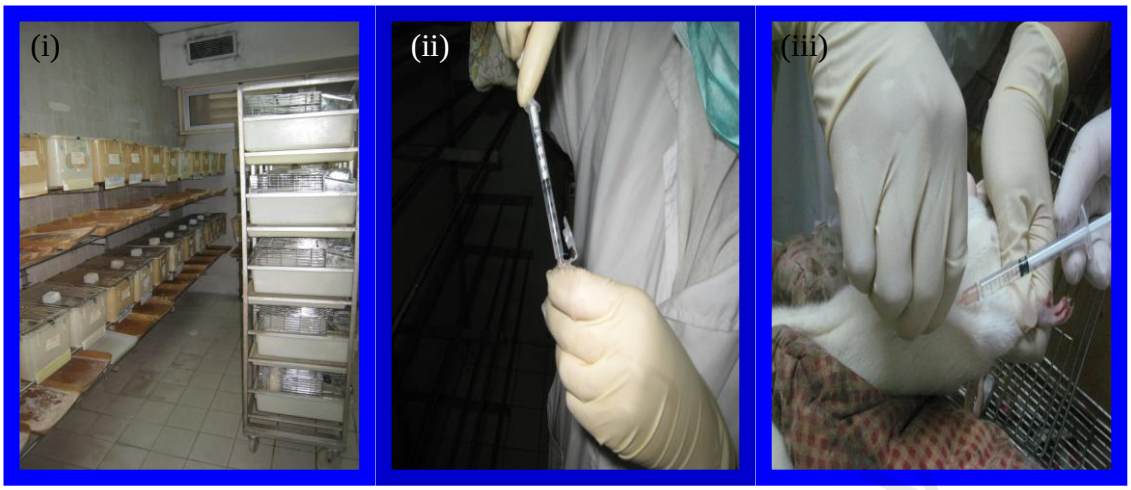


Figure 3.9: Rats caging and induction of diabetes mellitus in rats.

i) Each rats was housed separately (one rat per cage).

ii) Streptozocin was freshly prepared by dissolving STZ powder in 0.1M Sodium citrate buffer; pH 4.5.

iii) The freshly prepared STZ solution was injected intraperitoneally within 10 minutes.

3.7 Screening *via* Oral Glucose Tolerance Test

3.7.1 Oral Glucose Tolerance Test (OGTT) in STZ-diabetic rats and normal rats

The diabetic rats were allowed to fast overnight at least for 10 hours. The next morning, blood glucose was measured (0 minute). Then, the diabetic rats were intragastric fed with aqueous plant extracts at a dose of 500 mg/kg (preliminary studies using a dose 250 mg/kg showed no significant effect) or Glibenclamide (positive control) (5 mg/kg b.w) (A. Kumar et al., 2008; Meenakshi, et al., 2010). Sterile distilled water 4 ml/kg per body weight was given to normal healthy rats as negative control. The blood glucose was measured 30 minutes after feeding, and then the rats were fed with glucose (2 g/kg) per body weight (Ortiz-Andrade, et al., 2008). Blood glucose was measured again at 60, 90 and 120 minutes after glucose feeding (Diniz et al., 2008). Note: All the extracts have been confirmed to be non-toxic in preliminary experiments conducted previously (results not shown).

The same procedure was carried out using normal rats to make a comparison on effects of extracts. The steps involved in Oral Glucose Tolerance Test procedure are outlined in *Figure 3.10.*

Table 3.8: Animal grouping for Oral Glucose Tolerance Test (OGTT) in STZ-diabetic rats and normal rats.

Group	Treatment (n=6/group)
A	Normal rats + Sterile distilled water (4 ml/kg)
B	Normal rats + *LL* (500 mg/kg)
C	Normal rats + Glibenclamide (5 mg/kg)
D	Diabetic + Sterile distilled water (4 ml/kg)
E	Diabetic + *LL* (500 mg/kg)
F	Diabetic + Glibenclamide (5 mg/kg)

Abbreviations:

LL stands for *Leucaena leucocephala* dry fruit water crude extract and n stands for number of rat.

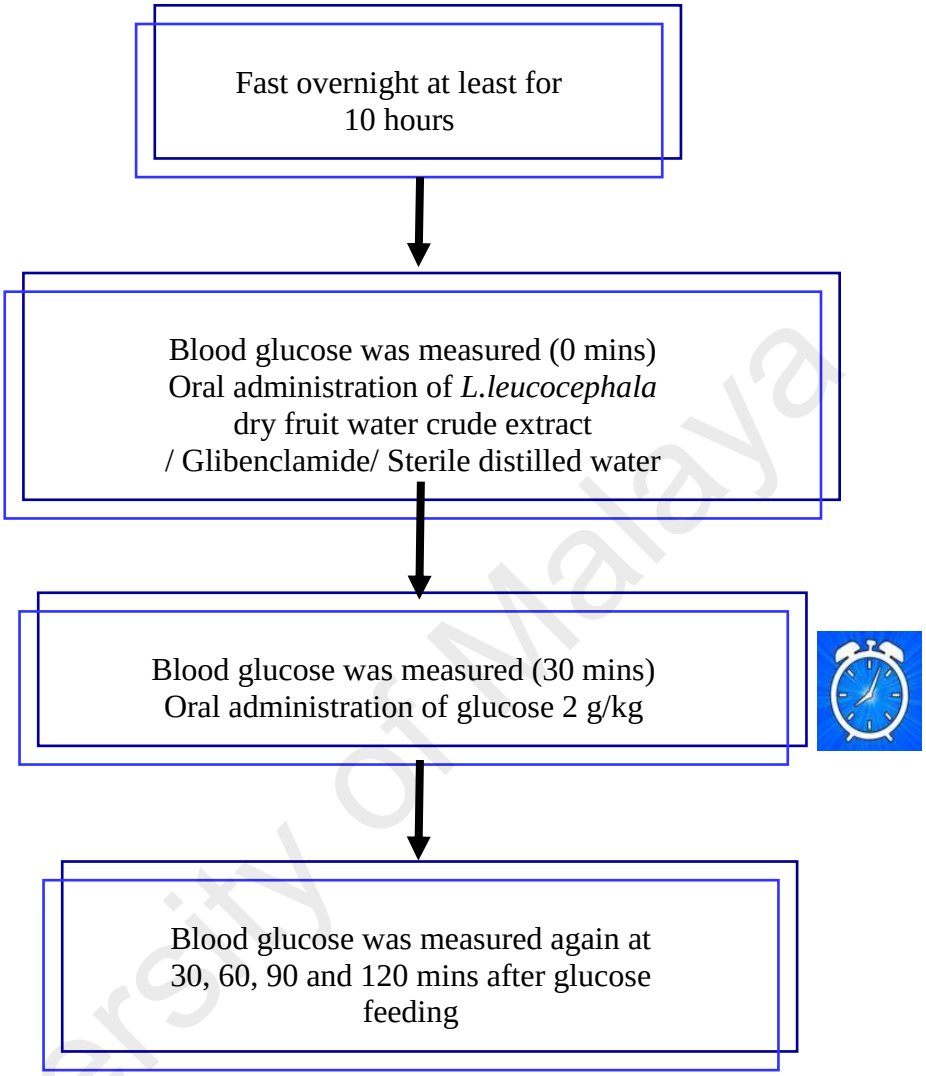


Figure 3.10: Schematic overview of Oral Glucose Tolerance Test (OGTT) in STZ-diabetic rats and in normal rats.

3.8 Assessment of *Leucaena leucocephala* extract for

3.8.1 Chronic study

L. leucocephala dry fruit water crude extract were tested for *in vivo* anti-diabetic and antioxidant properties. The STZ-induced diabetic rats were given treatment (extract) once daily by intragastric tube for 30 days (Guerrero-Analco et al., 2007) as *Table 3.9.*

3.8.2 Sub-chronic study

L. leucocephala partially purified extracts (water-hexane, water-ethyl acetate and water-aqueous extracts) were further assessed for anti-diabetic properties for seven days in STZ-diabetic rats at two doses of 50 mg/kg and 25 mg/kg as *Table 3.10.* This step would be necessary to confirm the efficacy of the purified extract as an anti-diabetic agent in the rat model.

Table 3.9: Animal grouping for anti-diabetic and antioxidant study of *Leucaena leucocephala* dry fruit water crude extract.

Group	Treatment (n=6/group)
A	Normal Control
B	Diabetic + Sterile distilled water (4 ml/kg)
C	Diabetic + *LL* (250 mg/kg)
D	Diabetic + *LL* (500 mg/kg)
E	Diabetic + Glibenclamide (1.25 mg/kg)

Abbreviations:

LL stands for *Leucaena leucocephala* dry fruit water crude extract and n stands for number of rat.

Table 3.10: Animal grouping for anti-diabetic and antioxidant study of *Leucaena leucocephala* partially purified water-solvents extracts.

Group	Treatment (n=6/group)
A	Normal Control
B	Diabetic + 0.9 % Normal Saline (4 ml/kg)
C	Diabetic + *LL* water-hexane partially purified extract (25 mg/kg)
D	Diabetic + *LL* water-ethyl acetate partially purified extract (25 mg/kg)
E	Diabetic + *LL* water-aqueous partially purified extract (25 mg/kg)
F	Diabetic + *LL* water-hexane partially purified extract (50 mg/kg)
G	Diabetic + *LL* water-ethyl acetate partially purified extract (50 mg/kg)
H	Diabetic + *LL* water-aqueous partially purified extract (50 mg/kg)
I	Diabetic + Glibenclamide (1.25 mg/kg)

Abbreviations:

LL stands for *Leucaena leucocephala* dry fruit water crude extract and n stands for number of rat.

3.8.3 Sample preparations

After completion of treatment, the rats were sacrified humanly. The rats were anesthesized using diethyl ether (Schwetz & Becker, 1971) and subjected to cervical dislocation.

Serum was collected in sterile vacutainer tubes with no anticoagulant or additives. Fibrin was allowed to clot for 30 minutes, follow by low speed centrifugation at 4410 xg for five minutes. Aliquots of serum in micro-centrifuge tubes were stored at -70 °C for measurements of insulin and adiponectin levels.

The tissue samples of organs namely; liver, kidney, brain and pancreas (*Figure 3.11*) were placed in 50 ml Falcon tube containing 12.5 ml ice-cold 0.9 % sodium chloride as a rinsing buffer. The tissues were trimmed and weighed. To one gram of tissue, 8 ml ice-cold PBS buffer, 20 mM, pH 7.4, was added and then homogenized. The homogenates were centrifuged at 17640 xg for 15 minutes. Aliquots of the resulting supernatant were stored at -70°C. The *in vivo* antioxidant level and the extent of oxidative damage were measured using the following assays; ferric reducing antioxidant power, advanced oxidation protein product, malondialdehyde and glutathione peroxidase.

3.8.4 Biochemical analysis

3.8.4.1 Measurement of fasting blood glucose

At day 1, 7, 15 and 30 the fasting blood glucose concentrations were measured using an Accu-chek Advantage II Glucometer and compatible blood glucose test strips. Every time before using the Glucometer, it was calibrated with Accu- chek calibrator strips to make sure it is functioning well. The percentage of variation of glycemia was

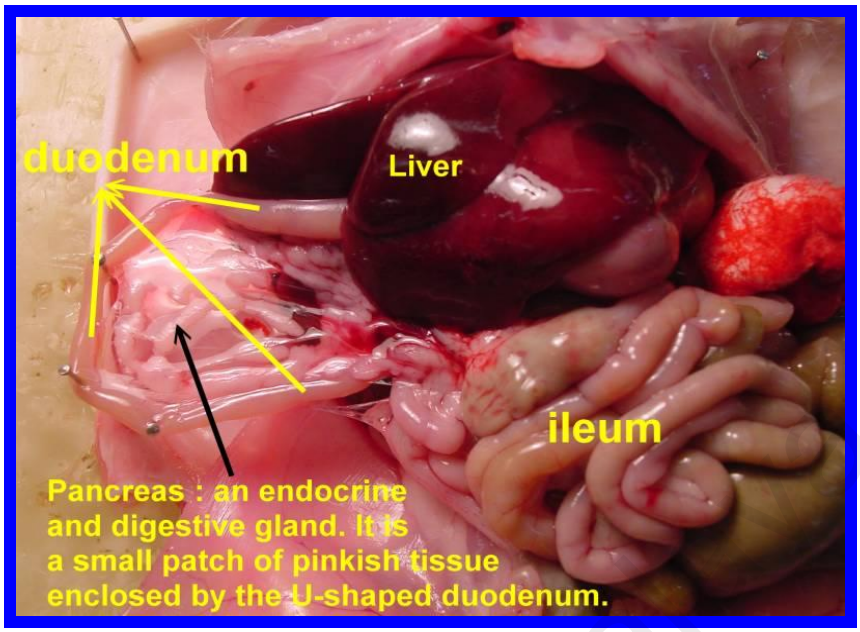
calculated (Ortiz-Andrade, et al., 2008) based on the formula below; where x were either day 7, 15 or 30.

$$\% \text{ Variation of glycemia} = \frac{\text{Day}_x - \text{Day}_1}{\text{Day}_1} \times 100$$

3.8.4.2 Measurement of body weight before and after treatment

The body weight in gram of all the rats were measured before and after tests performed by using a digital weight scale. The changes in body weight was calculated using the following formula;

$$\frac{\text{Body weight on Day 30 - Body weight on Day 1}}{\text{Body weight on Day 1}} \times 100$$



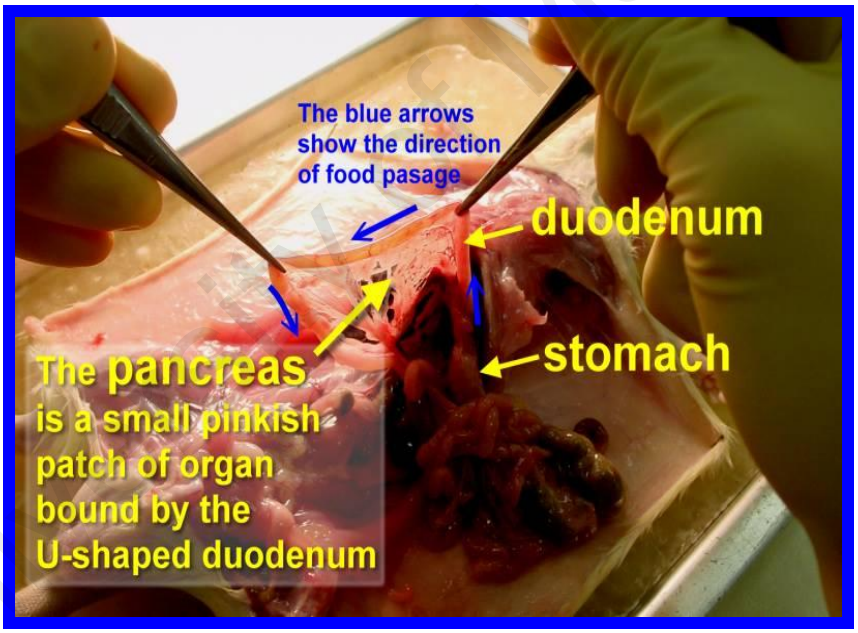


Figure 3.11: Location of rats pancreas.

(illustration cited from (cccmkc, 2006))

3.8.4.3 Measurement of Serum Insulin Level

3.8.4.3.1 Principle of the assay

The insulin assay is based on sandwich ELISA principle. The insulin antigen from the serum samples will bind to the rat capture antibody, pre-coated in the test wells. Antibodies added subsequently, bind the antigen and this is followed by the addition of enzyme-linked secondary antibody which binds to the detecting antibody. Lastly, addition of substrate commences enzyme reaction that leads to formation of product that is detectable.

3.8.4.3.2 General procedure

Insulin test was performed by using Rat Insulin ELISA kit from Linco Research (USA). All the reagents were pre-warmed at room temperature prior to assay. Each well of the microtiter plate was washed thrice with 300 µl of diluted wash buffer. The residual washed buffer was removed carefully by inverting the plate and tapping it gently. Then, 10 µl Assay Buffer was added into the wells, followed by 10 µl Matrix Solution. Rat Insulin Standards (0.2, 0.5, 1, 2, 5, 10 ng/ml), controls and samples 10 µl each were added into the respective wells. Detection antibody 80 µl was added to each well and the plate was sealed and incubated at room temperature for two hours on a micro-titer plate shaker 400-500 rpm. After the incubation, solutions were aspirated from the wells and any residual solution was removed by gentle tapping. This was followed by rinsing thrice with diluted wash buffer. Enzyme Solution (100 µl) was added to each well and the plate was sealed and incubated at room temperature for 30 minutes on micro-titer plate shaker with moderate shaking. After incubation, solutions were aspirated from the plate and any residual solution was removed by gentle tapping.

The coated wells were washed six times with diluted wash buffer. Substrate Solution (100 µl) was added to each well, the plate was sealed and shaken on micro-titer plate shaker for 15 minutes and at this stage blue color formed in standard wells and this changed to yellow after the addition of 100 µl Stop Solution. The absorbances were read at 450 nm within five minutes. All samples were analyzed in triplicate in a single run.

3.8.4.3.3 Calculation

A standard curve of insulin, absorbance versus concentration (ng/ml) was plotted (*Appendix B i and ii*) and the concentration of test samples was determined using the standard curve.

3.8.4.4 Measurement of Adiponectin Level

3.8.4.4.1 Principle of the assay

Adiponectin test was performed by using Rat Adiponectin ELISA assay kit from BioVision Research Products (USA). Poly-clonal antibody specific for rat adiponectin is allowed to coat the wells. Adiponectin present in samples binds to the antibody. The bound adiponectin is then captured by anti-rat adiponectin monoclonal antibody. The addition of Horse Reddish Peroxidase conjugated rat IgG and substrate developes a color which can be measured spectrophotometrically.

3.8.4.4.2 General procedure

The reagents were brought to room temperature and were prepared according to the manual provided with the kit. Standards concentrations (0, 0.375, 0.75, 1.5, 3, 6, 12, 24 ng/ml) were prepared using the standard adiponectin and diluent provided. The samples were diluted to dilution factor 1000 using the diluent. Standards or control or

sample (100 µl) were added into its respective wells. The plate was incubated at 37 °C for an hour. Solution was removed and the coated wells were washed thrice with 250 µl of wash solution. Secondary antibody (100 µl) was added and the plate was incubated again at 37 °C for an hour. The solution was removed and the plate was washed again five times. Detector (100 µl) was added to each well and was incubated again at 37 °C for an hour. The solution was removed and the plate was washed again five times. 100 µl of substrate solution was added to each well and the plate was incubated in the dark at room temperature for 20 minutes. After the incubation, 100 µl of stop solution was added to each well. The absorbance was read at 450 nm.

3.8.4.4.3 Calculation

A standard curve of adiponectin absorbances versus concentration was plotted (*Appendix C i and ii*). The adiponectin concentration of samples were calculated using quadratic equation derived from interpolation of the regression curve. The adiponectin concentration calculated were multiplied with the dilution factor to obtain the original concentration of samples.

3.8.5 Antioxidant assays

The tissue homogenates were diluted appropriately liver 40X, kidney 20X, pancreas 10X and brain 10X prior to the antioxidant assays.

3.8.5.1 Ferric Reducing Antioxidant Power (FRAP) Assay

3.8.5.1.1 Principle of the assay

FRAP assay is a well-known method for measuring the total antioxidant power. The ferric was reduced to ferrous ion at low pH that gives rise to the development of a

blue colored complex which has maximum absorption at 593 nm (Benzie & Strain, 1996). This color development indicates that a reductant (antioxidant) is present in the sample. Hence, the color intensity of FRAP is linearly related to the molar concentration of the total antioxidants present in the sample. The absorbance of each sample was compared and calculated with the standard curve obtained from iron (II) sulphate-heptahydrate ($FeSO_4.7H_2O$) reaction with the FRAP reagent.

$$Fe^{2+}\ TPTZ \longrightarrow Fe^{3+}\ TPTZ$$

3.8.5.1.2 General Procedure

The FRAP assay was performed in 96 well plate. Sample (10 µl) was added to 300 µl of FRAP reagent. The reagent comprising of sodium acetate buffer (300 mM, pH 3.6) tripyridyltriazine or TPTZ (10 mM in 40 mM HCL) and ferric chloride (20 mM) was mixed in a ratio of 10:1:1. Sodium acetate buffer was prepared using sodium acetate trihydrate, glacial acetic acid and distilled water. The reaction was allowed to proceed at 37 °C under kinetic mode. Absorbance reading was taken at zero and fourth minute at 593 nm using a micro-plate reader. All samples were analyzed in triplicate in a single run.

3.8.5.1.3 Calculation

The absorbance difference was calculated as follows:

$$\frac{(Abs_{sample}-Abs_{blank})\ \text{at 4 minutes} - (Abs_{sample}-Abs_{blank})\ \text{at 0 minute}}{Abs_{standard}} \times [\text{standard}]$$

Determination of reductant concentration was determined from the standard curve of ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) solutions (0, 200, 400, 600, 800, 1000 µM) (*Appendix D i and ii*). The FRAP values of homogenate samples were expressed in µmol mg^{-1} protein while the plant extracts were expressed as µM in 1mg extract.

3.8.5.2 Antioxidant Protein Product (AOPP) Assay

3.8.5.2.1 Principle of the assay

Advanced oxidation protein products assay was conducted as described by Witko-Sarsat *et.al.,* (1996). AOPPs are formed during oxidative stress by the action of chlorinated oxidants, mainly hypochlorous acid and chloramines which are produced by myeloperoxidase in activated neutrophils (Witko-Sarsat, et al., 1996).

3.8.5.2.2 General Procedure

Sample (18 µl) was added to 200 µl reagent into a 96 well micro-plate. The reagent contained phosphate buffered saline, 50 % acetic acid and 1.16 M potassium iodide solution in 81:15:4 ratio. Absorbance reading was taken using micro-plate reader at 340 nm. Chloramine-T solutions of known concentrations (0, 100,200, 300, 400, 500 µM) (*Appendix E i and ii*) were used as standards for comparison. The amount of damaged protein (AOPP) present in samples was expressed in chloramines unit (µM).

3.8.5.2.3 Calculation

The sample value was calculated as follows:

$$\frac{Abs_{sample}}{Abs_{standard}} \quad X \quad [Chloramine\ T_{standard}]\ (\mu M)$$

The AOPP levels of homogenate samples were expressed in µmol mg^{-1} protein.

3.8.5.3 Lipid Peroxidation (MDA) Assay

3.8.5.3.1 Principle of the assay

This assay was based on the formation of thiobarbituric reactive substances (TBARS) which absorbs at 532 nm. The substance formed is a 2:1 of thiobarbituric acid

(TBA) and MDA. In the pre-treatment procedure, trichloroacetic acid (TCA) precipitation was used to produce a protein free extract and the heating procedure at 100 °C allowed the reaction between MDA and TBA to occur. The MDA-TBA_2 formation is initiated by the nucleophilic attack involving carbon-5 of TBA onto carbon-1 of MDA. This is followed by dehydration and finally the intermediate MDA-TBA is attacked by a second molecule of TBA in a similar reaction.

3.8.5.3.2 General Procedure

TCA (0.5 ml, 15% v/v) was added to 0.1 ml of sample in a microcentrifuge tube. The resulting precipitate was removed by centrifugation. The supernatant was transferred into a screw capped glass tube. TBA (1.0 ml, 1% w/v) was added and mixed well before placing in a boiling water bath for 10 minutes. The tubes were cooled, centrifuged and the absorbance of the supernatant was measured using spectrophotometer at 532 nm. The standard absorption curve for MDA quantification were prepared using 1,1,3,3-tetraethoxypropane (*Appendix F i and ii*).

3.8.5.3.3 Calculation

The sample value can be calculated from the formula:

$$\frac{Abs_{sample} - Abs_{blank}}{Abs_{standard}} \quad X \quad [1,1,3,3\text{-tetraethoxypropane}_{standard}]\ (\mu M)$$

The MDA values of homogenate samples were expressed in μmol mg^{-1} protein.

3.8.5.4 Glutathione Peroxidase Assay

3.8.5.4.1 Principle of the assay

GPx enzymes catalyze the reduction of hydrogen peroxide to water and organic peroxides to the corresponding stable alcohols using glutathione. This assay is an

indirect measurement of the activity of cellular glutathione peroxidase. The GPx enzyme activity were monitored using the oxidation of NADPH to $NADP^+$ and the absorbance can be measured at 340 nm, spectrophotomerically.

$$2GSH + H_2O_2 \xrightarrow{\text{c-GPx}} ROH + GSSG + 2H_2O$$

$$GSSG + NADPH + H^+ \longrightarrow 2\,GSH + NADP^+$$

3.8.5.4.2 General Procedure

Glutathione Peroxidase assay was performed by using BioxyTech GPx-340, Oxis Research product (USA). The reagents were prepared according to the instruction manual provided with the kit. The samples were diluted 10X using the assay buffer provided. In each well of a micro-titer plate, 75 µl of assay buffer, 75 µl of NADPH reagent and 15 µl of sample were added. The assay was commenced with the addition of Tert-Butyl hydroperoxide. The absorbance was measured at 340 nm at an interval of 30 seconds for three minutes.

3.8.5.4.3 Calculation

The average rate of decrease in absorbance per minute was calculated. The absorbance unit was converted to nmol using the extinction coefficient of NADPH. The value were expressed in nmol NADPH/ min/ mg.

3.8.5.5 Bradford Assay

3.8.5.5.1 Principle of the assay

The Bradford protein assay is a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein (Kruger, 2009). The method is simple, fast, highly sensitive and can be used to estimate small amount of

protein concentration up to the range of 100-1000 μg/ml protein. Coomasie blue or Coomassie brilliant blue G-250 (CBBG) is a type of dye used in this method made by the addition of : 0.0035 % (w/v) CBBG, 3.5 % (v/v) ethanol and 7.5 % (v/v) phosphoric acid. The dye exists in three forms: cationic, neutral, and anionic. Although the anion is not freely present at the dye reagent pH, it is this form that complexes with protein. Dye binding requires a macromolecular form with certain reactive functional groups. Interactions are mainly with arginine rather than primary amino groups; the other basic (His, Lys) and aromatic residues (Try, Tyr, and Phe) give slight responses. The binding behavior is attributed to Van der Waals forces and hydrophobic interactions. The Bradford protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when bound to protein.

3.8.5.5.2 General Procedure

The Coomassie blue dye reagent was purchased from Bio-Rad. The dye was diluted with distilled water in a ratio of 1:5, then filtered using Whatman paper as well as centrifuged at 3500 rpm for five minutes. The BSA standard was prepared to yield final concentrations of 0.05 to 0.5 mg/ml sample or standard (10 μl) were added in the wells of a micro-plate which was followed by the addition of Bradford reagent (200 μl). The absorbance was measured after five minutes incubation at room temperature at 595 nm within an hour.

3.8.5.5.3 Calculation

The sample value can be calculated using the following formula:

$$\frac{Abs_{sample} - Abs_{blank}}{Abs_{standard}} \quad X \quad [BSA_{standard}]\ (\mu M)$$

The Bradford standard curve was plotted (*Appendix G).*

3.8.6 Histological assessment

3.8.6.1 Hematoxylin and Eosin staining

3.8.6.1.1 Principle of the assay

Hematoxylin and Eosin (H & E) staining are the most commonly used technique in histology. The haematoxylin stains acidic structures a purplish blue. Nuclei, ribosomes and rough endoplasmic reticulum have a strong affinity for this dye owing to their high content of DNA and RNA, respectively. In contrast, Eosin is an acidic dye which stains basic structures red or pink. Most cytoplasmic proteins are basic and hence cytoplasm usually stains pink or pinkish red. In general, nuclei stain blue and cytoplasm stains pink or red.

3.8.6.1.2 General Procedure

After the rats were sacrificed, major organs such as liver and kidney were immediately removed and fixed into 10 % buffered formalin overnight. The next day, the tissues were trimmed and placed in a cassette followed by automated tissue processing. Next, tissues were embedded with paraffin, trimmed and sectioned into 5 μm thick sections using a rotary microtome. Lastly, the sections were stained with Hematoxylin and Eosin and mounted with distyrene, plasticizer, xylene (DPX).

3.8.6.1.3 Evaluation

The degree of liver and kidney damage were examined under light microscope with magnification of 100X and 400X. The structures were compared with the histology of the liver and kidney from normal control group rats. The images were taken using

Dino Eye Capture System camera at original magnification of 10×10. The liver sections were graded numerically to assess their histological features. Acute liver injury was evaluated by a histologist: central vein, hepatocytes and arrangements, no fatty changes, RBC infiltration, lymphocytes infiltration, dilated sinusoid, RBC congestion, shrunken nuclei were used as criteria, and a combined score of histological features was given for each liver section. For kidney; glomerulus/capsular space, dilated capillaries and glomerulosclerosis were evaluated. The parameters were graded from scores 0-6, with 0 indicating no abnormality, 1-2 indicating mild injury, 3-4 indicating moderate injury and 5-6 indicating severe liver injury (Wang, et al., 2008; Wills & Asha, 2006). The histological appearance of liver is shown and discussed in *Section 4.3.9* (*Figure 4.12*) and *Section 4.4.9* (*Figure 4.22*) and kidney *Section 4.3.10 (Figure 4.13)* and *Section 4.4.10 (Figure 4.23).*

3.9 Identification of bioactives compound

3.9.1 High Performance Liquid Chromatography (HPLC)

Both HPLC and LC-MS were performed by BST Consultancy and Services Sdn. Bhd (868522-X).

3.9.1.1 Principle of the assay

High performance liquid chromatography is a chromatography method that separates non-volatile compounds by stationary and mobile phase concepts. The specific method used in this study was Reversed-Phase Chromatography (RPC). RPC uses non-polar column (stationary phase) and water-miscible organic solvent (mobile phase). RPC molecules separation was based on hydrophobicity (SchluTer, 1999) which involves binding of hydrophobic molecules onto a hydrophobic solid support (e.g column coated with silica C18) in a polar mobile phase (e.g acetonitrile) (Rafferty, Siepmann, & Schure, 2011). The solutes are eluted using aqueous buffer (e.g water-acetic acid), followed by organic solvent (acetonitrile) in increasing gradient elution (increasing molecular hydrophobicity) over a period of time. The peak identification was based on the retention time and the diode array detector (DAD) spectrum against the blank control in the chromatogram.

3.9.1.2 General Procedure

Equipment used in this study was HPLC Agilent 1100 Series equipped with auto sampler, fraction collector and HPChem system. Method used was Reverse Phase Chromatography where separation of compounds was based on hydrophobic property. Column used is Agilent XDB C18 with column diameter of 4.6 mm, column length 250 mm and pore particle size 5.0 µm. Solvent (mobile phase) used was gradient elution of

acetonitrile in buffer solution (water- 0.25 acetic acid) as shown in *Table 3.11*. The flow rate was 1.2 ml/min. The DAD detects the absorption in UV to VIS range therefore, wavelength was set at UV (190 nm, 230 nm, 254 nm and 280 nm) for acquiring chromatograms.

Leucaena leucocephala water-hexane partially purified was prepared by dissolving 100 mg of extract in 5 ml water in volumetric flask. Solution was then filtered by using SRP-4 membrane 0.45 μm. The stock solution 20 mg/ml was kept in fridge 4 °C. Sample was injected into HPLC at 100 μl for 18 times respectively. The was run at 191 nm, 230 nm, 254 nm and 280 nm UV wavelengths.

3.9.2 Liquid Chromatography Mass Spectrophotometry (LC-MS)

3.9.2.1 Principle of the assay

Liquid Chromatography Mass Spectophotometry (LC-MS) is a combination of liquid chromatography with mass spectrophometer. It gives a molecular weight information after separation of compounds and quantity and purity of sample. Ultra Performance Liquid Chromatography (UPLC) was used in this study because it provides higher speed and higher separation performances (Neue, 2007).

A mass spectrophotometer must contain ion source, mass analyzer and detector. Electrospray ionisation source (ESI) is an ion source and operation in positive mode involved an addition of protons to analyte. ESI works well with moderately polar molecules and gives high yield without degradation (Barnett, Handy, & Horlick, 2000). Mass analyzer function to separate ions according to m/z ratio and a quadrupole type was used to maintain the low pressure of the collision gas induced dissociation, therefore, could transmit most of the fragment ions that are produced . A detector used

in this study was Time of flight (TOF) which accelerates ions through a high voltage depending on mass to charge ratio (m/z) and convert ions to electrons. One of the concepts applied is mass to charge ratio (m/z ratios) where the lighter ions move faster and reache the detector earlier than heavier ions. TOF has high mass accuracy in the determination of molecular weight.

Liquid samples were pumped through metal capillary (at voltage 3-5kV) which forms fine spray of charged droplets at the tip of capillary. Application of nitrogen and heat evaporated the droplets rapidly and at the same time, the residual electrical charge on the droplets was transferred to the analytes. The ionised analytes move to the high vacuum of mass spectrophotometry.

3.9.2.2 General Procedure

Equipment used in this study is Acquity ™ Ultra Performance Liquid Chromatography (UPLC)-PDA system coupled to Synapt High Definition Mass Spectrometry (HDMS) quadrupole-orthogonal acceleration time-of-flight (TOF) detector equipped with an ESI source. The column used was Acquity BEH C 18, with inner column diameter of 2.1 mm, column length 50 mm and pore particle size 1.7 μm UPLC column. The injection volume was 3 μl with flow rate 0.5ml/min and the solvents used were water, 0.1 % formic acid and acetonitrile as shown in *Table 3.12.*

Two milligrams of *L. leucocephala* water-hexane partially purified was dissolved in two millilitre methanol in a volumetric flask. Then, the solution was filtered using SRP-4 membrane (0.45 mm). The stock 1mg/ml was kept at 4 °C until used. The procedure was run at 191 nm, 230 nm, 254 nm and 280 nm wavelengths.

Table 3.11: Solvent and buffer solution proportion used in the HPLC study.

Acetonitrile (%)	Water-0.25 Acetic acid (%)
0	100
0	100
30	70
35	65
40	60
50	50
100	0
100	0

Table 3.12: Solvent and buffer solution proportion used in the LC-MS study.

Time	Water-0.1% Formic acid	Acetonitrile-0.1% Formic acid
0.00	100	0
0.42	100	0
0.83	70	30
1.25	65	35
1.67	60	40
2.08	50	50
2.50	0	100
4.50	50	50

3.10 STATISTICAL ANALYSIS AND SOFTWARE

3.10.1 Analyses

All experiment were carried out in triplicate. The Student's T-test was used to analyze between two groups where a test group was compared over a control group. One-Way ANOVA test was performed for more than two groups over many variate studies. Data were expressed as Mean ± Standard Error Mean (SEM). In statistical significance comparisons * denotes $p < 0.05$, **denotes $p < 0.01$ and *** $p < 0.001$ unless stated otherwise.

3.10.2 Software

Analyses of Mean ± Standard Error Mean (SEM) were calculated using Microsoft ® Office 2003 Excel software. The graphs were designed used Microsoft Office 2003 Excel and GraphPad Prism version 5-02 for Windows, Graph Pad Software, San Diego California USA.

CHAPTER FOUR : RESULTS AND DISCUSSION

4.1 Analyses for nutrient content of *Leucaena leucocephala*

Data tabulated in *Table 4.1*, analytes that has high nutrition were vitamin B_2, calcium and magnesium as one gram of the extract could give more than enough recommended by RDA. The vitamin B_2 supplied 143.85 % , calcium provides 554.97 % and magnesium supplied 135.12 %.

Vitamin B_2 (Riboflavin) is a yellow crystalline pigment and a water-soluble vitamin. It functions as a coenzyme in the oxidative processes of carbohydrates, fats and proteins by combining with specific flavoprotein. Vitamin B_2 exists in meat, eggs, dairy products and leafy vegetables. Small amount of riboflavin are found in the liver and kidney, but it is not stored to any degree in the body. Therefore, must be supplied regularly in the diet.

Calcium is needed for muscle contraction, blood vessel expansion and contraction, secretion of hormones and enzymes, and transmitting impulses throughout the nervous system. Less than 1 % of total body calcium is needed to support these functions, remaining 99 % of the body's calcium supply is stored in the bones and teeth. Milk, yogurt, and cheese are rich sources of calcium, non dairy sources include vegetables, such as chinese cabbage, kale, and broccoli.

Table 4.1: Food nutrition analysis results.

Test parameter	Test method	Unit	Result
Carbohydrate	By calculation	g/100 g	45.5
Protein	Kjeldahl method	g/100 g	25.0
Total fat	Solvent extraction	g/100 g	1.5
Total sugars	Lane & Eynon	g/100 g	38.0
Dietary fibre	AOAC 985.29	g/100 g	10.8
Trans fatty acid	GC	g/100 g	Not detected (< 0.01)
Vitamin A	HPLC	mg/100 g	Not detected (< 0.01)
Vitamin B_1	HPLC	mg/100 g	0.07
Vitamin B_2	HPLC	mg/100 g	1.87
Vitamin B_3	HPLC	mg/100 g	3.46
Vitamin C	HPLC	mg/100 g	Not detected (< 0.05)
Vitamin E	HPLC	mg/100 g	0.01
Sodium (as Na)	ICP-OES	mg/100 g	85.57
Calcium (as Ca)	ICP-OES	mg/100 g	5549.7
Magnesium (as Mg)	ICP-OES	mg/100 g	540.58
Zinc (as Zn)	ICP-OES	mg/100 g	4.65
Potassium (as K)	ICP-OES	mg/100 g	2445.9
Manganese (as Mn)	ICP-OES	mg/100 g	2.27
Selenium (as Se)	ICP-OES	mg/100 g	Not detected (< 0.02)

Abbreviations:

AOAC stands for Association of Official Agricultural Chemists, where an enzymatic-gravimetric method for analyzing total dietary fibre was adopted from. GC stands for gas chromatography, HPLC stands for high performance liquid chromatography and ICP-OES stands for inductively coupled plasma atomic emission spectroscopy.

4.2 Assessment of *Leucaena leucocephala* for

4.2.1 Effect of *Leucaena leucocephala* dry fruit water crude extract on gene expression in rat adipocytes.

An investigation at molecular level was also verified by inspection on relative expression of genes that regulate glucose in insulin signalling pathway. Total RNA was extracted as method in *Section 3.6.3*, then transcribed into complimentary DNA (cDNA) as described in *Section 3.6.4* and *Section 3.6.5* respectively.

Insulin hormone regulates glucose level by increasing glucose uptake in adipocyte and skeletal muscle and promotes synthesis and storage of carbohydrates, lipid and protein in the cells (Saltiel & Kahn, 2001). In this study, *L. leucocephala* extract used was 10 μg/ml because it corresponded to 50% effective concentration in glucose uptake activities and adipogenesis (Lim, 2007).

In the gene expression study, *L. leucocephala* extract stimulated GLUT-4 gene expression ($p < 0.01$) and this implied an insulinic property of the extract (*Figure 4.2*). GLUT 4 is an insulin regulated glucose transporter present in adipose tissues and striated muscle (skeletal and cardiac) which is responsible for insulin-regulated glucose uptake. The presence of insulin and *L. leucocephala* extract, stimulated the redistribution of GLUT 4 from intracellular sites to the extracellular. At plasma membrane, GLUT 4 facilitates the glucose uptake by passive diffusion into muscle and fat cells. The finding clearly reflects the ability of both insulin and *L. leucocephala* extract to enchance glucose uptake by 2.5-fold and 2.0-fold.

The two opposing biochemical processes, lipolysis and lipid synthesis, are controlled by different enzymes and hormones. Accordingly, the level of expression of hormone sensitive lipase (HSL), the rate-limiting enzyme responsible for the intracellular triglycerides hydrolysis and fatty acid mobilization in adipose tissue, was

increased by *L. leucocephala* treatment in the present study. Insulin, as an antilipolytic agent down-regulated HSL gene expression by 4.4 fold whereas *L. leucocephala* extract up-regulated expression of HSL by one-fold (p <0.05). The finding was comparable with lipolysis activity of this extract at a dose of10 μg/ml (Lim, 2007). *L. leucocephala* extract triggers cyclic AMP pathway to activate protein kinase A (PKA) and then HSL.

AKT and PI3K genes are components in insulin tyrosine kinase pathway; where the underlying mechanisms and roles remain unclear (Cong et al., 2007; Hajduch, Litherland, & Hundal, 2001). In this study, AKT, PI3K and SREBP gene expressions were down-regulated (p <0.01),(p <0.05) and (p <0.01) respectively. The down-regulation might be regulated at the post-transcriptional level to manage glucose uptake in cells (Chai, Lim, Kanthimathi, & Kuppusamy, 2011). Glucose regulation in adipocytes requires only a small activation of AKT gene for glucose uptake activity (Whiteman, Cho, & Birnbaum, 2002). Down-regulation of PI3K could possibly reduce insulin-stimulated adiponectin secretion (Pereira & Draznin, 2005). However, the stimulation of adiponectin depend on multiple factors such as genetic and environmental as well as includes fasting condition. Decrease in SREBP gene expression implies impaired lipogenesis in adipocytes. In the present study, down-regulation of AKT, PI3K and SREBP1 genes may be associated with greater lipolysis effect exerted by *L. leucocephala* extract than its adipogenesis activity in rat adipocytes at concentration of 10 μg/ml.

Taken together, *L. leucocephala* extract stimulated glucose uptake *via* increase GLUT-4 expression and promotes lipolysis at dose of 10 μg/ml but regulates adipogenesis at higher concentration. In conclusion, *L.leucocephala* dry fruit water crude extract at ideal concentration with increased glucose uptake as well as balanced

adipogenesis and lipolytic effects would be valuable in the management of Type II diabetes mellitus.

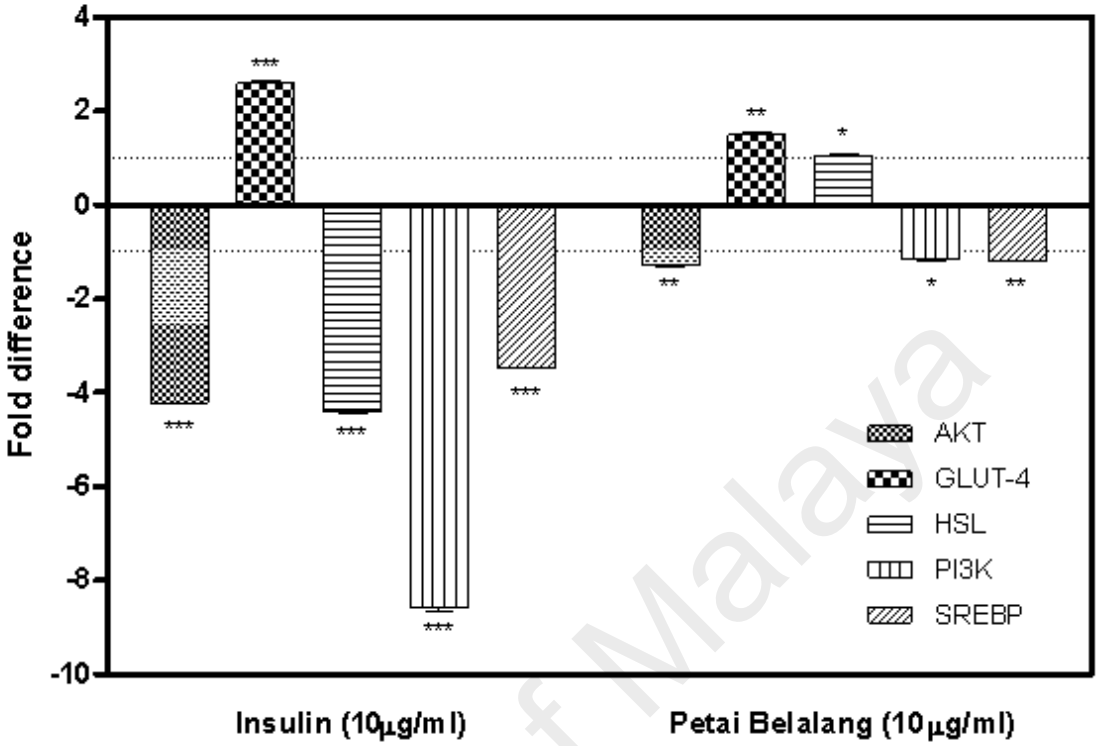


Figure 4.1: Effect of *Leucaena leucocephala* dry fruit water crude extract on gene expression in adipocyte cells.

Results are expressed as fold difference over positive control (insulin) at a dose 10 µg/ml. Results presented are mean ± SD. Fold variations less than 0 were expressed as negative numbers. Range between +1 and -1, which corresponded to no changes are labelled by dotted lines. Statistical difference was calculated based on the mean ΔC_T values by Student's T-test. ∗ denotes one-fold difference, ∗∗ denotes two-fold difference, ∗∗∗ denotes three-fold difference.

Abbreviations: Protein kinase B (AKT), glucose transporter 4 (GLUT-4), hormone sensitive lipase (HSL), phosphoinositide 3-kinases (PI3K) and sterol regulatory element–binding protein (SREBPs).

4.2.2 Effect of *Leucaena leucocephala* dry fruit water crude extract on Oral Glucose Tolerance Test in normal rats.

Leucaena leucocephala was prepared as stated in method *Section 3.3.1* and a single dose of 500 mg kg $^{-1}$ was administered *via* intragastric route to the normal rats and the OGTT was conducted as described in the method *Section 3.8*. *L. leucocephala* dry fruit water crude extract showed improved tolerance to glucose at 60 minutes compared to negative control ($p < 0.05$) (*Figure 4.2*).

Different mechanisms of action to reduce high blood glucose levels using plant extracts already exist. Some plants exhibit properties similar to the well-known sulfonylureas like glibenclamide; they reduce blood glucose in normoglycemic animals (Davis & Granner, 2001; Ivorra, Paya, & Villar, 1989). Some other plant extracts exhibit properties like biguanides such as metformin, which is an antihyperglycemic compound; do not affect blood glucose in normal state (Bailey, Day, Turner, & Leatherdale, 1985; DeFronzo & Goodman, 1995; Stumvoll, Nurjhan, Perriello, Dailey, & Gerich, 1995). Several anti-diabetic drugs do not act by stimulating activity of insulin, but act on extra-pancreatic actions such as increasing in glucose utilization and reduced hepatic gluconeogenesis.

Leucaena leucocephala dry fruit water crude extract slow the glucose absorption at 60 minutes (Eseyin, Ebong, Ekpo, Igboasoiyi, & Oforah, 2007). The slower / reduced glucose absorption could help the body to control blood glucose from postprandial hyperglycemia.

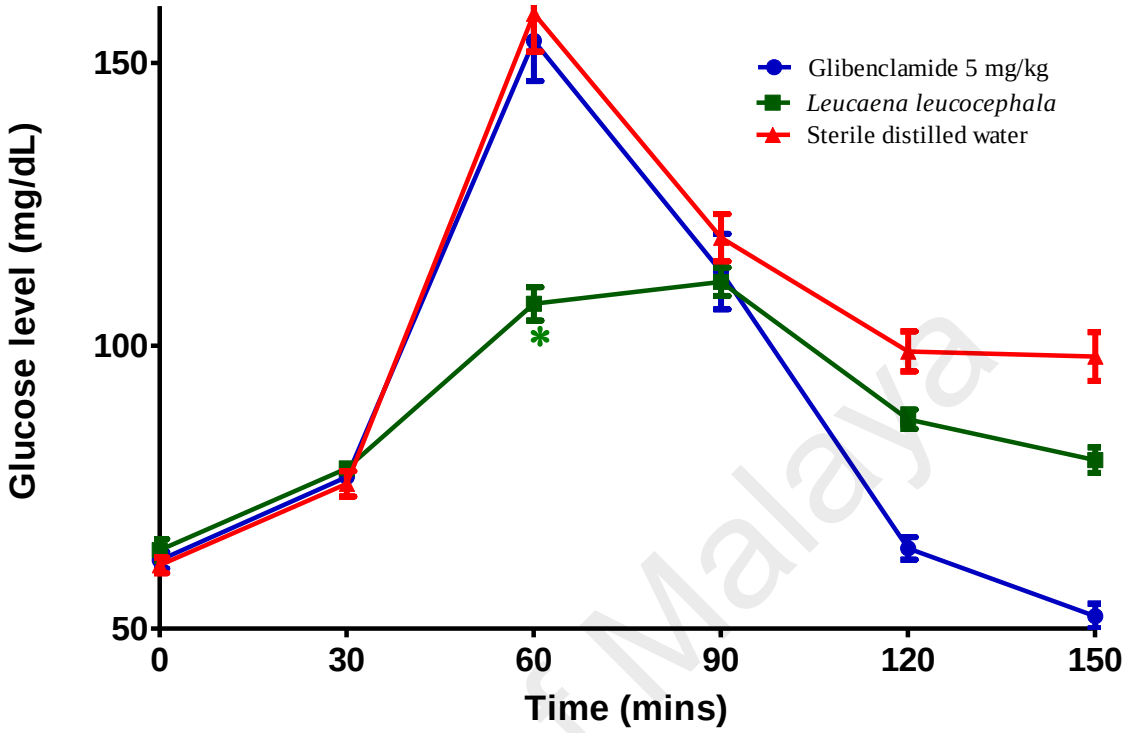


Figure 4.2: OGTT of normal rats pretreated with 500 mg kg^{-1} *Leucaena leucocephala* dry fruit water crude extract.

Each plotted colored lines represent glucose absorption in normal rats after oral administration of different treatment. Result is presented as mean ± SEM. Statistical difference was compared to negative control at different treatment hours using Student's T-test; * denotes $p < 0.05$.

4.2.3 Effect of *Leucaena leucocephala* dry fruit water crude extract on Oral Glucose Tolerance Test in Streptozocin-induced diabetic rats.

In diabetic rats OGTT, at the dose of 500 mg kg^{-1}, *L. leucocephala* dry fruit water crude extract showed a mild, but significant improvement at 150 minutes ($p < 0.05$) (*Figure 4.3*).

In OGTT of diabetic rats, the blood glucose levels in the glibenclamide (5 mg/kg)-treated group throughout the study were similar to those of the vehicle-treated group while in OGTT in normal rats pretreated with the glibenclamide; the blood glucose levels were significantly reduced at 60 and 120 minutes, compared to the vehicle. In a similar study, researcher claimed that glibenclamide did not produce a significant glucose clearance in STZ-induced diabetic rats; and this is contrary to the action of metformin (Zhang & Tan, 2000). Glibenclamide is a sulfonylurea derivative which causes hypoglycemia by stimulating pancreatic β-cells to release more insulin, and inhibiting glucagon secretion. The effect requires a functional β-cells, thus it can lower blood glucose level in non-diabetic subjects better than in diabetic subjects.

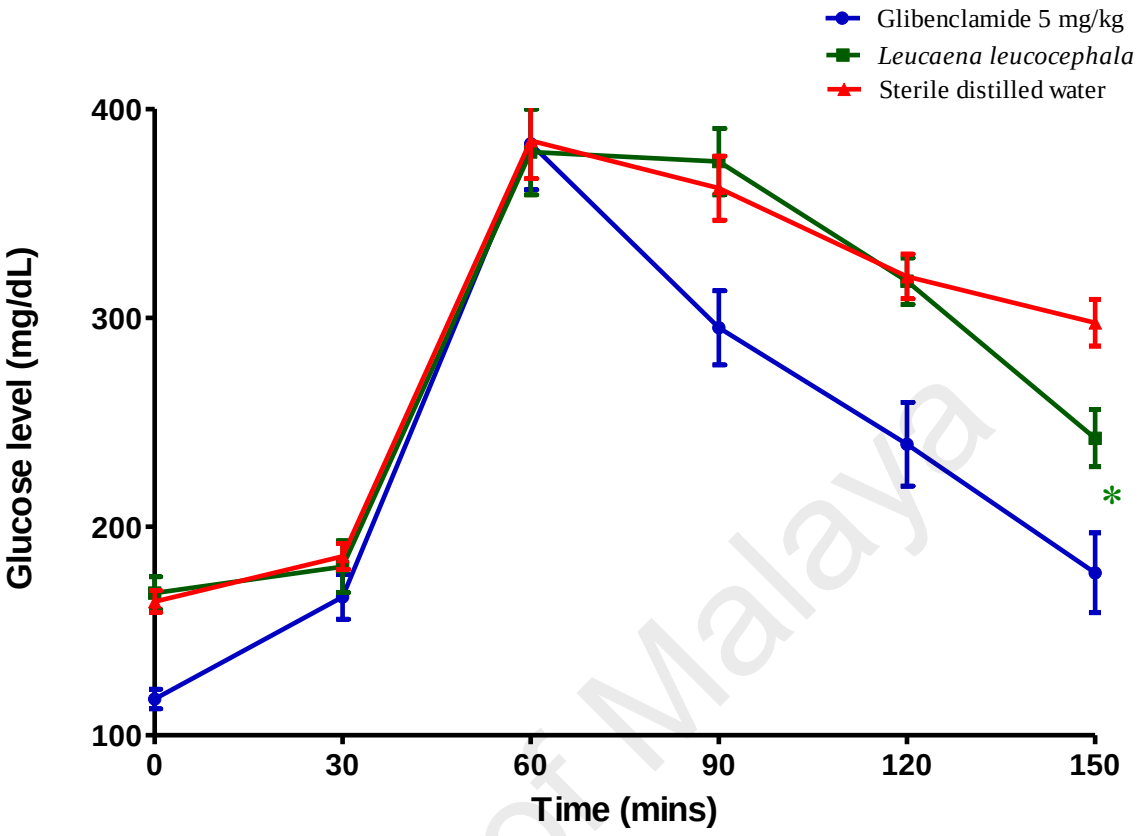


Figure 4.3: OGTT of diabetic rats pretreated with 500 mg kg^{-1} *Leucaena leucocephala* dry fruit water crude extract.

Each plotted colored lines represent glucose level in diabetic rats after oral administration of different treatment. Result is presented as mean ± SEM. Statistical difference was compared to negative control using Student's T-test; * denotes $p < 0.05$.

4.2.4 Justification of *Leucaena leucocephala* dry fruit water crude extract for further study

Leucaena leucocephala was selected for further studies because *L. leucocephala* exhibited a promising result obtained from gene expression study in adipocyte cellular model. Thus, the first reason of the selection of *L. leucocephala* for detailed study was due to significant up-regulation of Glucose Transporter 4 (GLUT-4). The significant GLUT-4 gene expression states that *L. leucocephala* dry fruit water crude extract could stimulate the uptake of circulating blood glucose *via* insulin signalling pathway.

Secondly, *L. leucocephala* dry fruit water crude extract showed improvement in glycemic index in both OGTT in normal and diabetic rats. Thirdly, previous *in vitro* research in our laboratory showed that *L. leucocephala* at dose 0.1 µg/ml promoted mild lipogenesis (13 %) and high lipolysis activity in adipocyte cell culture (Lim, 2007), this implies the potential of anti-obesity and anti-hyperglycemia.

4.3 Assessment of *Leucaena leucocephala* dry fruit water crude extract for anti-diabetic and antioxidant properties in rats

4.3.1 Effect of *Leucaena leucocephala* dry fruit water crude extract on glucose level in Streptozocin-induced diabetic rats.

Figure 4.4 shows the effect of *L. leucocephala* dry fruit water crude extract on glucose level in Streptozocin-induced diabetic rats over a period of 30 days. Diabetic rats administered with sterile distilled water (negative control) showed worsening of hyperglycemic state ($p < 0.01$); with increasing glucose levels from day 1 (312.29 ± 17.20 mg/dL) until day 30 (480.16 ± 73.46 mg/dL). The positive control group, treated with Glibenclamide at dose of 1.25 mg kg^{-1} showed a reduction of glycemic value on day 15 (210.88 ± 32.25 mg/dL) compared with day 1 (282.19 ±14.87 mg/dL) ($p < 0.05$). Diabetic rats treated with *L. leucocephala* extract in the dose range of 1.25-250 mg/kg showed significant reduction of blood glucose when compared with untreated diabetic group of the corresponding experiment duration. Generally, *L. leucocephala* extract at both doses appeared to prevent the glucose level from worsening. However, it did not reduce the blood level into normoglycemic state. Meanwhile, the healthy non-diabetic rat group showed normal blood glucose level throughout the period of study (day 1- day 30). During the study period, the untreated diabetic rats (only distilled water) showed significant increase in fasting blood glucose level but in normal rats; fasting blood glucose remained within the normal range.

Monitoring blood glucose is an effective way to reduce diabetic complications in diabetic patients (Chase et al., 1989). Controlling diet is essential to reduce the hyperglycemia (Gutierrez, Akhavan, Jovanovic, & Peterson, 1998). In Glibenclamide treated group, the decrease in blood glucose occur by the stimulation of the existing β-

cells to secrete more insulin (Aston-Mourney, Proietto, Morahan, & Andrikopoulos, 2008; Fuhlendorff et al., 1998). The observed reduction in glucose level in *L. leucocephala* 500 mg kg^{-1} rats treated group was showed similar pattern as in glibenclamide treated group and this finding was also supported by the results of OGTT in diabetic rats (*Figure 4.4*). The prevention of the glucose level from worsening might produce less oxidative stress-induced protein damage (Yazdanparast, Ardestani, & Jamshidi, 2007).

Abbreviations: Normal rats treated with sterile distilled water (N+ sdH_2O), diabetic rats treated with sterile distilled water (D+ sdH_2O), diabetic rats treated with *Leucaena leucocephala* at dose of 250 mg per kg (D+ LL 250 mg/kg), diabetic rats treated with *Leucaena leucocephala* at dose of 500 mg per kg (D+ LL 500 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D + G 1.25 mg/kg).

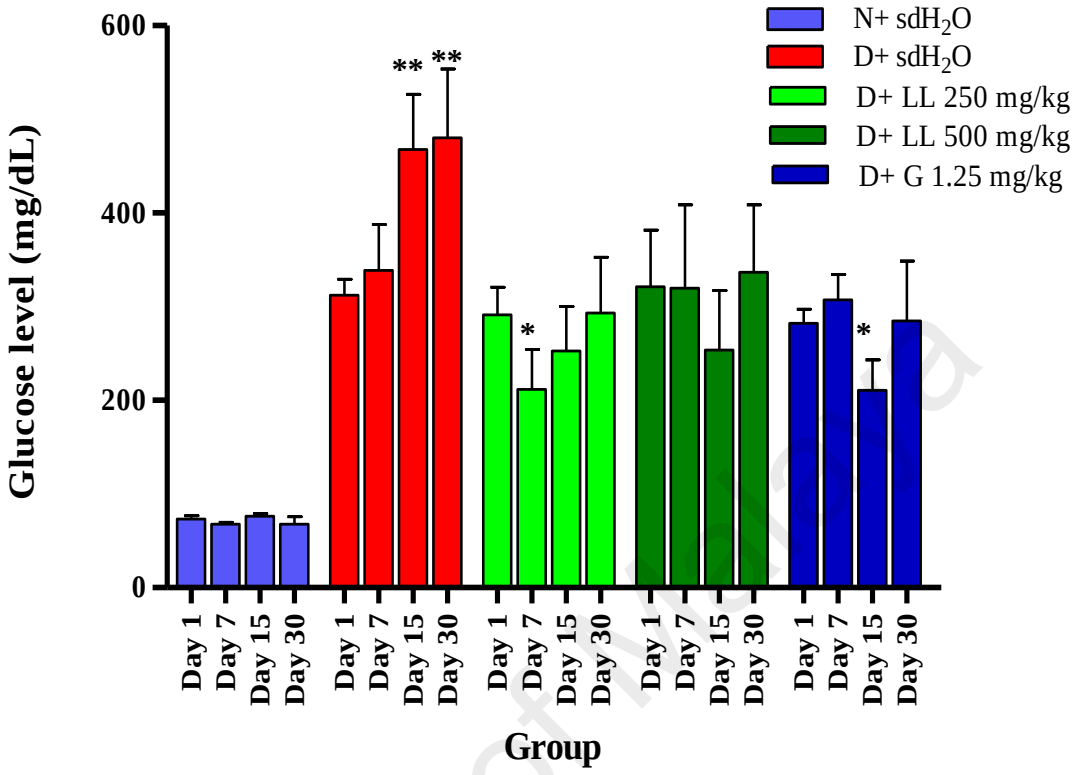


Figure 4.4: Effect of *L. leucocephala* dry fruit water crude extract on glucose level in STZ-induced diabetic rats.

The changes in blood glucose were measured in milligram per decilitre on days 1, 7, 15 and 30. Each coloured bar represents a different group. Comparisons were made between different days in each groups. Results present are mean ± SEM. Statistical significance was calculated based on Student's t-test; ** denotes $p < 0.01$, * denotes $p < 0.05$.

4.3.2 Effect of *Leucaena leucocephala* dry fruit water crude extract on body weight in Streptozocin-induced diabetic rats.

Figure 4.5 shows the effect of *L. leucocephala* dry fruit water crude extract on percentage change in body weight in Streptozocin-induced diabetic rats. Both concentration of *L. leucocephala* extract demonstrated gradual increase in body weight from day 1 treatment until day 30 while Glibenclamide group showed a decreased as much as -3.22 %. In normal rats, the increment of body weight was 31.88 % contrary to negative control group -22.17 %.

The results show that there was reduction in weight after the rats become diabetic similar results were found by Sarkhail et. al., (2007). *Leucaena leucocephala* treatment at both dose restored almost 44-55 % (32-35 g) weight compared to normal rats, while in diabetic postive control rats there were reduction -10 % (-8 g) compared to normal rats body weight after 30 days treatment. The decrease in body weight in diabetic rats suggests that the loss or degradation of structural proteins was due to diabetes, and the structural proteins are known to contribute to the body weight (Rajkumar, Srinivasan, Balasubramanian, & Govindarajulu, 1991).

Type I Diabetes patients experience body weight loss due to the absence of insulin production (Khan, 2004), which is needed to utilize the glucose. When the body is unable to use glucose, it is excreted in urine. As a result, despite having an increased appetite, the patients may continue to lose weight (Kahn, Hull, & Utzschneider, 2006). In this study, untreated diabetic rats experienced significant weight loss ($p < 0.01$). Weight loss occur in poorly managed diabetics where absence of insulin or insufficient amount of insulin with excessive amount of glucose triggers the release of triglycerides from adipose tissue and catabolism of amino acids in muscle tissue which eventually

lead to ketoacidosis. The restoration of body weight in *L. leucocephala* groups was probably due to restoration of insulin production or sensitisation to insulin.

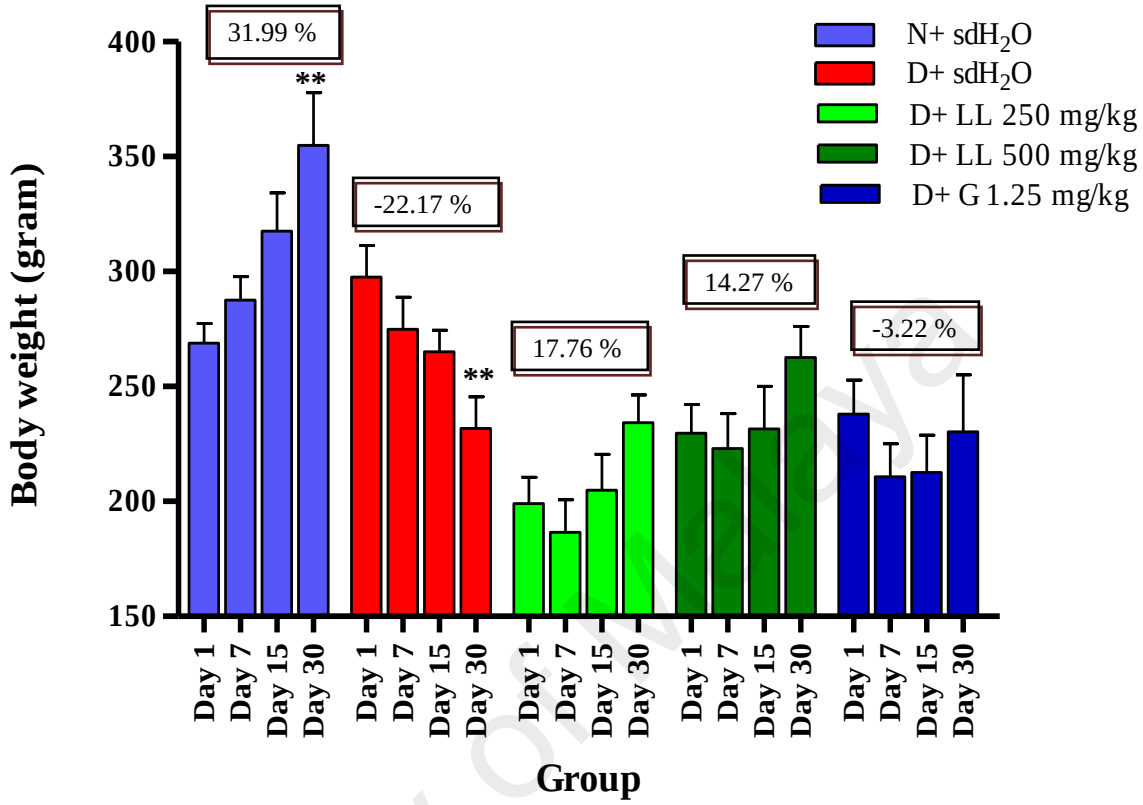


Figure 4.5: Effects of *L. leucocephala* dry fruit water crude extract on percentage change in body weight in STZ-induced diabetic rats.

The changes of body weight were calculated based on formula in the *Section 3.9.4.2*. The results were calculated between day 1 and 30 in each group and compared with negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; ** denotes $p < 0.01$.

Abbreviations: Normal rats treated with sterile distilled water (N+ sdH_2O), diabetic rats treated with sterile distilled water (D+ sdH_2O), diabetic rats treated with *Leucaena leucocephala* at dose of 250 mg per kg (D+ LL 250 mg/kg), diabetic rats treated with *Leucaena leucocephala* at dose of 500 mg per kg (D+ LL 500 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D + G 1.25 mg/kg).

4.3.3 Effects of *Leucaena leucocephala* dry fruit water extract on insulin production in Streptozocin-induced diabetic rats.

Figure 4.6 shows the effect of *L. leucocephala* dry fruit water crude extract on insulin level (after 30 days treatment) in Streptozocin-induced diabetic rats. In diabetic rats treated with *L. leucocephala* 500 mg kg^{-1} a significantly increase in insulin level ($p < 0.05$) compared to the level in negative control (distilled water).

Most diabetes drugs widely used are 'secretagogues' that trigger insulin release by direct action on the K^+ ATP channel of the pancreatic beta cells or 'sensitizers' that reduce liver glucose output and increase uptake of glucose by the periphery or enhance production of mRNAs of insulin dependent enzymes. This showed that *L. leucocephala* has 'insulin stimulation potential' that works like 'secretagogues'.

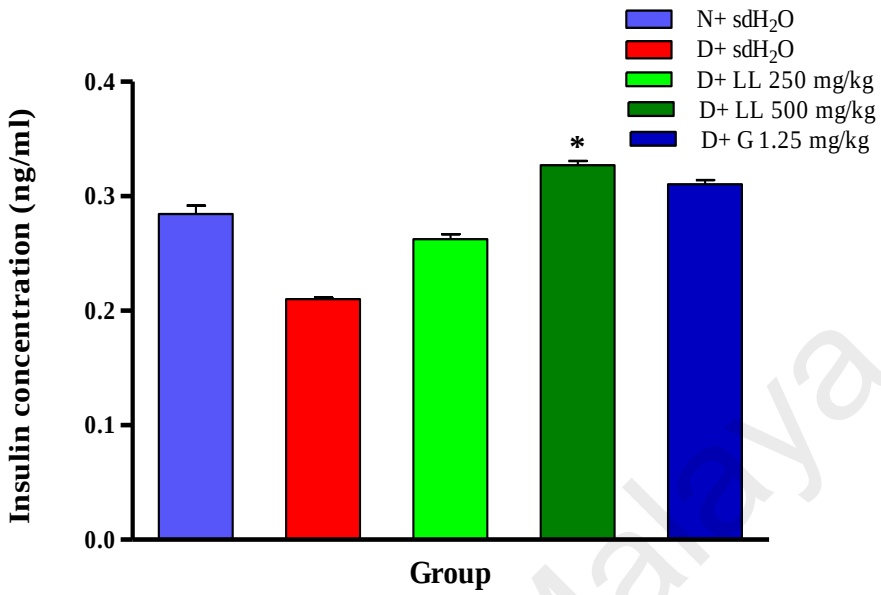


Figure 4.6: Effects of *L. leucocephala* dry fruit water crude extract on serum insulin level in STZ-induced diabetic rats.

The insulin test was performed on serum rats pooled samples using method in *Section 3.9.4.3*. Comparison were made between each groups and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; * denotes $p < 0.05$.

Abbreviations: Normal rats treated with sterile distilled water (N+ sdH_2O), diabetic rats treated with sterile distilled water (D+ sdH_2O), diabetic rats treated with *Leucaena leucocephala* at dose of 250 mg per kg (D+ LL 250 mg/kg), diabetic rats treated with *Leucaena leucocephala* at dose of 500 mg per kg (D+ LL 500 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D + G 1.25 mg/kg).

4.3.4 Effects of *Leucaena leucocephala* dry fruit water extract on production of adiponectin in Streptozocin-induced diabetic rats.

Figure 4.7 shows the effect of *L. leucocephala* extract on adiponectin level in Streptozocin-induced diabetic rats. The *L. leucocephala* extract treated diabetic rats showed significantly higher level of adiponectin compared to the negative control (untreated diabetic) rats.

Adiponectin is an adipokine that modulates glucose regulation and fatty acid catabolism. Adiponectin has been shown to be inversely correlated with hyperglycemia in human (Blumer et al., 2008) and also inversely correlated with body fat percentage in adults (Yang et al., 2001). Interestingly, *L. leucocephala* stimulated more production of adiponectin in this *in vivo* model ($p < 0.05$). Fu, Luo, Klein, and Garvey (2005); in their *in vitro* study showed that adiponectin enhanced GLUT-4 gene expression by 78 % and increased GLUT 4 recruitment to the plasma membrane. The significant increase of adiponectin level observed in our study implies that the hyperglycemic state of the test "subject" correlates to the adiponectin level. Thus, *L. leucocephala* has a potential to serve as an adjuvant or alternative to the existing anti-diabetic agents.

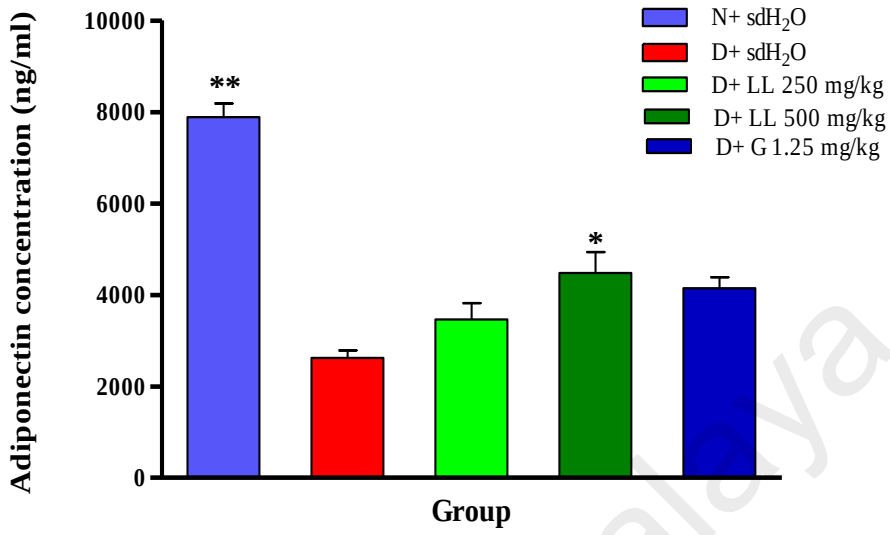


Figure 4.7: Effect of *L. leucocephala* dry fruit water crude extract on adiponectin level in STZ-induced diabetic rats.

The adiponectin test was performed using rat serum samples according to the method described in *Section 3.9.4.4.* Comparison were made between each groups and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; * denotes $p < 0.05$ and ** denotes $p < 0.01$.

Abbreviations: Normal rats treated with sterile distilled water (N+ sdH_2O), diabetic rats treated with sterile distilled water (D+ sdH_2O), diabetic rats treated with *Leucaena leucocephala* at dose of 250 mg per kg (D+ LL 250 mg/kg), diabetic rats treated with *Leucaena leucocephala* at dose of 500 mg per kg (D+ LL 500 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D + G 1.25 mg/kg).

4.3.5 Effects of *Leucaena leucocephala* dry fruit water crude extract on oxidative indices in pancreas of Streptozocin-induced diabetic rats.

Pancreas is a small organ located in the upper abdomen and adjacent to the small intestine. Pancreas functions as an exocrine organ in the digestive system that secretes pancreatic juice containing digestive enzymes and pass to the small intestine. It secretes insulin, glucagon, and somatostatin. Pancreas contains α-cells that secrete glucagon hormones which stimulates liver to breakdown glycogen from its cells therefore raises the level of glucose in the blood. It also secretes insulin that stimulates cells for glucose uptake and somatostatin that regulates secretion of glucagon and insulin.

Briefly, after a meal, the carbohydrates are digested and broken down into glucose and other sugars, which are absorbed into the bloodstream. As the blood glucose levels increase, beta cells in the pancreas response by secreting insulin into the blood. The glucose then passes into cells and the liver shuts down glycolysis. Between meals, insulin also prevents excessive release of glucose from the liver into the bloodstream. If blood glucose levels drop too low between meals, alpha cells in the pancreas release a hormone called glucagon. This hormone signals the liver to convert amino acids and glycogen into glucose which are then secreted into the blood.

Figure 4.8 a shows glutathione peroxidase (GPx) activity, an enzymatic antioxidant that was significantly decreased in untreated diabetic rats. Administration of *L. leucocephala* extract at both doses showed a significant increase in GPx activity compared to untreated diabetic rats ($p < 0.001$). These finding was similar to that reported by Bagri, Ali, Aeri, Bhowmik, and Sultana (2009) where GPx activity in the pancreas decreased in diabetic group increased with *Punica granatum* flower extract at dose of 250 mg kg^{-1} and 500 mg kg^{-1} treatment (Bagri, Ali, Aeri, Bhowmik, & Sultana, 2009).

Diabetic rats treated with Glibenclamide at a dose 1.25 mg kg^{-1} also showed significantly high glutathione peroxidase activity ($p < 0.001$) as compared to negative control.

Total antioxidant level in pancreas was significantly high ($p < 0.001$) in normal healthy rats but was reduced in diabetic (untreated) rats. Significant increased of total antioxidant in both doses of *L. leucocephala* treated diabetic rats ($p < 0.001$) were observed (*Figure 4.8 b*).

Diabetic untreated groups showed increased protein oxidation in pancreas and *L. leucocephala* at both doses reduced the protein oxidation as compared to negative control ($p < 0.001$). However, Glibenclamide treated group significantly showed higher protein oxidation in pancreas, even higher than negative control group ($p < 0.001$) (*Figure 4.8 c*). Meanwhile, the lipid peroxidation in pancreas of all diabetic rats treated with *L. leucocephala* and glibenclamide were restored to the levels of non-diabetic control rats ($p < 0.001$) (*Figure 4.8 d*). Similarly by Yazdanparast *et. al.,* (2007) reported that protein oxidation in pancreas of diabetic rats was significantly increased compared to control ($p < 0.05$) and treatment with 100 mg kg^{-1} *Achillea santolina* for a period of 30 days significantly reduced pancreas protein oxidation ($p < 0.05$) and lipid peroxidation in pancreas ($p < 0.05$).

The antioxidants in *L. leucocephala* confers protective effect by combating the radical attacks on protein and lipid. The protective effects on pancreas implicates the restoration of the remaining β-cells for survival. The pancreas is unique because cells somehow could regeneration of itself. *L. leucocephala* could stimulate more insulin production, that could be from the existing pancreatic cells or might be from regeneration of new cells. In view of the increase in insulin production (*Figure 4.6*), it is

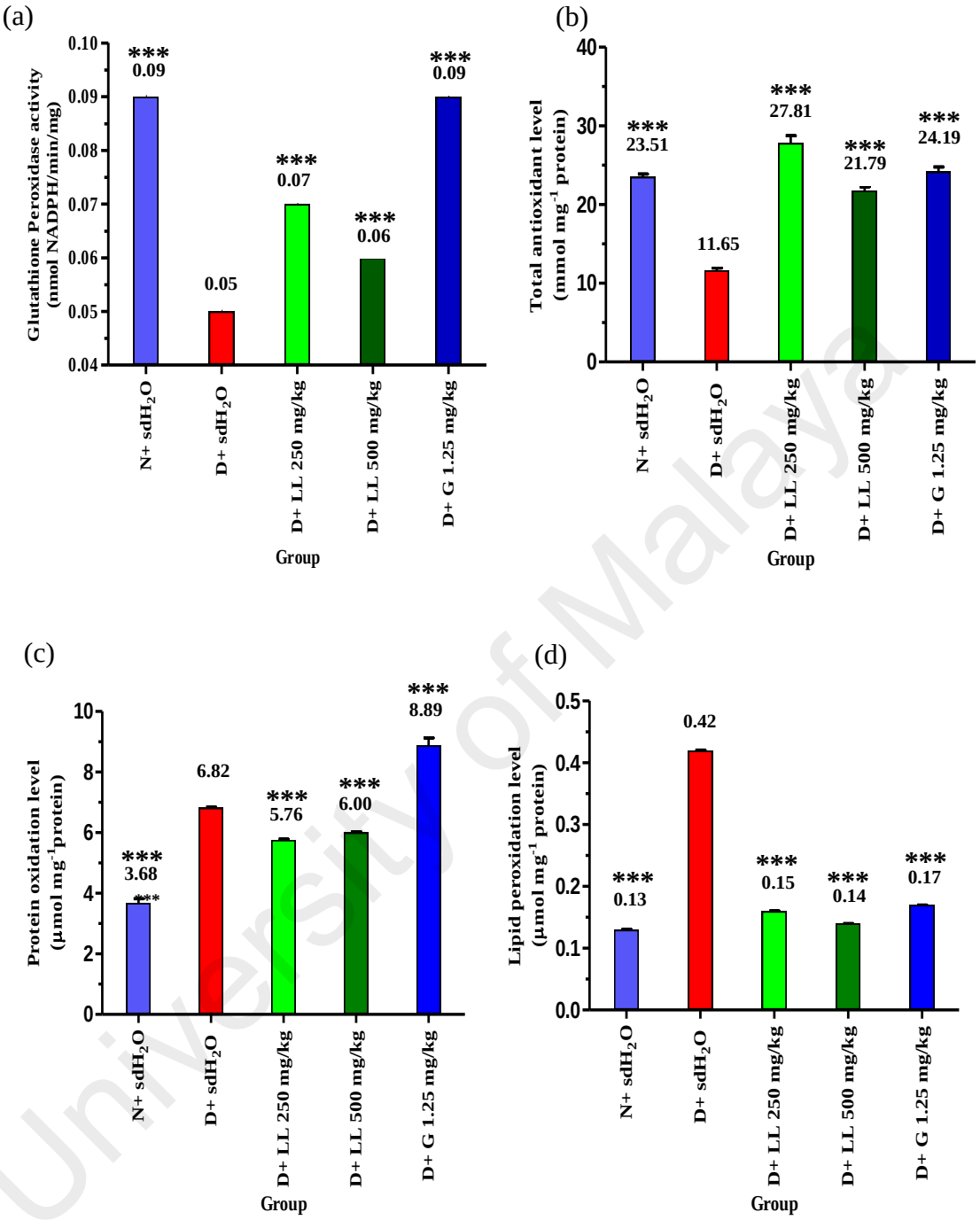
evident that *L. leucocephala* protects β-cells and its function.

The *in vivo* antioxidant protection conferred by *L. leucocephala* extract on pancreas in diabetic rats was due to high phenolic contents (Amarowicz, Naczk, & Shahidi, 2000) (data not shown).

Figure 4.8: Effect of *L. leucocephala* dry fruit water crude extract on enzymatic and non-enzymatic antioxidants pancreas tissue homogenate samples.

Figures shows the effect of *L. leucocephala* extract at 250 mg kg^{-1} and 500 mg $kg-^{1}$ on (a) Glutathione peroxidase activity level, (b) Total antioxidant level, (c) Protein oxidation and (d) Lipid peroxidation on rats pancreas in STZ-induced diabetic rats. The effect of *L. leucocephala* extract on oxidative indices namely Glutathione peroxidase method, FRAP method, protein oxidation (AOPP) and lipid peroxidation (TBARS) were measured according to the methods described in sections *3.9.5.4, 3.9.5.1, 3.9.5.2* and *3.9.5.3* respectively. Comparison were made between each group and negative control Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

Abbreviations: Normal rats treated with sterile distilled water (N+ sdH_2O), diabetic rats treated with sterile distilled water (D+ sdH_2O), diabetic rats treated with *Leucaena leucocephala* at dose of 250 mg per kg (D+ LL 250 mg/kg), diabetic rats treated with *Leucaena leucocephala* at dose of 500 mg per kg (D+ LL 500 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D + G 1.25 mg/kg).



4.3.6 Effects of *L. leucocephala* dry fruit water crude extract on oxidative indices in liver of Streptozocin-induced diabetic rats.

The liver maintains normal blood glucose concentration during fasting and postprandial states. In diabetic patients, the loss of insulin stimulates glycogenolysis and increase the hepatic glucose production. In animal diabetic models, it can be used to monitor the effects of potentially hepatotoxic drugs eg. Troglitazone.

The total antioxidant gives an overall antioxidant status in the body and synergistic effects of antioxidants provide greater protection against free radical attacks compared to any single antioxidant. It reflects the capacity of the body to overcome oxidative stress. However, the method used in this study (FRAP method) applicable for measurement of non-enzymatic total antioxidant only. In liver, enzymatic markers also could provide an insight in hepatocellular injury. However, the total antioxidant in the liver could be interpreted in comparison with control groups.

In untreated diabetic rats group, GPx activity was significantly reduced compared to normal rats ($p < 0.001$), similar to the report by Sarkhail *et. al*., 2007 ($p < 0.001$). *L. leucocephala* at a dose of 500 mg kg^{-1} showed high GPx activity compared to the negative control ($p < 0.001$) however, *L. leucocephala* at a dose of 250 mg kg^{-1} showed significant low level of GPx activity ($p < 0.001$) (*Figure 4.9 a).* The FRAP level in diabetic rat liver was negligeable but significantly increased in *L. leucocephala* treated diabetic rats (*Figure 4.9 b*). Thus, it is pertinent to suggest that *L. leucocephala* extract contains high reducing properties that could increase total antioxidant in the liver.

L. leucocephala at a dose of 250 mg kg^{-1} managed to reduce the protein oxidation in diabetic liver, as effectively as Glibenclamide treated group ($p < 0.001$) and attenuated lipid peroxidation ($p < 0.001$) (*Figure 4.9 c*).

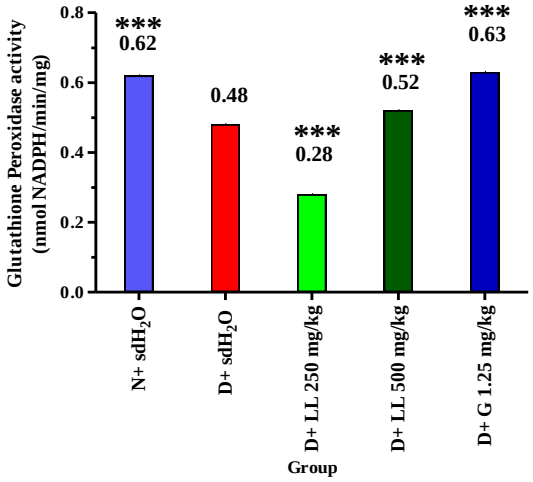
However, at a dose of 500 mg kg^{-1} *L. leucocephala* showed an increase of protein oxidation ($p < 0.001$) exceeded the positive control group and also in lipid peroxidation ($p < 0.001$) but still lower than Glibenclamide treated group (*Figure 4.9 c and d*). Lipid peroxidation in liver of untreated diabetic rats was significantly increased ($p < 0.001$) (Dewanjee, Das, Sahu, & Gangopadhyay, 2009; Jeong-Sook, 2005; Sarkhail et al., 2007; Sepici-Dincel, Aclkgoz, Cevik, Sengelen, & Yesilada, 2007).

Briefly concluded that *L. leucocephala* at a dose of 250 mg kg^{-1} exerted a protective effect on protein and lipid in liver but *L. leucocephala* at dose of 500 mg kg^{-1} did not exert protection on protein and lipid peroxidation in the liver.This might be due to liver play an important role in many metabolic and detoxifying processes that produce large amount of free radicals (Luxon, 2006). An increased dosage of treatment (500 mg/kg) might bring more burden to the liver due to the more free radicals produced during catabolism of the components of the extract. Some plant antioxidant have been reported to exert prooxidant effects (damaging to the tissue) at higher concentrations (Sakihama, Cohen, Grace, & Yamasaki, 2002).

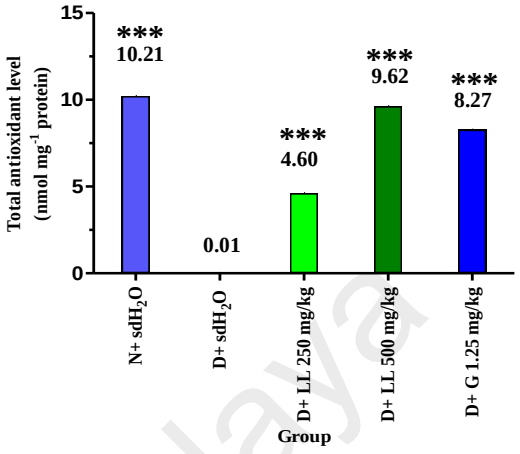
Figure 4.9: Effect of *L. leucocephala* dry fruit water crude extract on enzymatic and non-enzymatic antioxidants liver homogenates samples.

Figures shows the effect of *L. leucocephala* extract at 250 mg kg^{-1} and 500 mg kg^{-1} on (a) Glutathione Peroxidase activity level, (b) Total antioxidant level, (c) Protein oxidation and (d) Lipid peroxidation on rats liver in STZ-induced diabetic rats. The effect of *L. leucocephala* extract on oxidative indices namely Glutathione Peroxidase methods, FRAP method, protein oxidation (AOPP) and lipid peroxidation (TBARS) were measured according to the methods described in sections *3.9.5.4, 3.9.5.1, 3.9.5.2* and *3.9.5.3* respectively. Comparison were made between each groups and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

(a)



(b)



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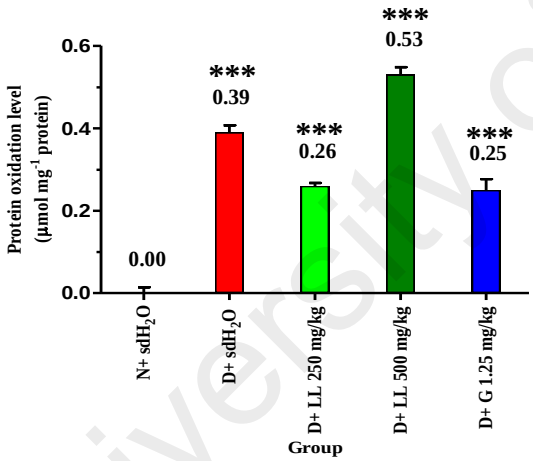

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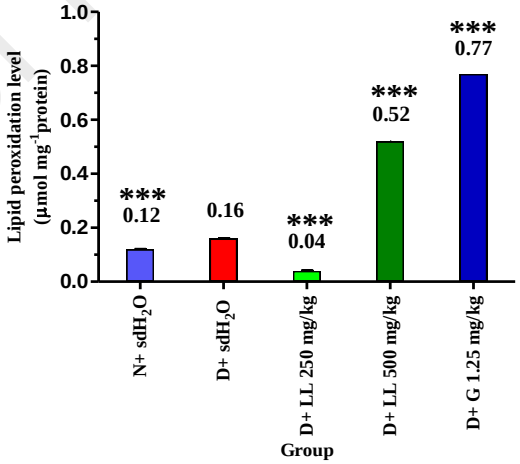


4.3.7 Effects of *Leucaena leucocephala* dry fruit water crude extract on oxidative indices in brain of Streptozocin-induced diabetic rats.

Brain is more susceptible to oxidative stress because it consumes high amount of oxygen, contains high level of polyunsaturated fatty acid and low level of antioxidant enzymes (Kapoor, Srivastava, & Kakkar, 2009). Schwann cells and axons are particularly sensitive to oxygen free radical damage where lipid peroxidation may increase cell membrane rigidity and impair cell function.

Many research had been done to investigate the implications of diabetes mellitus on the brain. Generally, diabetes patients experienced deficits on a wide range of cognitive tests including learning and memory, problem-solving, mental and motor speed. A study found that cerebral atrophy was significantly more frequent in patients with diabetes mellitus than in normal controls ($p > 0.005$) (Araki et al., 1994).

Glucose utilization is decreased in the brain during diabetes, providing a potential mechanism for increased vulnerability to acute pathological events. Hyperglycemia induces potential neuronal damage and this was observed following hypoxic / ischaemic events such as stroke (Makimattila et al., 2004). Hyperglycemia increases neuronal alterations and glial cell damage may be caused by temporary ischaemia. Chronic hyperglycemia contribute to nervous tissue damage and free radicals impair the central nervous system by attacking neurons, schwann cells and peripheral nerves.

The activity of GPx was significantly decreased in the brain of diabetic untreated rats, similarly found by Kapoor *et. al.*, (2009) and Pari and Latha, (2004) . GPx activity at both doses of *L. leucocephala* were significantly increased ($p < 0.001$) (*Figure 4.10 a*). Glibenclamide treated group also showed significant increased in GPx activity ($p < 0.001$), same found by Pari and Latha, (2004) which significantly increased ($p < 0.05$),

with dose 600 μg/kg treated on diabetic rats for 6 weeks. The total antioxidant level in diabetic untreated rats was significantly reduced meanwhile, at both doses of *L. leucocephala* the level was significantly increased ($p < 0.001$) (*Figure 4.10 b*). Diabetic untreated rats showed significantly high protein oxidation in contrast to normal rats. Protein oxidation in rats treated with *L. leucocephala* at both doses significantly reduced ($p < 0.001$) (*Figure 4.10 c*). In lipid peroxidation, diabetic untreated rats exhibited high lipid peroxidation ($p < 0.001$) compared to normal rats group. Diabetic rats treated with *L. leucocephala* at dose of 250 mg kg^{-1} exhibited decreased in lipid peroxidation ($p < 0.001$), but showed elevated level at dose of 500 mg kg^{-1} ($p < 0.001$) (*Figure 4.10 d*).

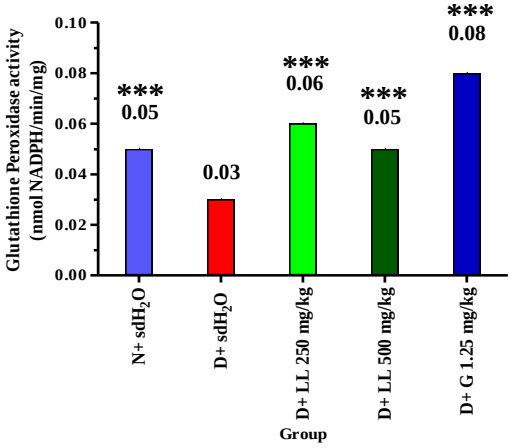
In general, *L. leucocephala* dry fruit juice water crude extract at a dose of 250 mg kg^{-1} exerted protective effects in protein and lipid of the brain as effectively as Glibenclamide at dose of 1.25 mg kg^{-1} but at dose of 500 mg kg^{-1} give less protection on lipid peroxidation in the brain of Streptozocin-induced diabetic rats.

Figure 4.10: Effect of *L. leucocephala* dry fruit water crude extract on enzymatic and non-enzymatic antioxidants brain homogenates samples.

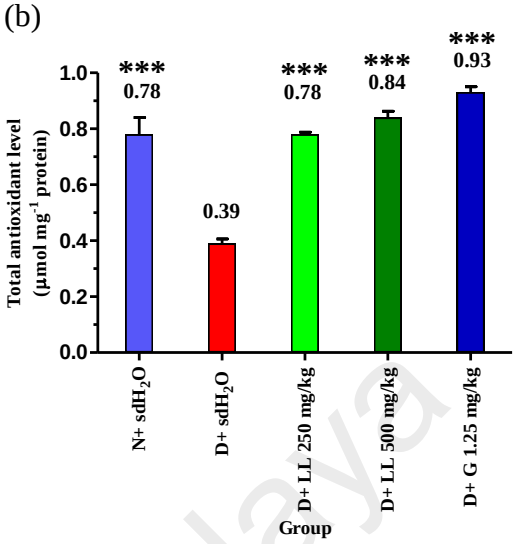
Figures shows the effect of *L. leucocephala* extract at 250 mg kg^{-1} and 500 mg kg^{-1} on (a) Glutathione Peroxidase activity level, (b) Total antioxidant level, (c) Protein oxidation and (d) Lipid peroxidation on rats brain in STZ-induced diabetic rats. The effect of *L. leucocephala* extract on oxidative indices namely Glutathione Peroxidase methods, FRAP method, protein oxidation (AOPP) and lipid peroxidation (TBARS) were measured according to the methods described in sections *3.9.5.4, 3.9.5.1, 3.9.5.2* and *3.9.5.3* respectively. Comparison were made between each groups and negative control Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

Abbreviations: Normal rats treated with sterile distilled water (N+ sdH_2O), diabetic rats treated with sterile distilled water (D+ sdH_2O), diabetic rats treated with *Leucaena leucocephala* at dose of 250 mg per kg (D+ LL 250 mg/kg), diabetic rats treated with *Leucaena leucocephala* at dose of 500 mg per kg (D+ LL 500 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D + G 1.25 mg/kg).

(a)



(b)



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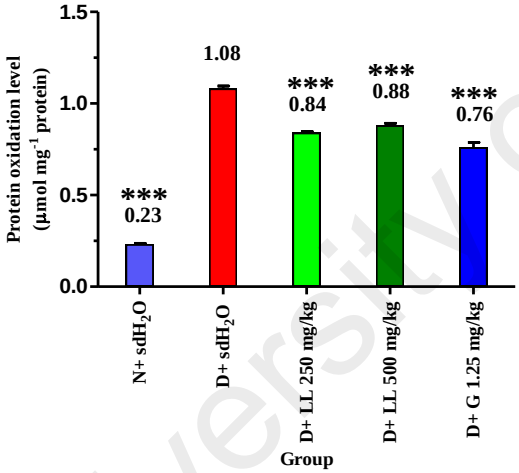
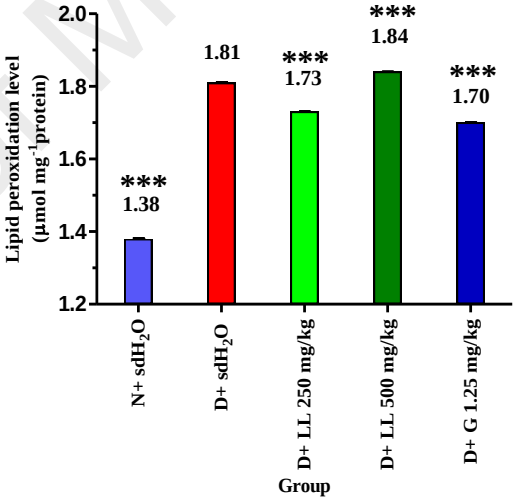


(d)



4.3.8 Effects of *L. leucocephala* dry fruit water crude extract on oxidative indices in kidney of Streptozocin-induced diabetic rats.

Kidney functions as the body's filtering system that removes wastes and toxins out of the blood and returns the cleaned blood back to the body. Inside each kidney is around one million nephrons. Each nephron consists of a small filter (glomerulus) attached to a tubule. Water that contains waste is separated from the blood by glomerulus and directed into the tubules. Much of the water is returned to the blood by the tubules, while the wastes are concentrated into urine. The urine is collected from the tubules by a funnel-like structure (renal pelvis). Then, the urine flows down in the ureter that joins each kidney to the bladder. Urine leaves the bladder *via* the urethra. Kidney failure is when kidneys are no longer able to remove waste and maintain the balanced level of fluid and salts in the body. Kidney failure is one of the complications of diabetes (Wiwanitkit, 2009).

When someone has diabetes, excess of glucose is present in the blood. Prolonged hyperglycemia condition will damage the glomerular that eventually leads to kidney failure. How hyperglycemia leads to the damage of the glomeruli is still unknown but hyperglycemia-induced oxidative stress and metabolic abnormalities might be involved (Kowluru, Abbas, & Odenbach, 2004). Many reports have indicated that diabetes patients are prone to nephropathy, independent of glycemic control (Wiwanitkit, 2009). Statistically, 30–40 % of patients with type I and 15 % with type II diabetes mellitus develop diabetic nephropathy (Ravid, Savin, Jutrin, Bental, Katz, & Lishner, 1993). A person with diabetes is susceptible to nephropathy whether they use insulin or not and the risk is dependant on genetic predisposition to hypertension (Krolewski et al., 1988). The risk of kidney failure is related to the length of time the person has diabetes. Diabetic nephropathy progresses steadily despite medical

intervention. However, treatment can significantly reduce the rate of damage and good metabolic control could delay the progression of nephropathy in diabetes ("The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group," 1993).

Brenner *et. al.,* (1982) proposed that diabetes with long-standing hyperglycemia leads to chronic renal vasodilation resulting in glomerular hyperfiltration, cellular injury, glomerulosclerosis, mesangial cell proliferation, and finally proteinuria and uremia. Hyperfiltration in diabetes patients is related to a reduction in both afferent and efferent arterial tone with increased renal blood flow and transcapillary pressure. Kidneys affected by diabetic nephropathy no longer work efficiently, and trace amounts of microalbuminuria will appear in the urine. Diabetic nephropathy patients will have increased renal plasma flow, increased glomerular filtration rate (GFR), and decreased renal vascular resistance.

In the present study, GPx activity in the kidney was significantly low in untreated diabetic rats ($p < 0.001$) but was significantly high in both *L. leucocephala* treated group ($p < 0.001$) (*Figure 4.11 a*). The FRAP level was significantly elevated in glibenclamide ($p < 0.001$) but in both doses of *L. leucocephala* treated groups the increased was not significant compared to diabetic untreated group (*Figure 4.11 b*). Diabetic rats exerted high protein oxidation in kidney (*Figure 4.11 c).* Suprisingly, *L. leucocephala* at dose 250 mg kg^{-1} showed increase in protein oxidation in the kidney but at a higher dose of 500 mg kg^{-1}, the level was comparable to the other groups. Diabetic untreated rats showed high lipid peroxidation in kidney ($p < 0.001$) (Kowluru *et al*., 2004) (*Figure 4.11 d*) similar to the report by Kapoor *et. al.,* (2009) and at both doses there was attenuation of lipid peroxidation in the kidney.

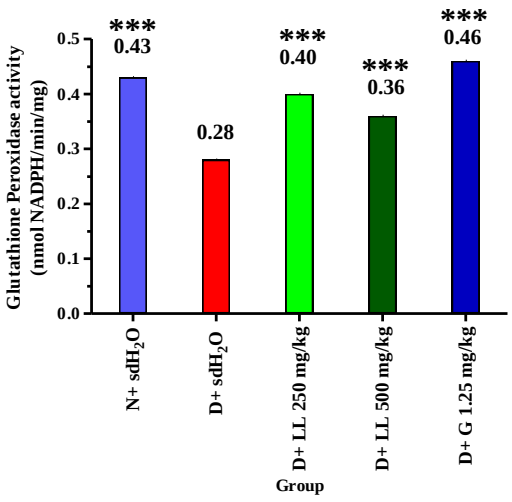
In general, *Leucaena leucocephala* dry fruit water crude extract showed a better protection against lipid peroxidation at a dose 500 mg kg^{-1}, but at lower dose of 250 mg kg^{-1} showed increased protein oxidation.

Figure 4.11: Effect of *L. leucocephala* dry fruit water crude extract on enzymatic and non-enzymatic antioxidants kidney homogenates samples.

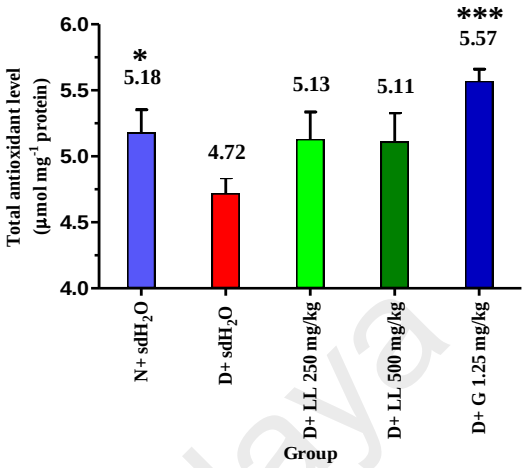
Figures shows the effect of *L. leucocephala* extract at 250 mg kg^{-1} and 500 mg kg^{-1} on (a) Glutathione peroxidase activity level, (b) Total antioxidant level, (c) Protein oxidation and (d) Lipid peroxidation on rats kidney in STZ-induced diabetic rats. The effect of *L. leucocephala* extract on oxidative indices namely Glutathione peroxidase methods, FRAP method, protein oxidation (AOPP) and lipid peroxidation (TBARS) were measured according to the methods described in sections *3.9.5.4, 3.9.5.1, 3.9.5.2* and *3.9.5.3* respectively. Comparison were made between each group and negative control Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

Abbreviations: Normal rats treated with sterile distilled water (N+ sdH_2O), diabetic rats treated with sterile distilled water (D+ sdH_2O), diabetic rats treated with *Leucaena leucocephala* at dose of 250 mg per kg (D+ LL 250 mg/kg), diabetic rats treated with *Leucaena leucocephala* at dose of 500 mg per kg (D+ LL 500 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D + G 1.25 mg/kg).

(a)



(b)



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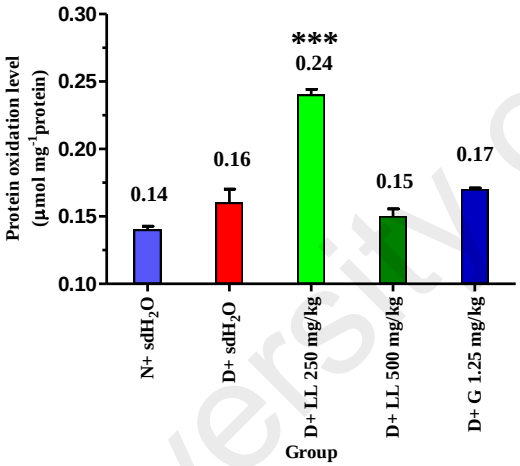

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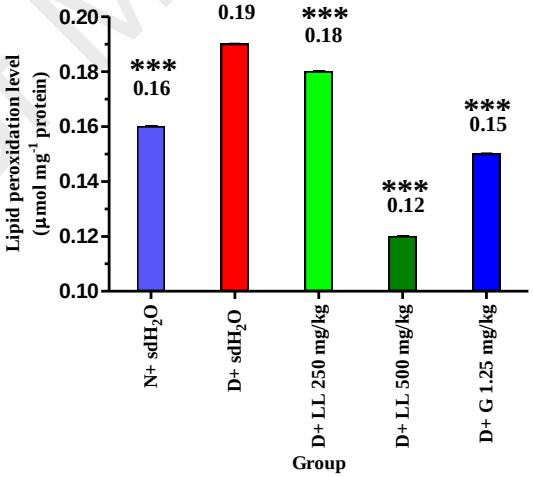


4.3.9 Effects of *L. leucocephala* dry fruit water crude extract on diabetic damages in liver pathology; histological evaluation.

Effects of *L. leucocephala* at dose of 250 mg kg^{-1} on diabetic rat liver showed normal central vein (blue arrow), normal hepatocytes and arrangements, no fatty change, very little RBC infiltration (red arrow) and little lymphocytes infiltration (white arrow) (*Figure 4.12 a*). At dose of 500 mg kg^{-1} exhibited normal central vein (blue arrow), normal hepatocytes, no fatty changes and very little RBC infiltration and dilated sinusoids (light orange arrow) (*Figure 4.12 d).* Effects of Glibenclamide 1.25 mg kg^{-1} on diabetic rats liver demonstrated moderate RBC congestion (red arrow) but less than negative control, lots of lymphocytes infiltration (white arrow) and very little fatty changes (yellow arrow). Meanwhile, effects of sterile distilled water 4 ml / mg kg^{-1} (placebo) on diabetic rats liver showed significant fatty changes (yellow arrow), lots of RBC congestion, lots of lymphocytes infiltration, cyst (brown arrow), shrunken nuclei (black arrow) and dilation sinusoids (light orange arrow) as shown in *Figure 4.12 e and b*.

The liver of control rats examined by light microscope revealed the same normal hepatic structure found in other mammals, that it is essentially formed of hepatic lobules. Each lobule is made up of radiating plates, strands of hepatic cells forming a network around a central vein (*Figure 4.12 a*). The liver strands with narrow sinusoids. These sinusoids have irregular boundaries composed of only a single layer of endothelial cells and large irregularly phagocytic cells, which are known as Kupffer cells. Outside the hepatic lobule at certain angle, lie the portal areas of connective tissue each including a hepatic portal vein, a branch of hepatic artery and a bile ductile .

Liver plays an important role in carbohydrate, fat and protein metabolism and

blood glucose level. Diabetes is known to produce substantial intracellular changes in most tissues including liver. The severity of the damage on the liver is based on duration of diabetes. Several research had shown that diabetes induced by Streptozocin exhibited loss of normal architecture of liver (Maisaa, 2007), dilation and inflammation in central and portal vein (Maisaa, 2007), severe fibrosis and leucocytic infiltration around the portal veins which appeared congested with blood (Maisaa, 2007), the sinusoids between hepatocytes were markedly dilated (NoorShahida, Wong, & Choo, 2009) with increased in Kupffer cells (Maisaa, 2007). The hepatocytes appeared to be suffering from certain degree of cloudily swelling with marked cytoplasmic vacuolations (Maisaa, 2007), nuclei of most cells revealed clear signs of pyknosis and karyolysis (Noor, Gunasekaran, Soosai, & Vijayalakshmi, 2008).

Noor *et. al.*, (2008) revealed that with Streptozocin dose of 30 mg kg^{-1}, diabetes rats showed shrunken nuclei, granular cytoplasm, dilation of sinusoids and inflammation in 3 weeks period of untreated diabetes and the effect was reduced with Aloe vera extract. The effect of treatment with *L. leucocephala* extract showed improvement in histological structure of liver sections of diabetic rats, pronounced in normalized appearance of liver lobules with strains of hepatocytes (*Figure 4.12 c and d*) compared with section of diabetic rats liver. The hepatocytes showed some degree of histological restoration, reduction in fat accumulation, less sinusoid dilation with decreased number of Kupffer cells and presence of less necrotic cells. This improvement was more pronounced in *L. leucocephala* 250 mg kg^{-1} treated rats.

Diabetes mellitus cause glycogen deposition, fibrosis and necroinflammatory (complications of therapy of diabetes) on a liver. The abnormalities of glucose homeostasis in diabetic patient cause hepatitis and hepatocellular carcinoma (Elgouhari, Zein, Hanouneh, Feldstein, & Zein, 2009).

Hepatic fat accumulation is a well-recognized complications of diabetes with a reported frequency of 40-70 %. Type I diabetes is not associated with fat accumulation if glycemia is well controlled, but type II diabetes may have a 70 % correlation regardless of blood glucose control and the degree of glycemic control does not correlate with the presence or absence of fat. Steatohepatitis is due to increased fat transport to the liver, enhanced hepatic fat synthesis, and decreased oxidation or removal of fat from the liver. This condition also can occur due to hepatotoxin, in addition to diabetes mellitus. In an animal model of type I diabetes, there is a high incidence of perisinusoidal hepatic fibrosis.

There is a rare association between the use of oral hypoglycemics and hepatic injury, but sulfonylureas can cause chronic hepatitis with necroinflammatory changes. Hepatocellular carcinoma may be associated with the development of hypoglycemia. Development of this hypoglycemia is related with the production of insulin-growth factor–II by hepatocellular carcinoma cells. The reason is still unclear.

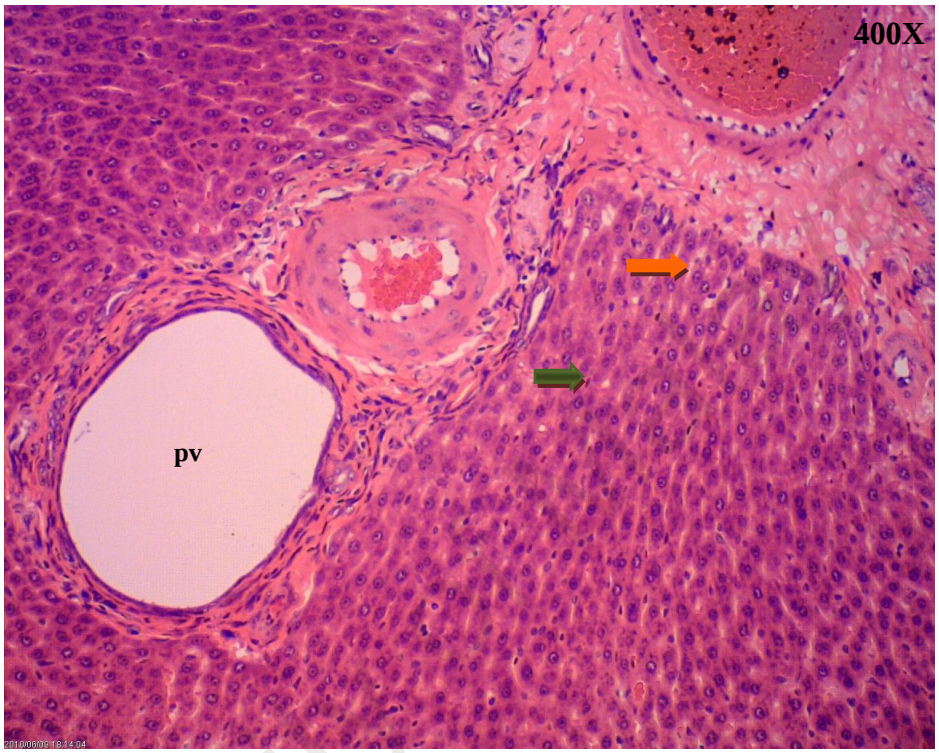
In summary, *Leucaena leucocephala* dry fruit water crude extract at both doses significantly reduced diabetic damages in liver pathology and more profoundly at a dose of 250 mg kg^{-1} and this finding was parallel with the effects of *L. leucocephala* in liver antioxidant status.

Figure 4.12: Effects of *L. leucocephala* dry fruit water crude extract at 250 mg kg^{-1} and 500 mg kg^{-1} on diabetic rats liver.

Panel (a)-(e) showed rats liver section stained with Hematoxylin and Eosin as described in method *Section 3.9.6*. The magnification was 400X for all panel. Panel (a) showed normal rats liver section. It showed normal hepatic structure, hepatic lobules, radiating plates or strands of cells, portal vein (pv), narrow sinusoids and Kupffer cells. Panel (b) showed the untreated diabetic rats liver. It showed lots of RBC congestion, increased in Kuppfer cells, karyolysis (ky) and pyknotic (pn) nuclei and dilation sinusoids.

Legends:
- Central vein
- Sinusoids
- Red blood cell infiltration
- Lymphocyte infiltration
- Kupffer cell
- Nuclei

(a)



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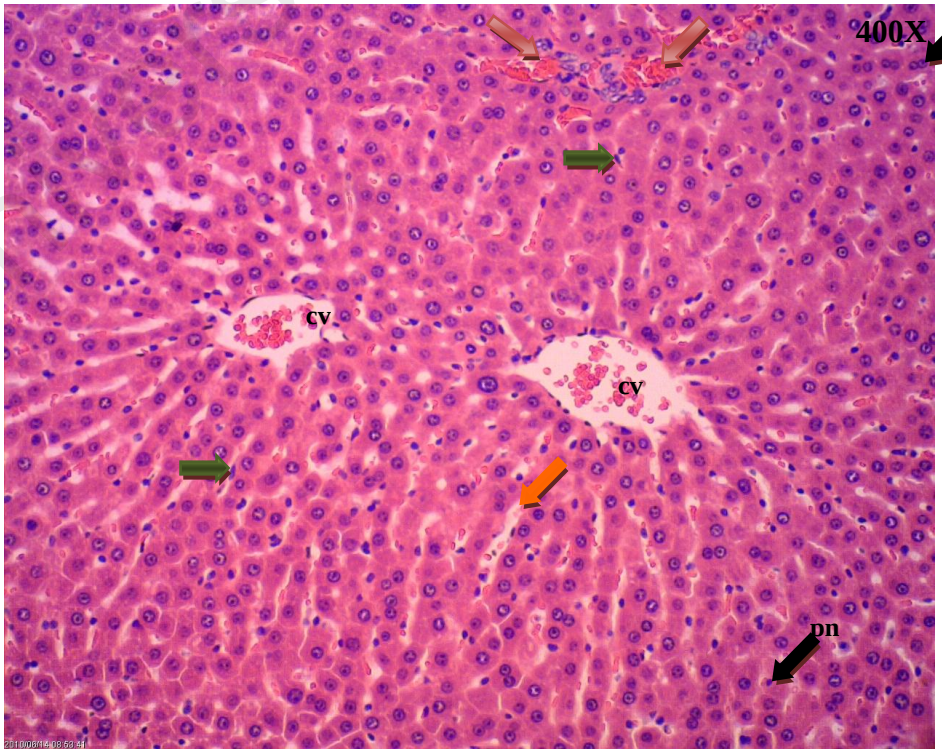
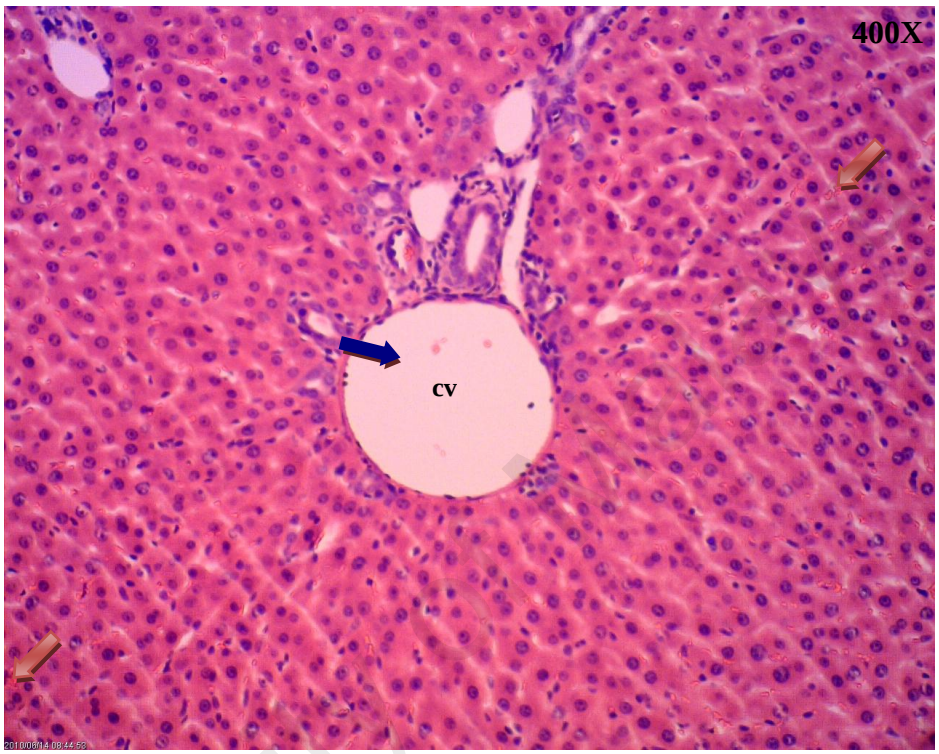


Figure 4.12: Effects of *L. leucocephala* dry fruit water crude extract at 250 mg kg^{-1} and 500 mg kg^{-1} on diabetic rats liver.

Panel (a)-(e) showed rats liver section stained with Hematoxylin and Eosin as described in method *Section 3.9.6*. The magnification was 400 X for all panel. Panel (c) showed the effects of *L. leucocephala* at 250 mg kg^{-1} on diabetic rats liver. Legends: normal central vein (cv), normal hepatocytes and arrangements, no fatty changes, very less RBC infiltration (red arrow). Panel (d) showed the effects of *L. leucocephala* at 500 mg kg^{-1} on diabetic rats liver. Legends: normal central vein (cv), normal hepatocytes, no fatty changes and very less RBC infiltration, dilated sinusoids (light orange arrow).

Legends:
- Central vein
- Sinusoids
- Red blood cell infiltration
- Lymphocyte infiltration
- Kupffer cell
- Nuclei

(c)



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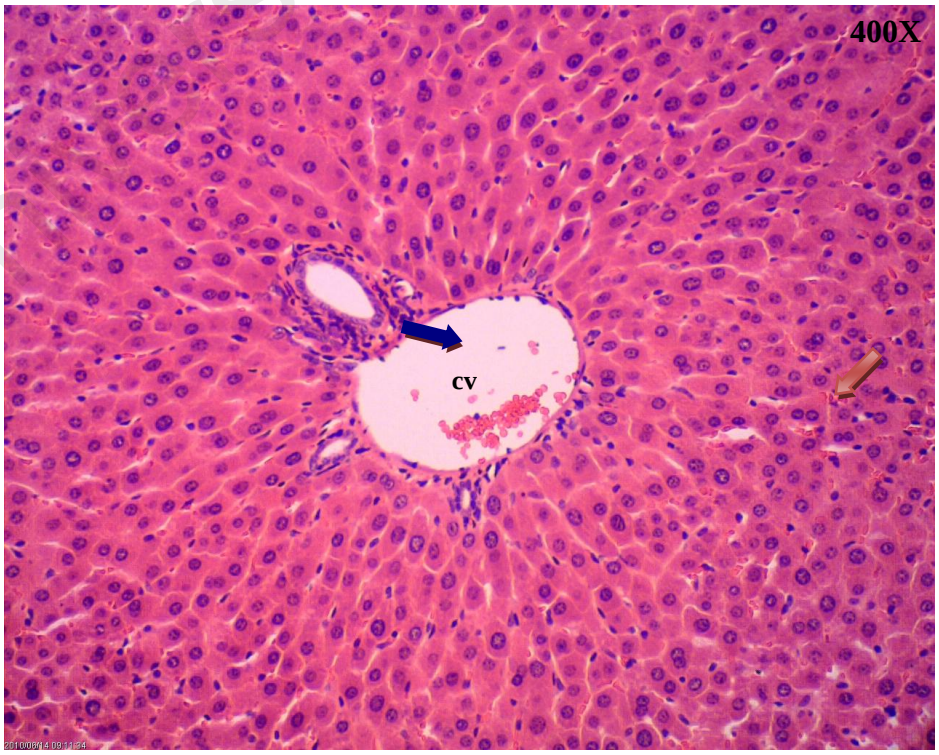
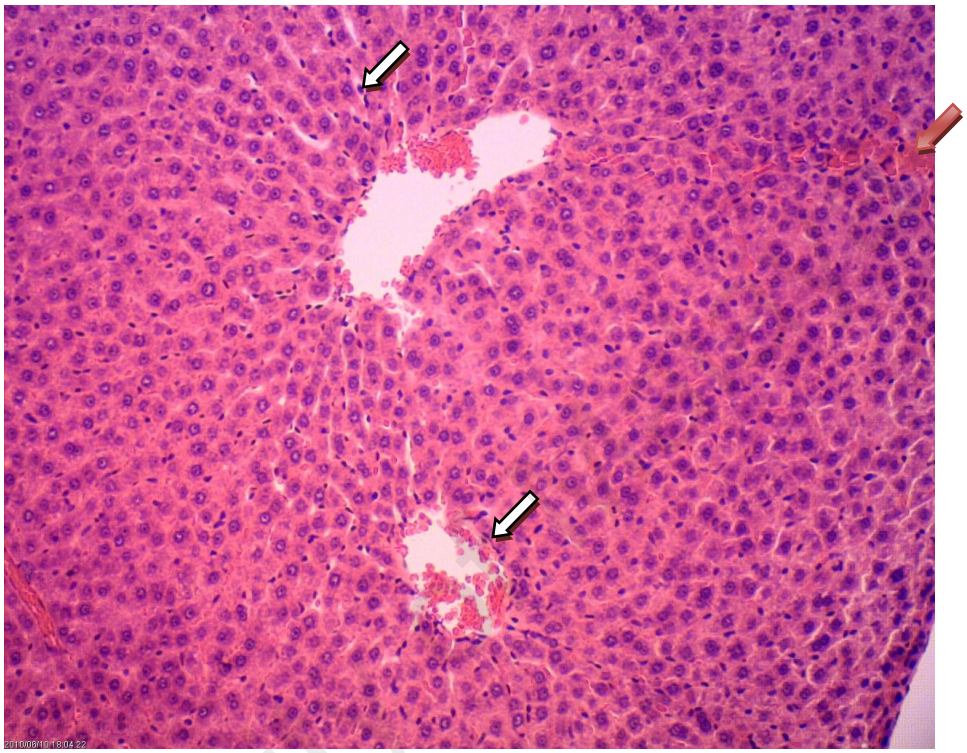


Figure 4.12: Effects of *L. leucocephala* dry fruit water crude extract at 250 mg kg^{-1} and 500 mg kg^{-1} on diabetic rats liver.

Panel (a)-(e) showed rats liver section stained with Hematoxylin and Eosin as described in method *Section 3.9.6*. The magnification was 400X for all panel. Panel (e) showed the effects of Glibenclamide 1.25 mg kg^{-1} on diabetic rats liver. Legends: moderate RBC congestion (red arrow) but less compared to negative control, lots of lymphocytes infiltration (white arrow).

Legends:
- Central vein
- Sinusoids
- Red blood cell infiltration
- Lymphocyte infiltration
- Kupffer cell
- Nuclei

(e)



4.3.10 Effects of *L. leucocephala* dry fruit water crude extract on diabetic damages in kidney pathology; histological evaluation.

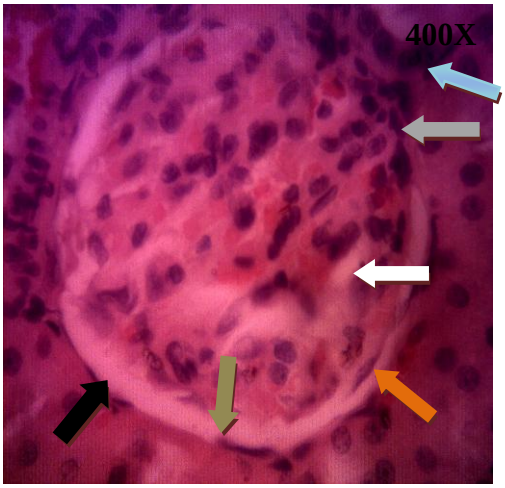
The kidney function was described in *Section 4.3.8*. In diabetes, vascular endothelium appears to be the initial site of injury. At the initial stage of diabetes kidney injury, glomerular lesions such as oedema, expansion of glomerular capillary lumen and enhanced glomerular perfusion will occur. Diffuse glomerulosclerosis is a thickening of the glomerular basement membrane due to the increased production with increased permeability. Hyalinization of the glomerulus will occur after an increase in a number of mesangial cells followed by the replacement of entire glomerulus with matrix. Later, the glomerular basement membrane, mesangial matrix and tubular basement membranes will thicken due to binding of albumins and non-specific protein. With time, the mesangial space becomes larger by deposits of proteins (collagen IV), initially diffuse, then become nodular. In renal vascular lesions, arteriolar sclerosis of both afferent and efferent arterioles at the glomerular pole will occur. *Figure 4.13* exhibits glomerular changes in different treatment groups in diabetic rats. *Figure 4.13* (a) shows normal glomerulus of rats. After 30 days treatment, the untreated diabetic group exhibited the worst dilation of capillaries and increase in capsular space size that seems to be due to loss of glomerular membrane (*Figure 4.13 b*). In *L. leucocephala* treated group dose 250 mg kg^{-1} showed protection from diabetes induced vascular and glomerulus changes (*Figure 4.13 c*) but, *L. leucocephala* at dose of 500 mg kg^{-1} exerted slight dilation of capillaries (*Figure 4.13 d*). In Glibenclamide treated group (1.25 mg kg^{-1}) showed enlargement of glomerulus/capsular space and dilated capillaries. Thus, in general, *L. leucocephala* dry fruit water crude extract significantly reduced diabetic damages in kidney pathology and more profoundly at dose of 250 mg kg^{-1}.

Figure 4.13: Effects of *Leucaena leucocephala* dry fruit water crude extract at 250 mg kg^{-1} and 500 mg kg^{-1} on diabetic rats' kidney.

Panel (a)-(e) showed rats kidney section stained with Hematoxylin and Eosin as described in method *Section 3.9.6*. The magnification was 400X for all panel. Panel (a) showed the effects of sterile distilled water 4 ml mg kg^{-1} (placebo) on normal rats kidney. Legends: normal capillaries (white arrow), normal capsular space (black arrow), normal podocytes (orange arrow), macula densa (blue arrow), parietal blade of Bowman's capsule (green arrow), mesangial cells (grey arrow). Panel (b) showed the untreated diabetic rats kidney. Legend: moderately dilated of capillaries (white arrow) and increased in capsular space (black arrow). Panel (c) showed the effects of *Leucaena leucocephala* at dose of 250 mg kg^{-1} on diabetic rats' kidney. Panel (d) showed the effects of *Leucaena leucocephala* at 500 mg kg^{-1} on diabetic rats' kidney. Legend: slightly dilated of capillaries (white arrow). Panel (e) showed the effects of Glibenclamide 1.25 mg kg^{-1} on diabetic rats kidney. Legends: enlargement of glomerulus / capsular space (black arrow) and dilated capillaries (white arrow).

Legends:
- capillaries
- capsular space
- podocytes
- macula densa
- parietal blade of Bowman's capsule
- mesangial cell

(a)



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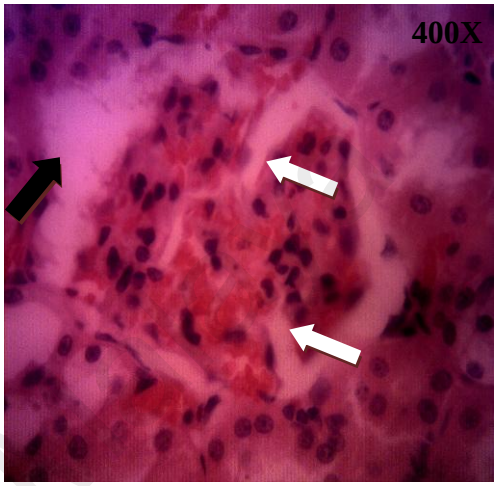

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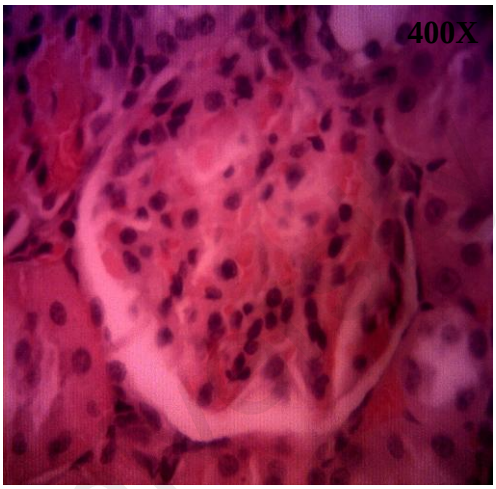
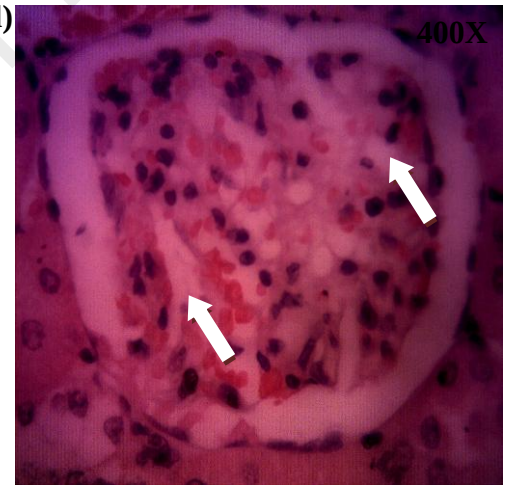
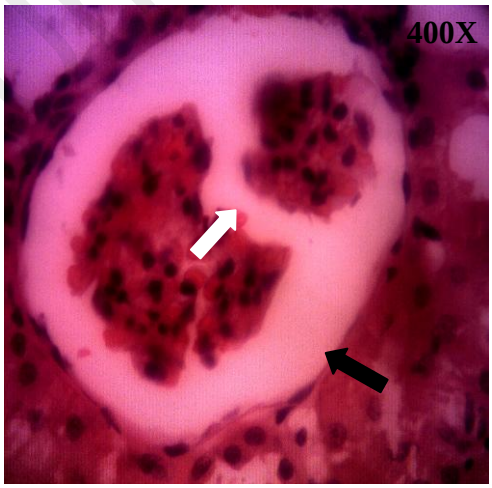


(d)



(e)



4.4 Assessment of *Leucaena leucocephala* water-solvents partially purified extract for anti-diabetic and antioxidant; sub-chronic study.

4.4.1 Effect of *L. leucocephala* water-hexane partially purified extract on glucose level in Streptozocin-induced diabetic rats; sub-chronic study.

Figure 4.14 showed increment of glucose level in all groups of *Leucaena leucocephala* treated rats ($p < 0.001$) excluding diabetic group treated with *L. leucocephala* water-hexane partially purified extract at dose of 25 mg kg^{-1} ($p < 0.001$). The Glibenclamide treated group also showed significant reduction after seven day treatment ($p < 0.001$). The percentage of changes in glucose level in the group was -57.79 %, even better than Glibenclamide treated group -22.04 %. The result presented in *Figure 4.15* exhibited that *L. leucocephala* water-hexane partially purified extract at dose of 25 mg kg^{-1} was effective in controlling blood glucose level in diabetic rats. This confirmed *L. leucocephala* water-hexane partially purified extract contained the most bioactive compound and 25 mg kg^{-1} is an effective hypoglycemic dose.

Abbreviations: Normal rats treated with normal saline (N+ Normal saline), diabetic rats treated with normal saline (D+ Normal saline), diabetic rats treated with *Leucaena leucocephala* partially purified water-hexane at dose of 25 mg per kg (D+ LL He 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-ethyl acetate at dose of 25 mg per kg (D+ LL Et 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-aqueous at dose of 25 mg per kg (D+ LL Aq 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-hexane at dose of 50 mg per kg (D+ LL He 50 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-ethyl acetate at dose of 50 mg per kg (D+ LL Et 50 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-aqueous at dose of 50 mg per kg (D+ LL Aq 25 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D+ G 1.25 mg/kg).

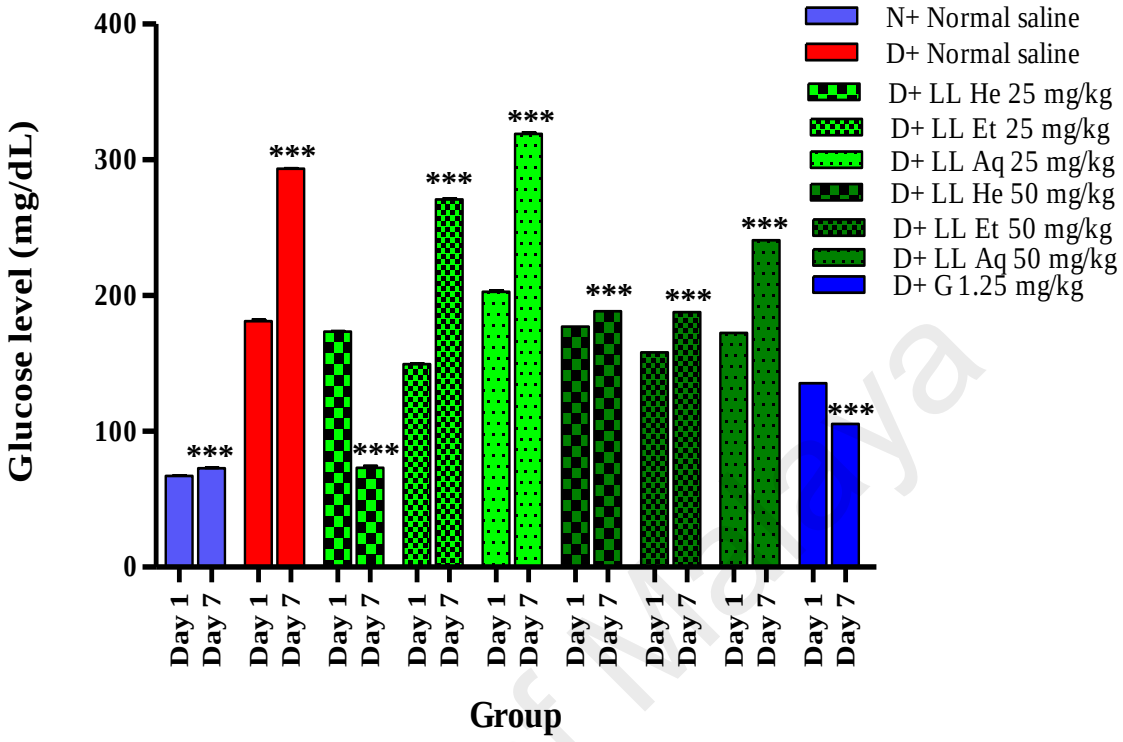


Figure 4.14: Effect of *L. leucocephala* water-solvents partially purified extracts on glucose level in STZ-induced diabetic rats; sub-chronic study.

The changes in blood glucose were measured in milligram per decilitre at days 1 and 7. Each different bar colour and pattern represented different group. The comparison made between different days in each group. Results present are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

4.4.2 Effect of *L. leucocephala* water-hexane partially purified extract on body weight in Streptozocin-induced diabetic rats; sub-chronic study.

Figure 4.15 shows percentage changes of body weight in STZ-induced diabetic rats in sub-chronic study. In diabetic groups treated with *L. leucocephala*, prevention of weight loss was observed (dose 50 mg kg^{-1} > dose 25 mg kg^{-1}). The reduction of body weight after seven days treatment was similar to that observed at the same period using crude water extract in chronic study.

In different solvent extraction, protection from losing weight was as followed;

LL Aq > *LL* Et > *LL* He

Leucaena leucocephala water-hexane partially purified extract at dose of 25 mg kg^{-1} significantly reduced blood glucose level but did not promote increment on body weight in short-term period (7 days). However, we could not predict the effect of long-term consumption. These findings could be used to control body weight as according to the patient status e.g.: obese with diabetes.

Abbreviations: Normal rats treated with normal saline (N+ Normal saline), diabetic rats treated with normal saline (D+ Normal saline), diabetic rats treated with *Leucaena leucocephala* partially purified water-hexane at dose of 25 mg per kg (D+ LL He 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-ethyl acetate at dose of 25 mg per kg (D+ LL Et 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-aqueous at dose of 25 mg per kg (D+ LL Aq 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-hexane at dose of 50 mg per kg (D+ LL He 50 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-ethyl acetate at dose of 50 mg per kg (D+ LL Et 50 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-aqueous at dose of 50 mg per kg (D+ LL Aq 25 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D+ G 1.25 mg/kg).

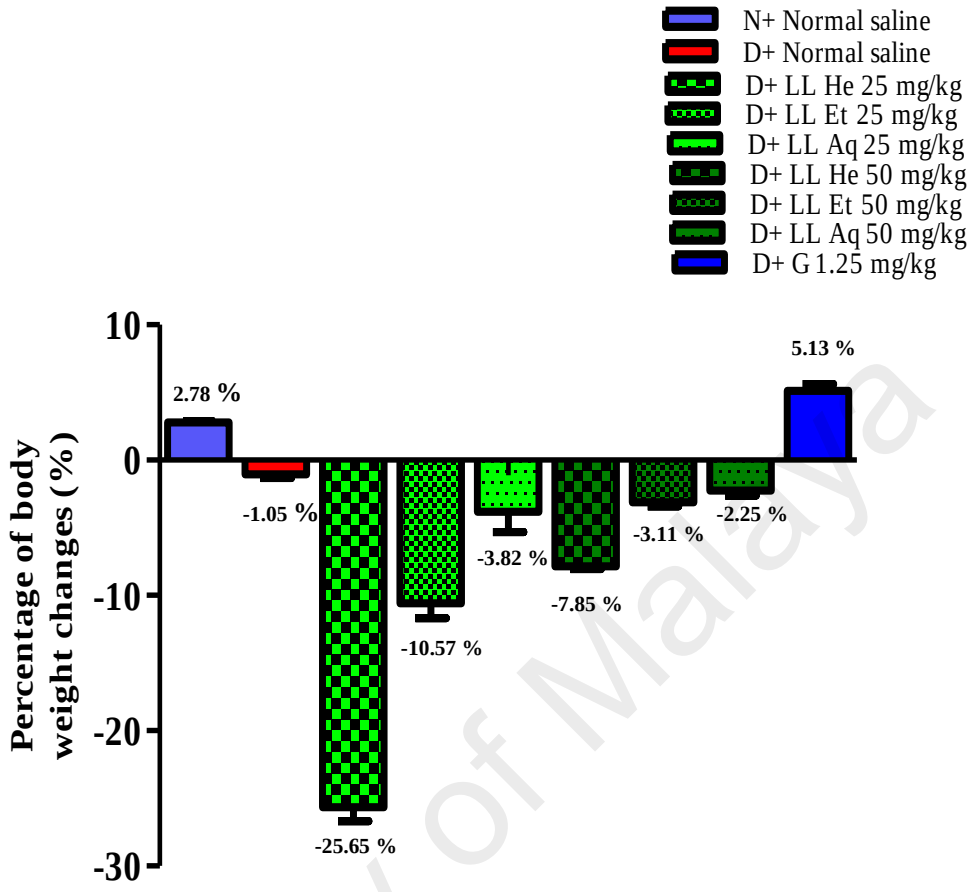


Figure 4.15: Effects of *L. leucocephala* water-solvents partially purified extracts on percentage change in body weight in STZ-induced diabetic rats; sub-chronic study.

Each different bar colour and pattern represent different group. The percentage of changes in body weight were calculated based on the formula shown in *Section 3.9.4.2*. The results were calculated between day 1 and 7 in each group and compared with negative control. Results presented are mean ± SEM.

4.4.3 Effect of *L. leucocephala* water-hexane partially purified extract on insulin level in Streptozocin-induced diabetic rats; sub-chronic study.

Figure 4.16 exhibits effect of *L. leucocephala* partially purified extracts on insulin level in STZ-induced diabetic rats in sub-chronic study. The diabetic rats treated with normal saline group (negative control) exhibited a reduction in insulin level contrary to normal rats group. In diabetic rats treated with both dose of *L. leucocephala* (50 mg kg^{-1} and 25 mg kg^{-1}) and Glibenclamide treated group showed a significantly raised insulin ($p < 0.001$) levels compared to negative control. The order of potency in descending order is as follows;

LL He 25 > G 1.25 > *LL* He 50 = *LL* Et 50 > *LL* Aq 50 > Normal rats

It was well-understood that secretion of more insulin production helps in cellular glucose-uptake which in turn reduced the hyperglycemia in diabetic rats. This insulin finding result was in line with the reduction in blood glucose level in *L. leucocephala* water-hexane partially purified at dose of 25 mg kg^{-1} and Glibenclamide treated groups.

Abbreviations: Normal rats treated with normal saline (N+ Normal saline), diabetic rats treated with normal saline (D+ Normal saline), diabetic rats treated with *Leucaena leucocephala* partially purified water-hexane at dose of 25 mg per kg (D+ LL He 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-ethyl acetate at dose of 25 mg per kg (D+ LL Et 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-aqueous at dose of 25 mg per kg (D+ LL Aq 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-hexane at dose of 50 mg per kg (D+ LL He 50 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-ethyl acetate at dose of 50 mg per kg (D+ LL Et 50 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-aqueous at dose of 50 mg per kg (D+ LL Aq 25 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D+ G 1.25 mg/kg).

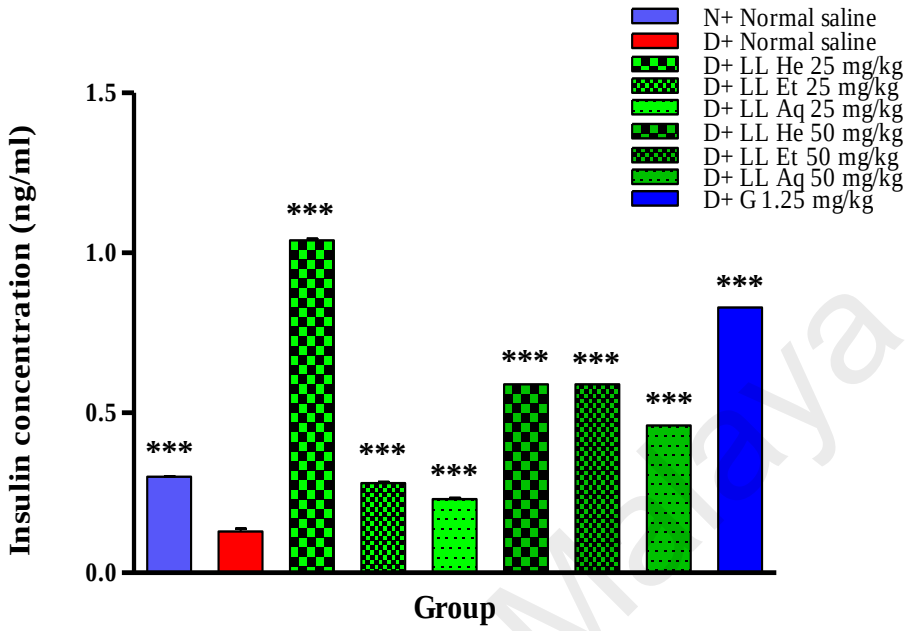


Figure 4.16: Effect of *L. leucocephala* water-solvents partially purified extracts on insulin level in STZ-induced diabetic rats; sub-chronic study.

The insulin test was performed on pooled serum samples using method in *Section 3.9.4.3*. Comparison were made between each group and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

4.4.4 Effect of *L. leucocephala* water-hexane partially purified extract on adiponectin concentration in Streptozocin-induced diabetic rats; sub-chronic study.

Figure 4.17 exhibits concentration of adiponectin in nanogram per millilitre in each group tested on serum pooled samples. Mostly, experimental rats experienced weight loss when they became diabetic (STZ-induced) due to lack of insulin and oxidative stress.

Previous *in vitro* lipolysis cell culture study showed that as the concentration of *L. leucocephala* crude fruit extract increased (0.1 to 100 μg/ml), percentage of lipolysis in adipocytes decreased (Lim, 2007). Based on this finding, lipolysis was expected to highly occur at dose 25 mg kg^{-1} than 50 mg kg^{-1}. Therefore, adiponectin was expected to be more concentrated at dose 50 mg kg^{-1} rather than 25 mg kg^{-1} because adiponectin was secreted exclusively by adipose tissue.

Adiponectin directly or indirectly affects obesity-linked diseases, however in this study, the rats was non-obese and without insulin resistance. Thus, in this case, the level of adiponectin reflects more the status of improvement or reduction in rats body weight.

Result showed that the diabetic group had significantly reduced adiponectin concentration (1137.65 ± 17.07 ng/ml) similar to the report by Guo and Zhao, (2007) in plasma samples. In contrast, normal rats showed highest adiponectin level (2934.13 ± 44.01 ng/ml). The diabetic rats treated with *L. leucocephala* water-hexane partially purified extract at both dose, *L. leucocephala* water-ethyl acetate partially purified (dose 25 mg kg^{-1}) and diabetic rats treated with glibenclamide group showed significantly reduced adiponectin concentration level ($p < 0.001$) compared to negative

control. There were no significant correlation between glucose regulation and adiponectin (p =0.321) and between insulin and adiponectin (p =0.358) in sub-chronic study. However, in this sub-chronic study, adiponectin concentration was positively correlated with percentage of changes in rats body weight (p <0.05) (*Appendix J*).

Abbreviations: Normal rats treated with normal saline (N+ Normal saline), diabetic rats treated with normal saline (D+ Normal saline), diabetic rats treated with *Leucaena leucocephala* partially purified water-hexane at dose of 25 mg per kg (D+ LL He 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-ethyl acetate at dose of 25 mg per kg (D+ LL Et 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-aqueous at dose of 25 mg per kg (D+ LL Aq 25 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-hexane at dose of 50 mg per kg (D+ LL He 50 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-ethyl acetate at dose of 50 mg per kg (D+ LL Et 50 mg/kg), diabetic rats treated with *Leucaena leucocephala* partially purified water-aqueous at dose of 50 mg per kg (D+ LL Aq 25 mg/kg), diabetic rats treated with glibenclamide at dose of 1.25 mg per kg (D+ G 1.25 mg/kg).

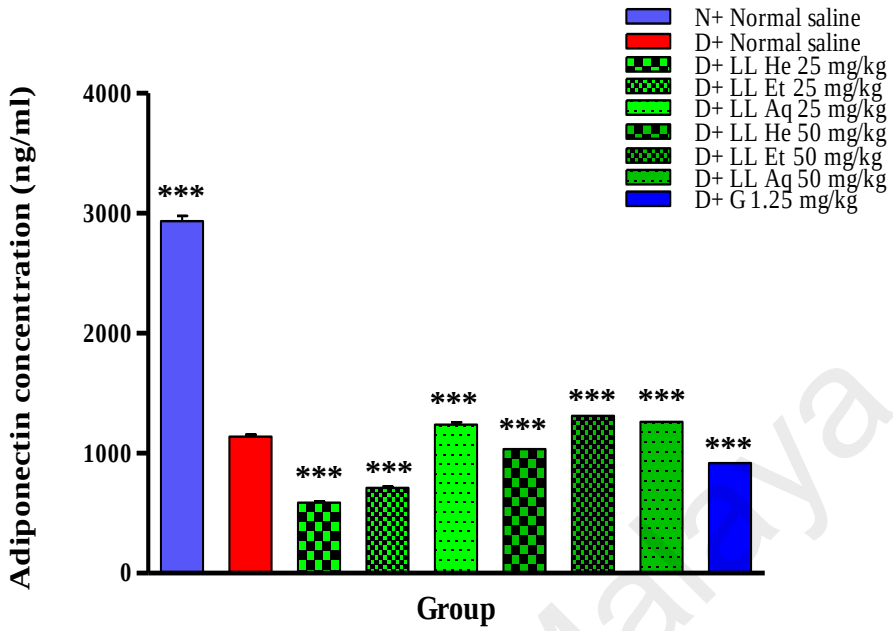


Figure 4.17 : Effect of *L. leucocephala* water-solvents partially purified extracts on adiponectin level in STZ-induced diabetic rats; sub-chronic study.

The adiponectin test was performed on pooled serum samples using method described in *Section 3.9.4.4*. Comparison were made between each group and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

Glutathione peroxidase in pancreas

G 1.25 > *LL* He 50 > *LL* Aq 50

17.54 > 9.39 > 5.78

Total antioxidant in pancreas

G 1.25 > *LL* He 25 > *LL* He 50 > *LL* Aq 25 > *LL* Et 25 > *LL* Aq 50 > *LL* Et 50

45.41 > 34.00 > 33.65 > 30.97 > 29.44 > 25.73 > 13.73

Protein oxidation in pancreas

LL Et 25 > *LL* He 25 > *LL* Aq 25 > G 1.25 > *LL* Aq 50 > N. saline > *LL* Et 50 > *LL* He 50

31.75 > 36.04 > 39.13 > 40.17 > 74.42 > 80.67 > 83.08 > 84.16

Lipid peroxidation in pancreas

LL He 25 > *LL* Et 25 > *LL* Aq 25 > *LL* Aq 50 > *LL* Et 50 > N. saline > G 1.25

10.27 > 20.44 > 23.00 > 24.15 >32.56 > 33.00 > 34.70

Figure 4.18 : The order of potency in descending order for glutathione peroxidase activity, total antioxidant, protein oxidation and lipid peroxidation level in pancreas; sub-chronic study.

The unit use for glutathione peroxidase are nmol NADPH/min/mg of protein and for all non-enzymatic antioxidants are µmol mg^{-1} protein.

Abbreviations: refer to page 153.

4.4.5 Effect of *L. leucocephala* water-hexane partially purified extract on oxidative indices in pancreas of Streptozocin-induced diabetic rats: sub-chronic study.

Figure 4.19 a shows effect of *L. leucocephala* water-solvents partially purified extracts on Glutathione peroxidase (GPx) level in pancreas of STZ-induced diabetic rats; sub-chronic study. Glutathione peroxidase activity was extremely increased in Glibenclamide diabetic rats treated group (17.54 nmol NADPH/min/mg) compared to diabetic rats treated with normal saline, that was almost zilch. The diabetic rats treated with *L. leucocephala* water-hexane partially purified and water-aqueous partially purified at dose of 50 mg kg^{-1} showed high activity of GPx (9.39 nmol NADPH/min/mg, $p < 0.001$ and 5.78 nmol NADPH/min/mg, $p < 0.001$ respectively).

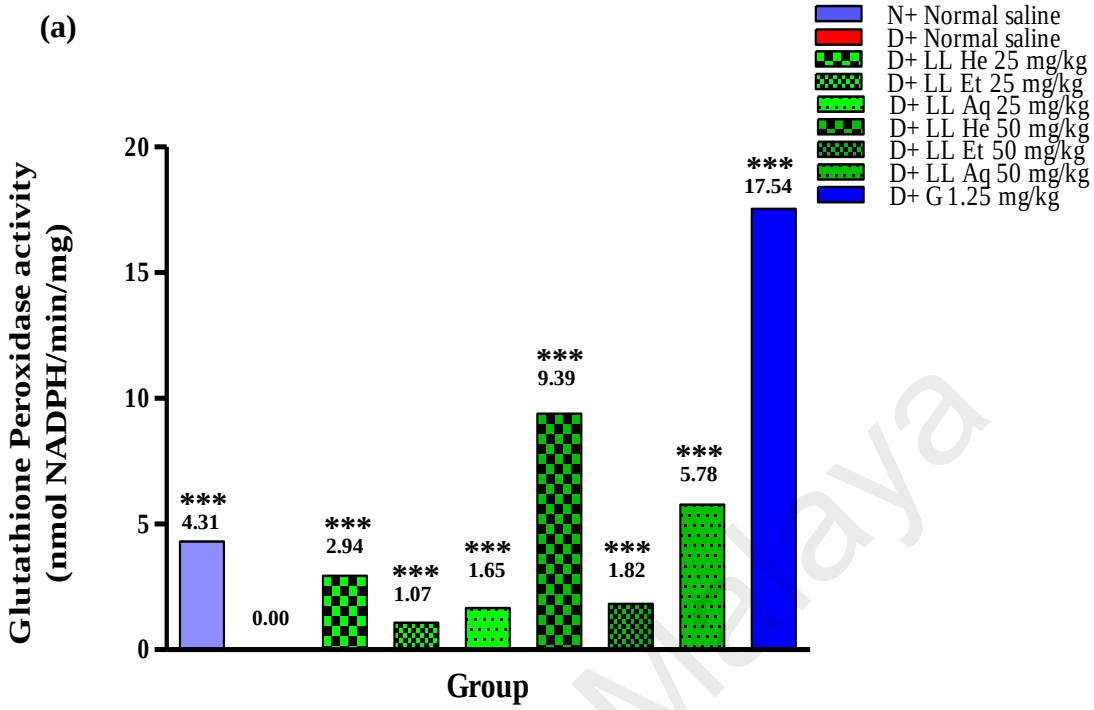
Figure 4.19 b, c and *d* shows total antioxidant, protein oxidation and lipid peroxidation in pancreas of STZ-induced diabetic rats; sub-chronic study. The total antioxidant shows high total antioxidant at both dose of *L. leucocephala* compared to negative control.. In protein oxidation, low level of protein oxidation shows better protection effect. Generally, *L. leucocephala* treated group at dose 25 mg kg^{-1} shows higher total antioxidant, low protein oxidation and low lipid peroxidation than 50 mg kg^{-1}.

In conclusion, among the different solvents and between two doses*; L. leucocephala* water-hexane partially purified at dose 25 mg kg^{-1} treated group posess the highest total antioxidant that could provide protection against protein and lipid damage in pancreas.

Figure 4.19: Effect of *L. leucocephala* water-solvents partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles on homogenized pancreas pooled samples; sub-chronic study.

The effect of *L. leucocephala* water-hexane partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles were measured on homogenized pancreas pooled samples by using Glutathione peroxidase methods (*Section 3.9.5.4*), total antioxidant using FRAP method (*Section 3.9.5.1*), protein oxidation using AOPP method (*Section 3.9.5.2*) and lipid peroxidation using MDA method (*Section 3.9.5.3*) in sub-chronic study. Panel (a) shows the effect of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on Glutathione peroxidase activity level in pancreas in STZ-induced diabetic rats. Panel (b) shows the effects of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats pancreas FRAP. Comparison were made between each group and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; ** denotes $p < 0.01$ and *** denotes $p < 0.001$.

Abbreviations: refer to page 153.



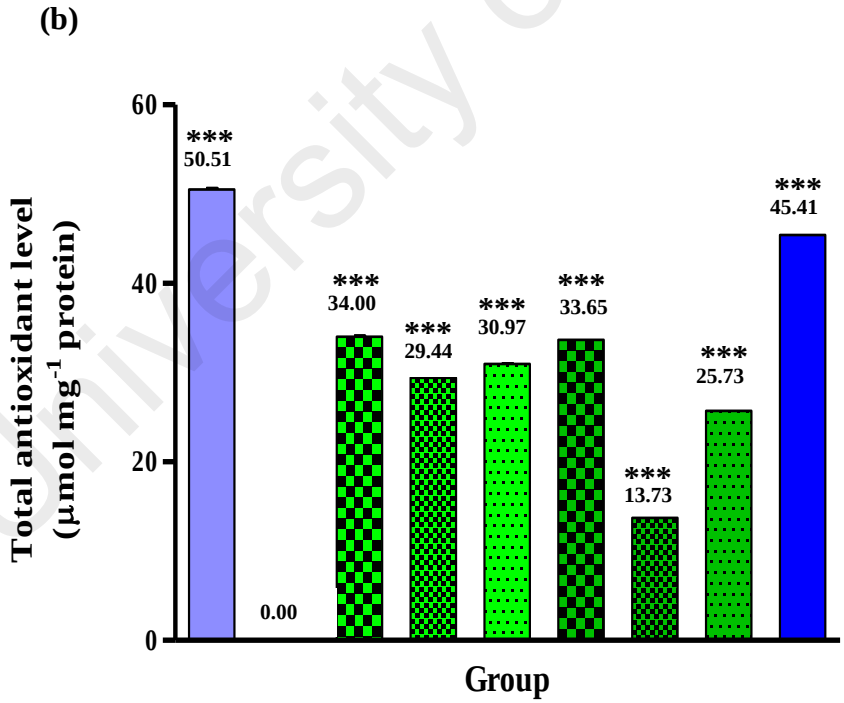
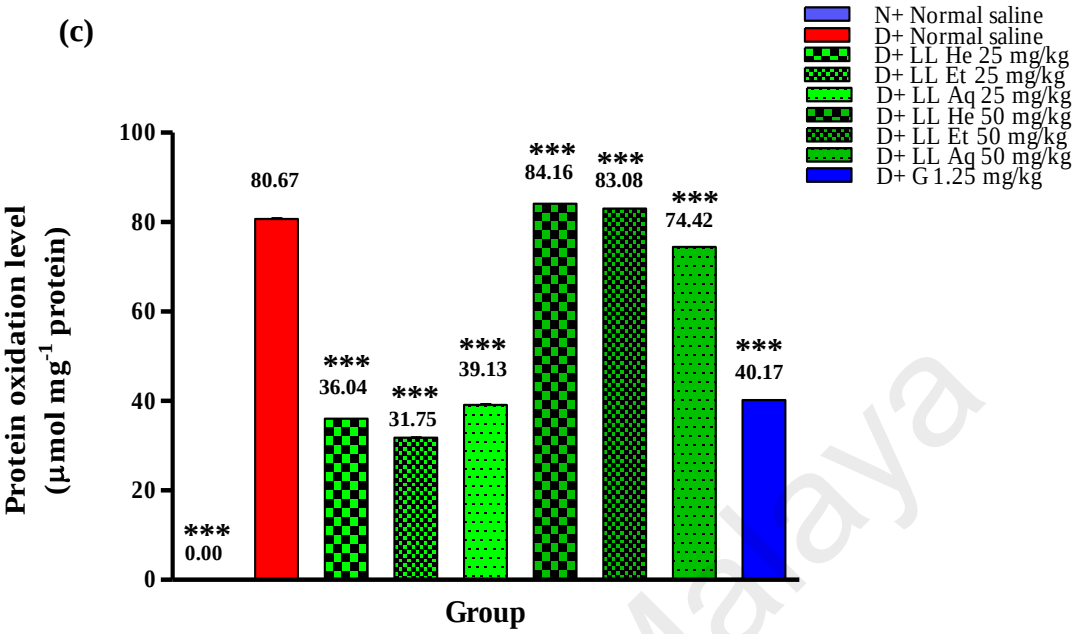


Figure 4.19: Effect of *L. leucocephala* water-solvents partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles on homogenized pancreas pooled samples; sub-chronic study.

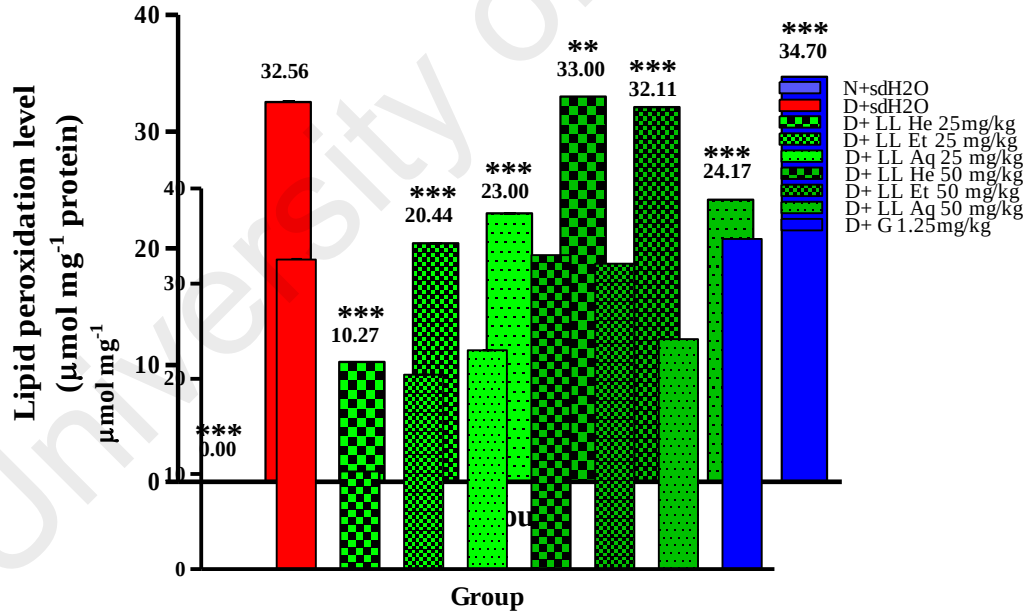
The effect of *L. leucocephala* water-hexane partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles were measured on homogenized pancreas pooled samples by using Glutathione Peroxidase methods (*Section 3.9.5.4*), total antioxidant using FRAP method (*Section 3.9.5.1*), protein oxidation using AOPP method (*Section 3.9.5.2*) and lipid peroxidation using MDA method (*Section 3.9.5.3*) in sub-chronic study. Panel (c) shows effect of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats pancreas AOPP. Panel (d) shows effects of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats pancreas MDA. Comparison were made between each groups and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; ** denotes $p < 0.01$ and *** denotes $p < 0.001$.

Abbreviations: refer to page 152

(c)



(d)



Glutathione peroxidase in liver

LL Aq 25 > *LL* He 50 > *LL* He 25 > *LL* Et 25

5.84 > 4.72 > 4.58 > 4.19

Total antioxidant in liver

LL He 50 > *LL* Et 50 > *LL* Aq 25 > *LL* Aq 50 > Normal Saline

4.04 > 3.64 > 3.09 > 2.97 > 2.74

Protein oxidation in liver

Normal control > *LL* Aq 50 > *LL* He 50 > Normal saline

0.79 > 0.94 > 1.94 > 2.14

Lipid peroxidation in liver

LL Aq 25 > *LL* Et 25 > *LL* Et 50 > G 1.25 > *LL* Aq 50

1.23 > 1.74 > 1.83 > 2.03 > 2.06

Figure 4.20 : The order of potency in descending order for glutathione peroxidase activity, total antioxidant, protein oxidation and lipid peroxidation level in pancreas; sub-chronic study.

The glutathione peroxidase unit is nmol NADPH/min/mg of protein and for all non-enzymatic antioxidants are µmol mg^{-1} protein.

Abbreviations: refer to page 153.

4.4.6 Effect of *Leucaena leucocephala* water-hexane partially purified extract on oxidative indices in liver of Streptozocin-induced diabetic rats; sub-chronic study.

Activity level of glutathione peroxidase in liver was increased in diabetic rats treated with water-hexane partially purified extract at dose of 50 mg kg^{-1} (4.72 nmol NADPH /min/mg) and all diabetic rats group treated with *L. leucocephala* water-solvents partially purified extracts at dose 25 mg kg^{-1}. However, diabetic rats treated with Glibenclamide showed low level of glutathione peroxidase activity (3.15 nmol NADPH /min/mg).

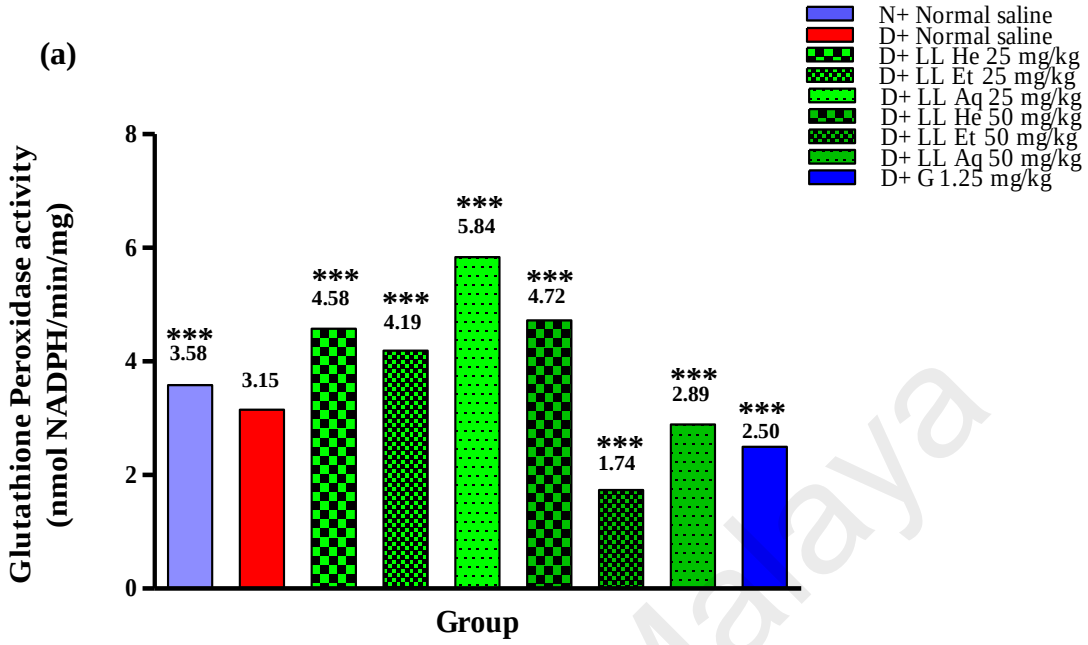
Meanwhile, total antioxidant levels in diabetic rats treated with *L. leucocephala* water-hexane and water-ethyl acetate partially purified at dose of 25 mg kg^{-1} exhibited low level of antioxidant compared to negative control. There was a very high protein oxidation showed in diabetic rats group treated with *L. leucocephala* water-ethyl acetate partially purified extracts at dose 50 mg kg^{-1}. *L. leucocephala* water-hexane partially purified at dose 25 mg kg^{-1} and 50 mg kg^{-1} showed no protection in lipid peroxidation in liver.

In conclusion, *L. leucocephala* water-hexane partially purified at dose of 50 mg kg^{-1} treated group has high glutathione peroxidase activity, high total antioxidant and showed protection against protein oxidation however exhibited less protection in lipid peroxidation in liver.

Figure 4.21: Effect of *L. leucocephala* water-solvents partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles on homogenized liver pooled samples; sub-chronic study.

The effect of *L. leucocephala* water-hexane partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles were measured on homogenized liver pooled samples by using Glutathione peroxidase methods (*Section 3.9.5.4*), total antioxidant using FRAP method (*Section 3.9.5.1*), protein oxidation using AOPP method (*Section 3.9.5.2*) and lipid peroxidation using MDA method (*Section 3.9.5.3*) in sub-chronic study. Panel (a) shows effect of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on Glutathione peroxidase activity level in liver in STZ-induced diabetic rats. Panel (b) shows effects of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats liver FRAP. Comparison were made between each group and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

Abbreviations: refer to page 153.



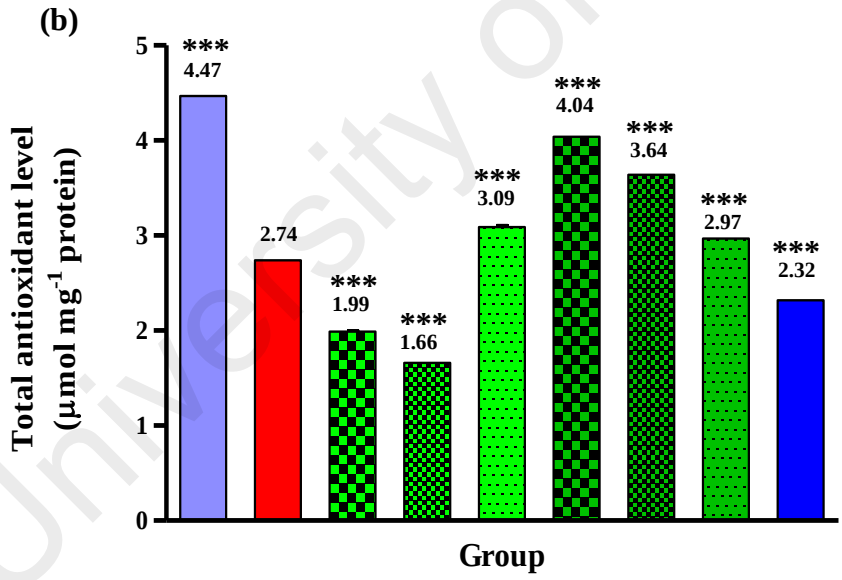
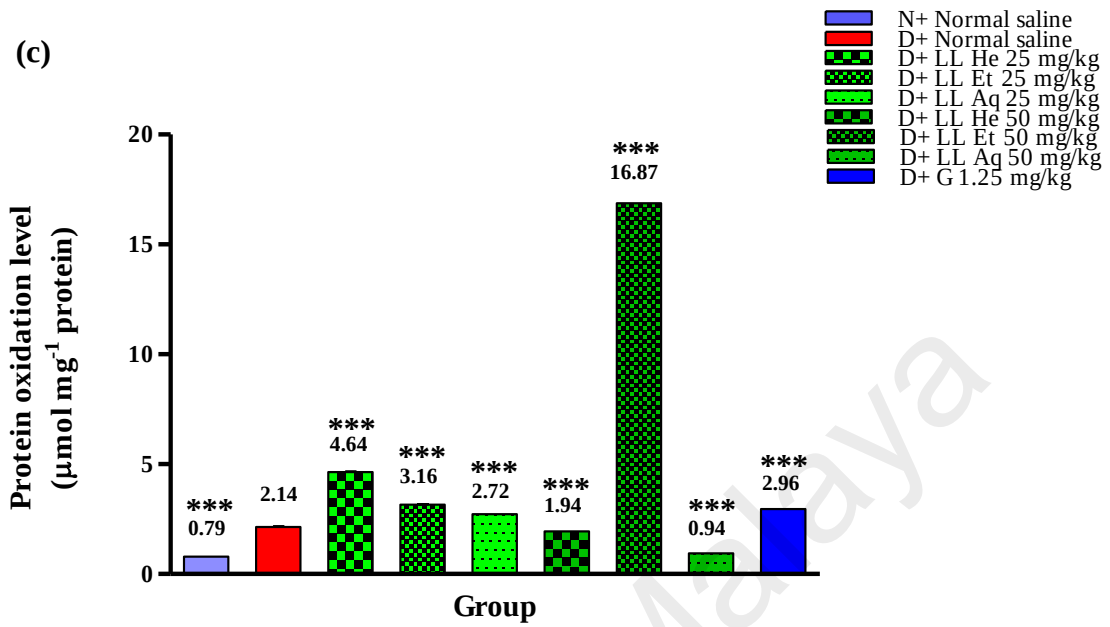
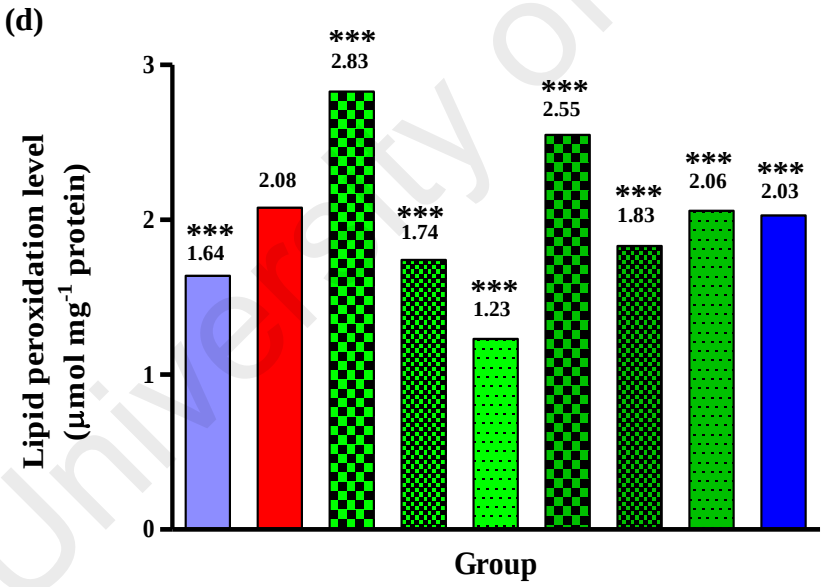


Figure 4.21: Effect of *L. leucocephala* water-solvents partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles on homogenized liver pooled samples; sub-chronic study.

The effect of *L. leucocephala* water-hexane partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles were measured on homogenized liver pooled samples by using Glutathione peroxidase methods (*Section 3.9.5.4*), total antioxidant using FRAP method (*Section 3.9.5.1*), protein oxidation using AOPP method (*Section 3.9.5.2*) and lipid peroxidation using MDA method (*Section 3.9.5.3*) in sub-chronic study. Panel (c) shows effect of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats liver AOPP. Panel (d) shows effects of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats liver MDA. Comparison were made between each group and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

Abbreviations: refer to page 153.





Glutathione peroxidase in brain

LL He 25 > *LL* He 50

0.21 > 0.16

Total antioxidant in brain

LL Aq 25 > *LL* Aq 50 > *LL* He 50 > *LL* Et 25 > *LL* Et 50 = *LL* He 25 > G 1.25

1.70 > 1.63 > 1.38 > 1.24 > 1.16 = 1.16 > 1.09

Protein oxidation in brain

LL Aq 25 > *LL* Et 25 > *LL* He 25 > *LL* Aq 50 = *LL* Et 50 > *LL* G 1.25 > *LL* He 50

1.32 > 2.59 > 3.82 > 6.51= 6.51 > 7.19 > 7.20

Lipid peroxidation in brain

LL Aq 25 > *LL* He 25 > *LL* Et 25 > *LL* Aq 50 > *LL* Et 50

2.19 > 2.22 > 2.53 > 2.67 > 3.15

Figure 4.22 : The order of potency in descending order for glutathione peroxidase activity, total antioxidant, protein oxidation and lipid peroxidation level in brain; sub-chronic study.

The glutathione peroxidase unit is nmol NADPH/min/mg of protein and for all non-enzymatic antioxidants are µmol mg^{-1} protein.

Abbreviations: refer to page 152.

4.4.7 Effect of *Leucaena leucocephala* water-hexane partially purified extract on oxidative indices in brain of Streptozocin-induced diabetic rats; sub-chronic study.

In glutathione peroxidase assay, only diabetic rats treated with *L. leucocephala* water-hexane partially purified extract at both dose (25 mg kg^{-1} and 50 mg kg^{-1}) showed glutathione peroxidase activity.

Meanwhile in FRAP assay, all *L. leucocephala* water-solvents partially purified extract treated group showed increased total antioxidant compared to diabetic treated with normal saline group. All *L. leucocephala* water-solvents partially purified extract treated group exerts reduction in protein oxidation and lipid peroxidation in the brain but the effects was more prominent at dose of 25 mg kg^{-1} and in *L. leucocephala* water-aqueous and water-hexane partially purified extract treated groups. Among different solvents and same dose, *L. leucocephala* water-hexane partially purified extract at dose 25 mg kg^{-1} treated group showed lowest total antioxidant. However, *L. leucocephala* water-hexane partially purified extract at dose 25 mg kg^{-1} treated group showed more protective effects against protein oxidation and lipid peroxidation than dose 50 mg kg^{-1}.

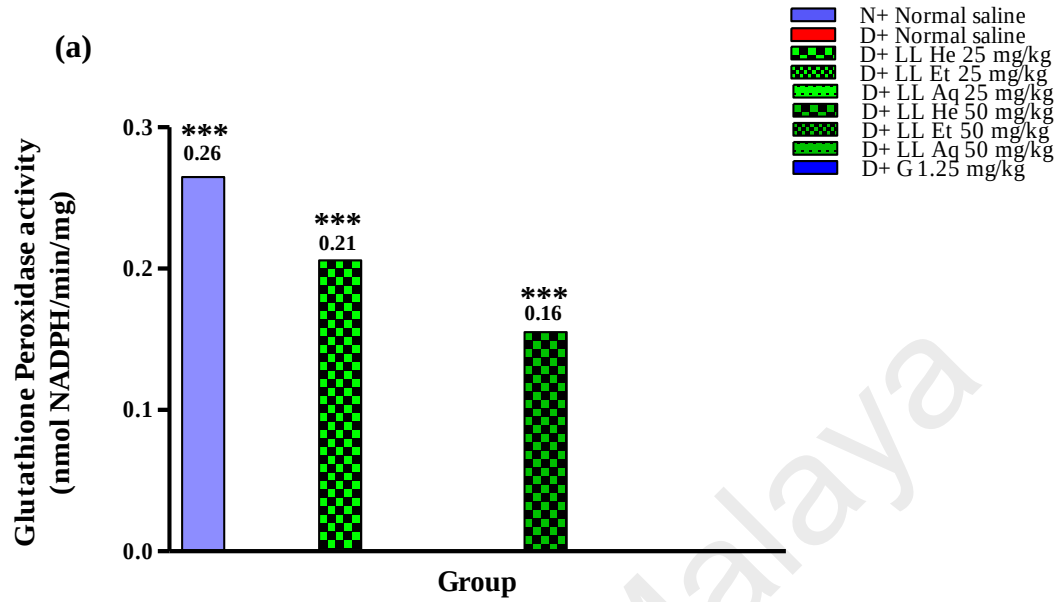
Therefore, *L. leucocephala* water-hexane partially purified extract at dose 25 mg kg^{-1} treated group showed high glutathione peroxidase activity, moderately total antioxidant, and protection against protein and lipid brain damage.

Figure 4.23: Effect of *L. leucocephala* water-solvents partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles on homogenized brain pooled samples; sub-chronic study.

The effect of *L. leucocephala* water-hexane partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles were measured on homogenized brain pooled samples by using Glutathione peroxidase methods (*Section 3.9.5.4*), total antioxidant using FRAP method (*Section 3.9.5.1*), protein oxidation using AOPP method (*Section 3.9.5.2*) and lipid peroxidation using MDA method (*Section 3.9.5.3*) in sub-chronic study. Panel (a) shows effect of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on Glutathione peroxidase activity level in brain in STZ-induced diabetic rats. Panel (b) shows effects of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats brain FRAP. Comparison were made between each group and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test.

*** denotes $p < 0.001$.

Abbreviations: refer to page 153.



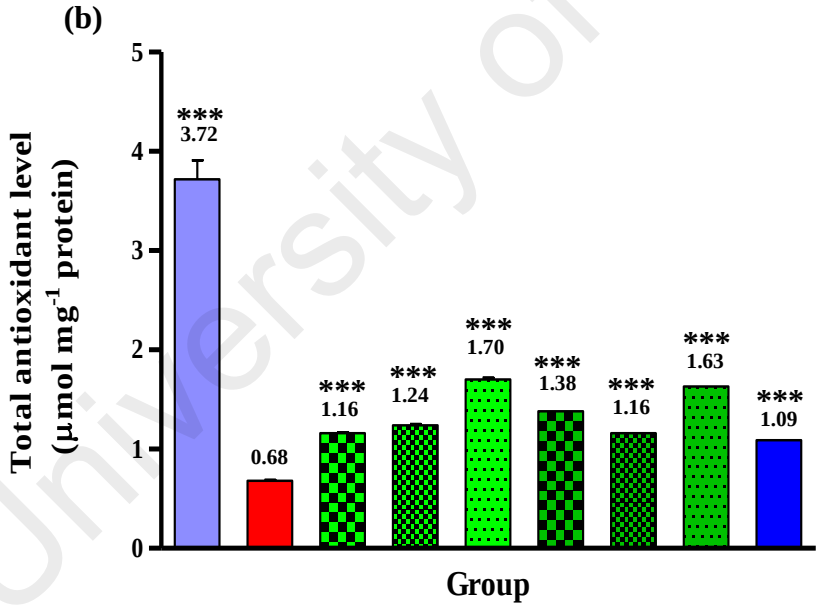
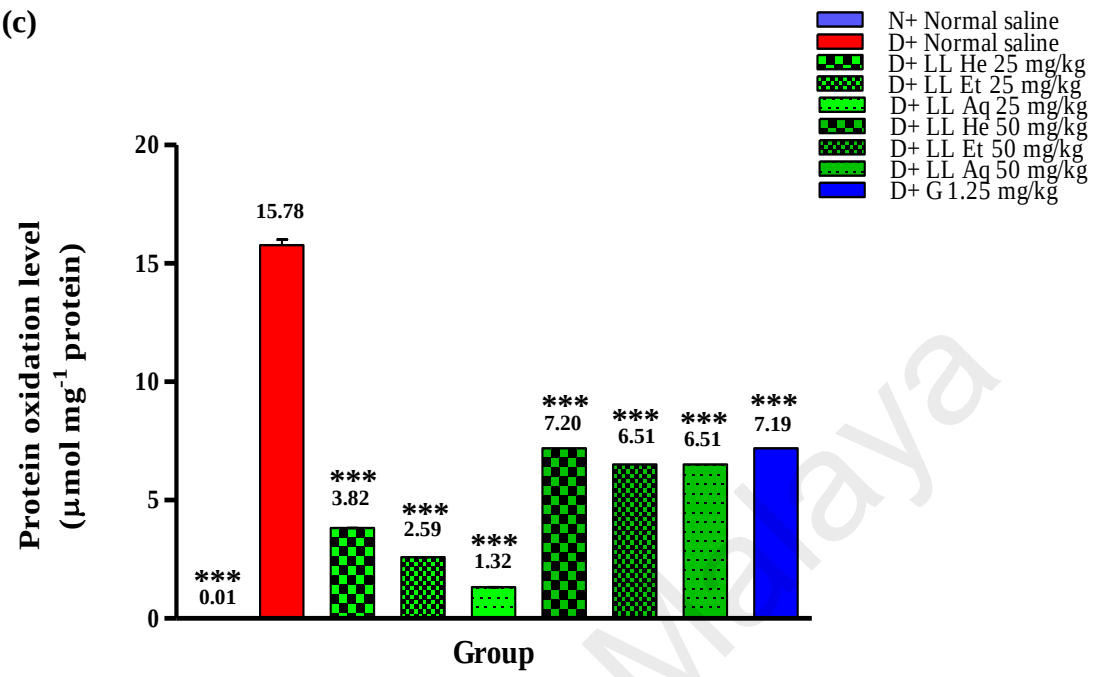


Figure 4.23: Effect of *L. leucocephala* water-solvents partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles on homogenized brain pooled samples; sub-chronic study.

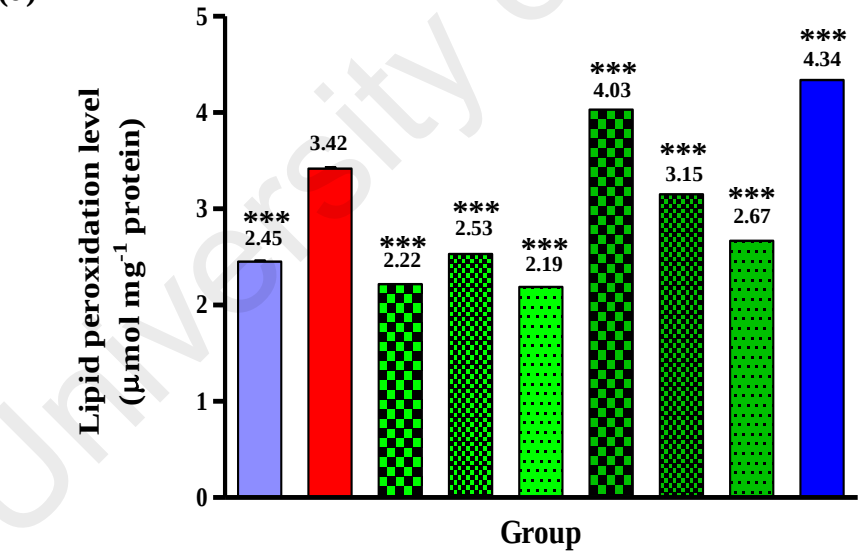
The effect of *L. leucocephala* water-hexane partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles were measured on homogenized brain pooled samples by using Glutathione peroxidase methods (*Section 3.9.5.4*), total antioxidant using FRAP method (*Section 3.9.5.1*), protein oxidation using AOPP method (*Section 3.9.5.2*) and lipid peroxidation using MDA method (*Section 3.9.5.3*) in sub-chronic study. Panel (c) shows effect of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats brain AOPP. Panel (d) shows effects of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats brain MDA. Comparison were made between each group and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

Abbreviations: refer to page 153.

(c)



(d)



Glutathione peroxidase in kidney

LL Et 50 > *LL* He 50 > *LL* Et 25 > *LL* Aq 50 > *LL* Aq 25 > *LL* He 25

1.73 > 1.65 >1.46 >1.29 >1.09> 0.96

Total antioxidant in kidney

LL Aq 50 > Normal control > *LL* Et 50 > *LL* He 50 = *LL* He 25 > G 1.25 > *LL* Aq 25

4.36 > 4.25 > 3.94 > 3.29 = 3.29 > 3.15 > 2.88

Protein oxidation in kidney

LL He 50 > *LL* Aq 25 > *LL* Et 50

1.57 > 1.66 > 2.17

Lipid peroxidation in kidney

LL He 50 = *LL* Et 50 > G 1.25 > *LL* Aq 25 > *LL* Aq 50

2.57 = 2.57 > 2.65 > 2.79 > 3.82

Figure 4.24 : The order of potency in descending order for glutathione peroxidase activity, total antioxidant, protein oxidation and lipid peroxidation level in kidney; sub-chronic study.

The glutathione peroxidase unit is nmol NADPH/min/mg of protein and for all non-enzymatic antioxidants are µmol mg^{-1} protein.

Abbreviations: refer to page 153.

4.4.8 Effect of *Leucaena leucocephala* water-hexane partially purified extract on oxidative indices in kidney of Streptozocin-induced diabetic rats; sub-chronic study.

In glutathione peroxidase assay, all group of *L. leucocephala* water-solvents partially purified extract exerts high glutathione peroxidase activities compared to negative control group. Glibenclamide-treated rats group (1.01 nmol NADPH/min/mg) and *L. leucocephala* water-hexane partially purified extract at dose 25 mg kg^{-1} (0.96 nmol NADPH/min/mg) showed high glutathione peroxidase activity than negative control group (0.87 nmol NADPH/min/mg) but less than normal control (1.04 nmol NADPH/min/mg).

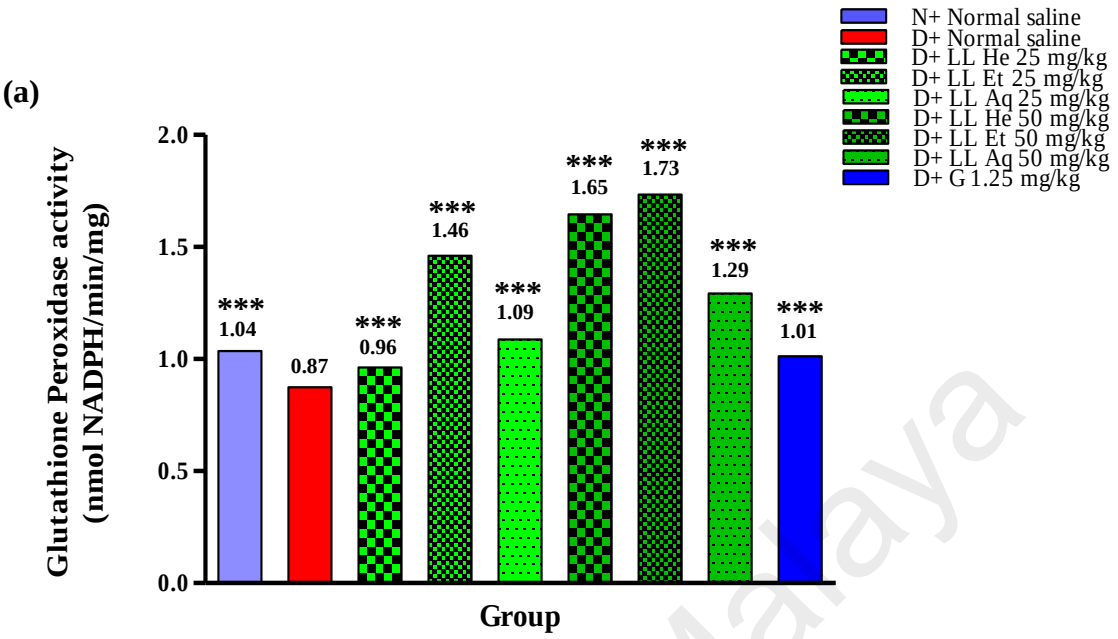
In total antioxidant (FRAP) assay, *L. leucocephala* water-hexane partially purified extract dose 25 mg kg^{-1} treated group exhibited highest total antioxidant among different solvent. Protective effects against protein oxidation in the kidney was shown in *L. leucocephala* water-hexane and water-ethyl acetate partially purified extract at dose 50 mg kg^{-1}, meanwhile similar protection was seen in *L. leucocephala* water-aqueous partially purified extract at dose 25 mg kg^{-1} treated group. *Leucaena leucocephala* water-hexane partially purified extract at dose 50 mg kg^{-1} treated group has demonstrated high glutathione peroxidase activity and total antioxidant, low protein oxidation and lipid peroxidation.

In summary, *L. leucocephala* water-hexane partially purified extract at dose of 50 mg kg^{-1} had offered best protection against damages in diabetic kidney.

Figure 4.25: Effect of *L. leucocephala* water-solvents partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles on homogenized kidney pooled samples; sub-chronic study.

The effect of *L. leucocephala* water-hexane partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles were measured on homogenized kidney pooled samples by using Glutathione peroxidase methods (*Section 3.9.5.4*), total antioxidant using FRAP method (*Section 3.9.5.1*), protein oxidation using AOPP method (*Section 3.9.5.2*) and lipid peroxidation using MDA method (*Section 3.9.5.3*) in sub-chronic study. Panel (a) shows effect of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on Glutathione peroxidase activity level in kidney in STZ-induced diabetic rats. Panel (b) shows effects of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats kidney FRAP. Comparison were made between each group and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

Abbreviations: refer to page 153.



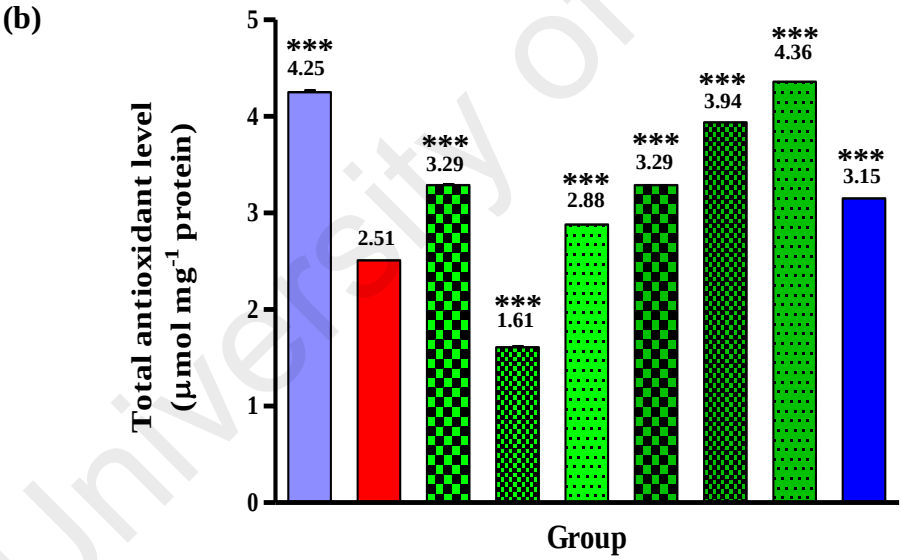
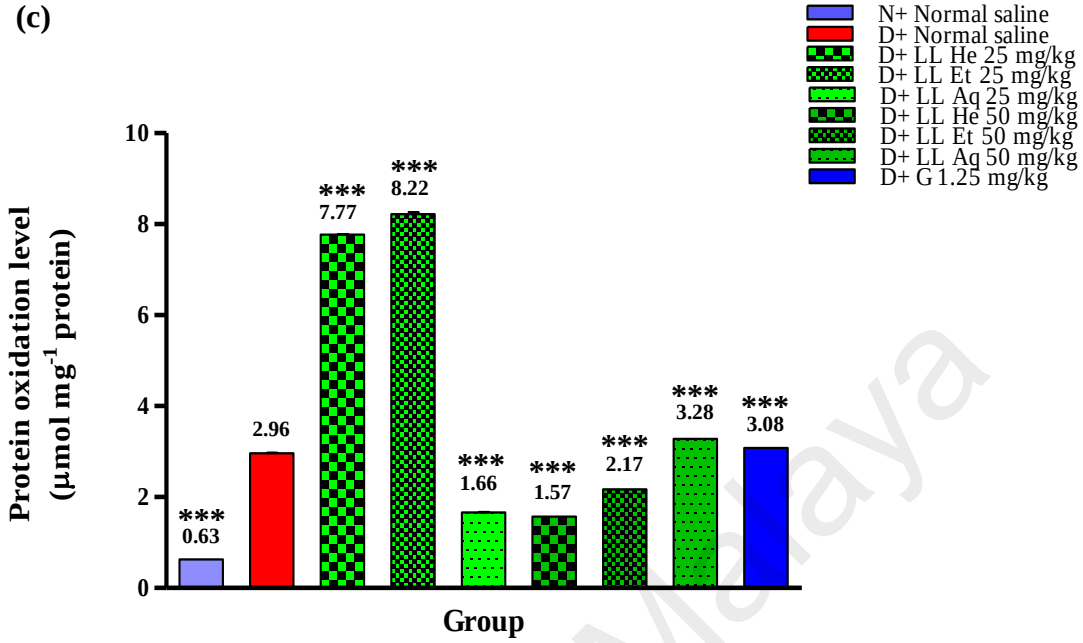


Figure 4.25: Effect of *L. leucocephala* water-solvents partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles on homogenized kidney pooled samples; sub-chronic study.

The effect of *L. leucocephala* water-hexane partially purified extracts on enzymatic and non-enzymatic antioxidants *in vivo* profiles were measured on homogenized kidney pooled samples by using Glutathione peroxidase methods (*Section 3.9.5.4*), total antioxidant using FRAP method (*Section 3.9.5.1*), protein oxidation using AOPP method (*Section 3.9.5.2*) and lipid peroxidation using MDA method (*Section 3.9.5.3*) in sub-chronic study. Panel (c) shows effect of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats kidney AOPP. Panel (d) shows effects of *L. leucocephala* water-hexane partially purified extracts at 25 mg kg^{-1} and 50 mg kg^{-1} on rats kidney MDA. Comparison were made between each group and negative control. Results presented are mean ± SEM. Statistical significance was calculated based on Student's t-test; *** denotes $p < 0.001$.

Abbreviations: refer to page 153.

(c)



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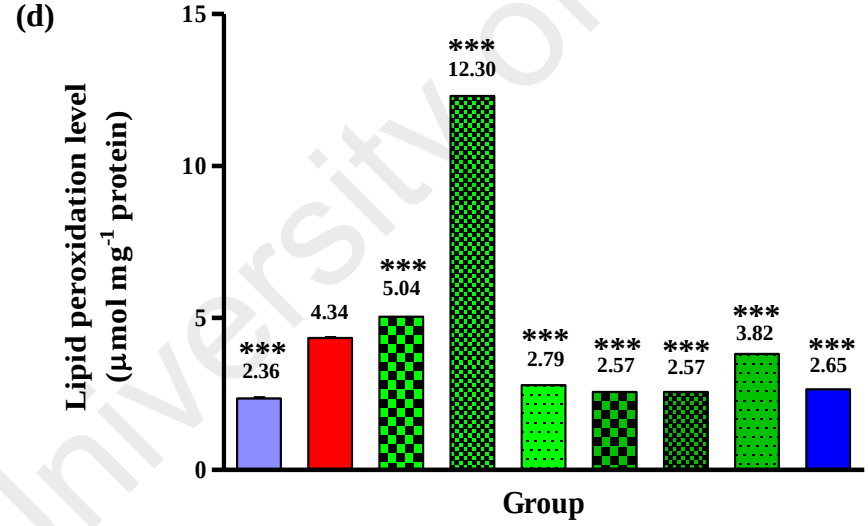


4.4.9 Effect of *Leucaena leucocephala* water-hexane partially purified extract on diabetic damages in liver pathology; sub-chronic study; histological examination.

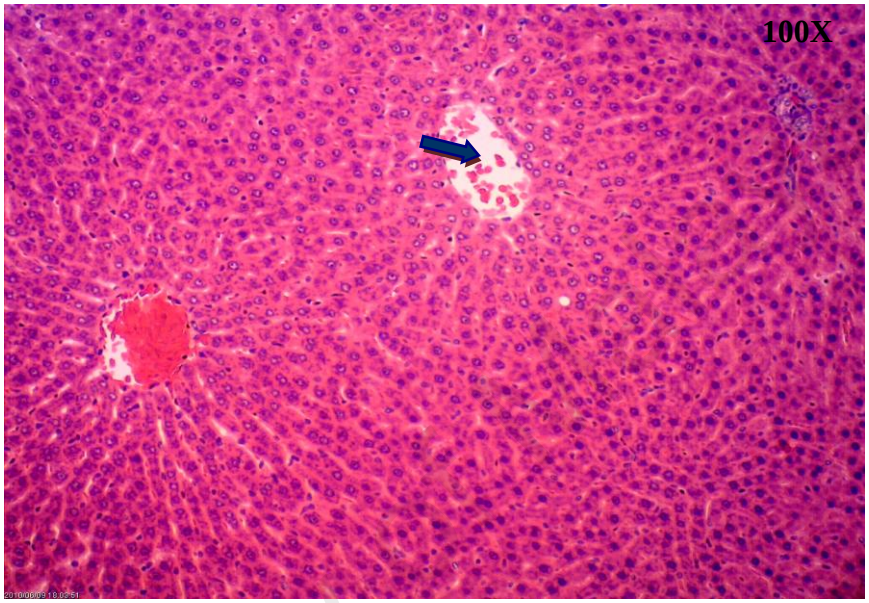
Figure 4.26 showed effects of *Leucaena leucocephala* water-hexane, water-ethyl acetate and water-aqueous partially purified extract at dose of 25 mg kg^{-1} and 50 mg kg^{-1} on diabetic rats liver. Normal healthy rats exhibit normal rats liver which can be view by normal central vein (blue arrow), normal hepatocytes and arrangements, no fatty changes, no RBC infiltration and no lymphocytes infiltration (*Figure 4.26 a*). In contrast, diabetic treated with normal saline rat liver showed dilated sinusoids, (light orange arrow), RBC infiltration (red arrow) and lymphocytes infiltration (white arrow) (*Figure 4.26 b*). *Leucaena leucocephala* water-hexane partially purified extract group at dose of 25 mg kg^{-1} showed RBC infiltration (*Figure 4.26 c*) whereas more prominent at dose of 50 mg kg^{-1} (*Figure 4.26 d*). *Leucaena leucocephala* water-ethyl acetate partially purified extract group at dose of 25 mg kg^{-1} exhibited less dilation of central vein, less RBC congestion and Kupffer cells (*Figure 4.26 e*) and at dose of 50 mg kg^{-1} showed moderately RBC congestion (red arrow), lymphocytes infiltration (white arrow), increase of Kupffer cells (green arrow) (*Figure 4.26 f*). *Leucaena leucocephala* water-aqueous partially purified extract at dose of 25 mg kg^{-1} showed markedly central vein dilated, RBC congestion and increased in Kupffer cells and at dose of 50 mg kg^{-1} showed worse RBC infiltration and worse dilated sinusoid (*Figure 4.26 g*). Glibenclamide-treated group (1.25 mg kg^{-1}) showed severe RBC infiltration (red arrow) (*Figure 4.26 h*).

Figure 4.26: Effects of *Leucaena leucocephala* water-solvents partially purified extracts at dose of 25 mg kg^{-1} and 50 mg kg^{-1} on diabetic rats liver; sub-chronic study.

Figure 4.26 (a)-(i) showed normal and diabetic rats liver section stained with Hematoxylin and Eosin as in method *Section 3.9.6*. The magnification was 100X for panels (a)-(i). Panel (a) showed normal rats liver. Legends: normal central vein (blue arrow), normal hepatocytes and arrangements, no fatty changes, no RBC infiltration and no lymphocytes infiltration. Panel (b) showed diabetic rats liver treated with Normal saline at dose of 4ml/kg. Legends; dilated sinusoids (light orange arrow), RBC infiltration (red arrow) and lymphocytes infiltration (white arrow).

Legends:
- Central vein
- Sinusoids
- Red blood cell infiltration
- Lymphocyte infiltration
- Kupffer cell

(a)



(b)

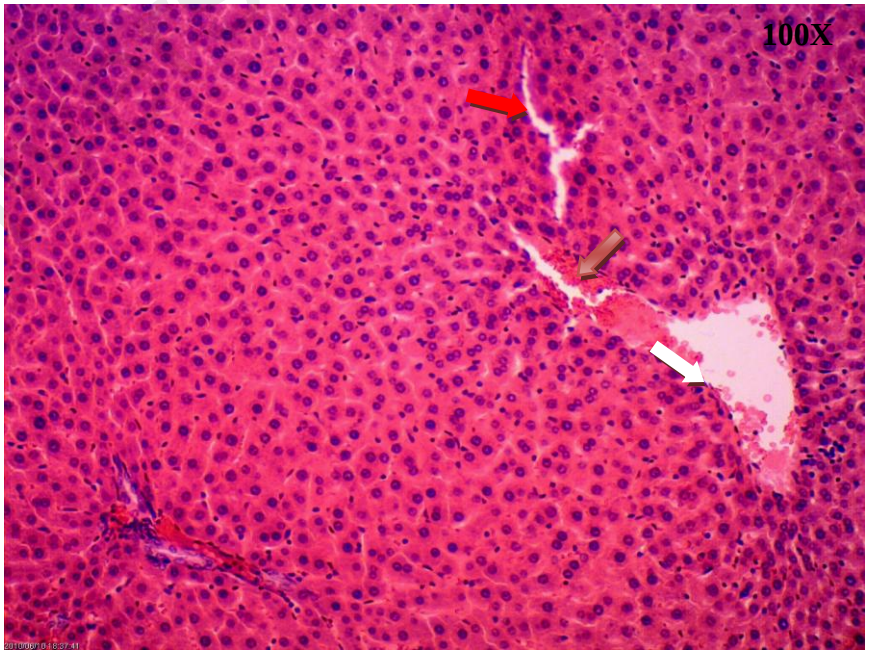
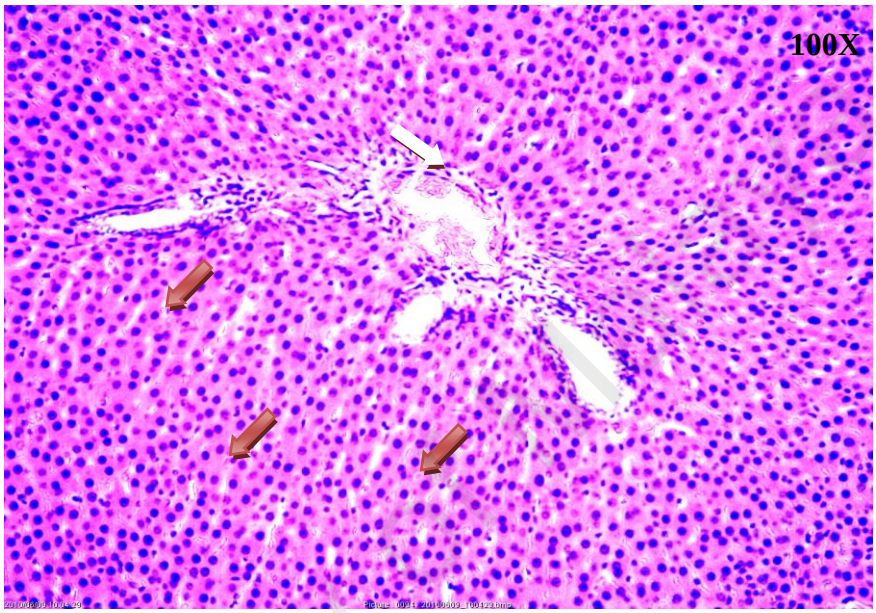


Figure 4.26: Effects of *Leucaena leucocephala* water-solvents partially purified extracts at dose of 25 mg kg^{-1} and 50 mg kg^{-1} on diabetic rats liver; sub-chronic study.

Figure 4.26 (a)-(i) showed normal and diabetic rats liver section stained with Hematoxylin and Eosin as in method *Section 3.9.6*. The magnification was 100X for panels (a)-(i). Panel (c) showed effects of *Leucaena leucocephala* water-hexane partially purified at dose of 25 mg kg^{-1} on diabetic rats liver. Legends: RBC infiltration (red arrow). Panel (d) showed effects of *Leucaena leucocephala* water-hexane partially purified at dose of 50 mg kg^{-1} on diabetic rats liver. Legends: RBC congestion (red arrow), lymphocytes infiltration (white arrow), Kupffer cells (green arrow).

Legends:
- Central vein
- Sinusoids
- Red blood cell infiltration
- Lymphocyte infiltration
- Kupffer cell

(c)



(d)

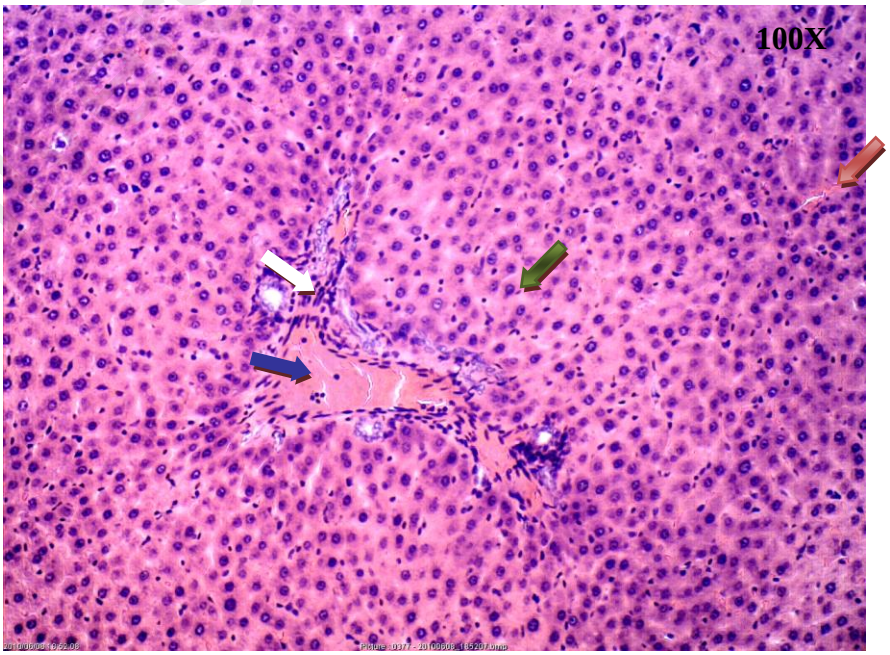
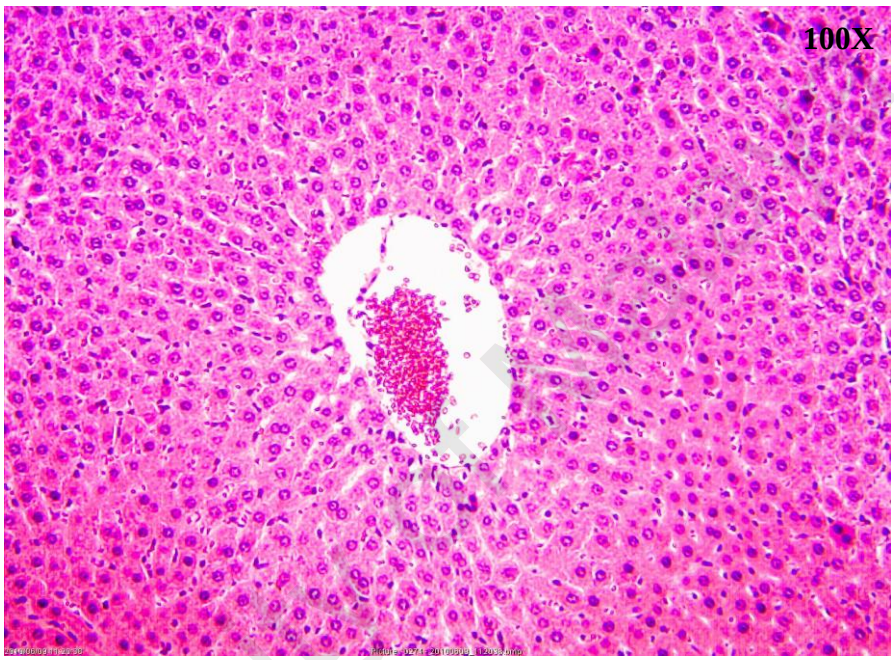


Figure 4.26: Effects of *Leucaena leucocephala* water-solvents partially purified extracts at dose of 25 mg kg^{-1} and 50 mg kg^{-1} on diabetic rats liver; sub-chronic study.

Figure 4.26 (a)-(i) showed normal and diabetic rats liver section stained with Hematoxylin and Eosin as in method *Section 3.9.6*. The magnification was 100X for panels (a)-(i). Panel (e) showed effects of *Leucaena leucocephala* water-ethyl acetate partially purified at dose of 25 mg kg^{-1} on diabetic rats liver. Legends: Central vein, RBC congestion and Kupffer cells. Panel (f) showed effects of *Leucaena leucocephala* water-ethyl acetate partially purified at dose of 50 mg kg^{-1} on diabetic rats liver. Legends: central vein, RBC congestion, Kupffer cells.

Legends:
- Central vein
- Sinusoids
- Red blood cell infiltration
- Lymphocyte infiltration
- Kupffer cell

(e)



(f)

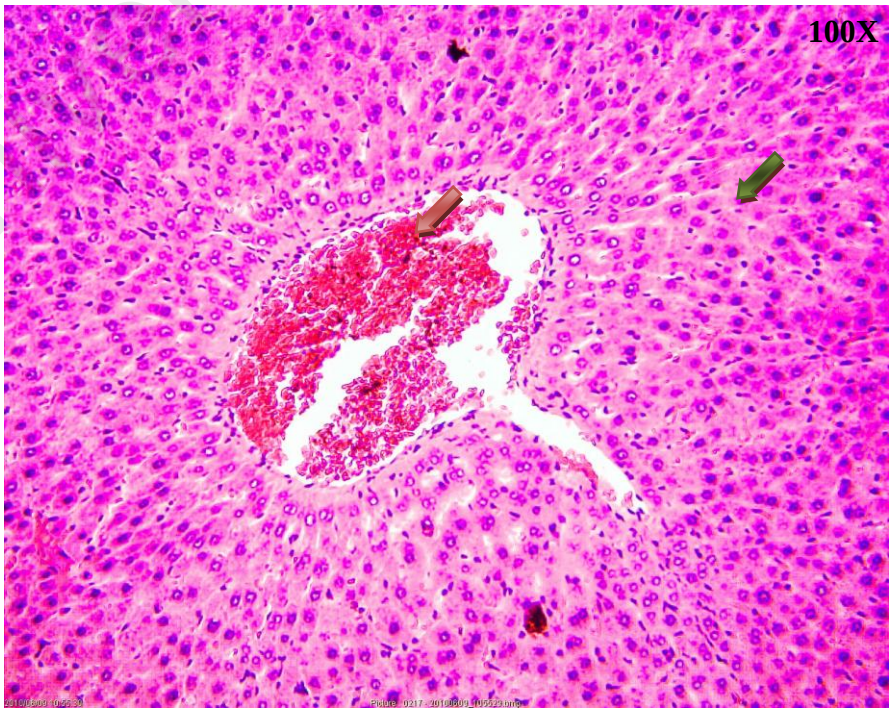
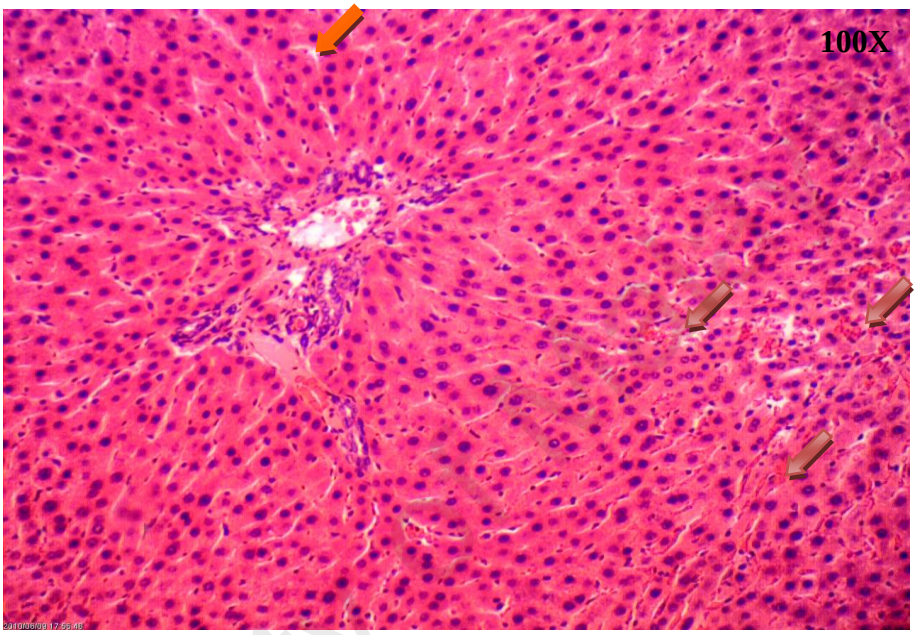


Figure 4.26: Effects of *Leucaena leucocephala* water-solvents partially purified extracts at dose of 25 mg kg^{-1} and 50 mg kg^{-1} on diabetic rats liver; sub-chronic study.

Figure 4.26 (a)-(i) showed normal and diabetic rats liver section stained with Hematoxylin and Eosin as in method *Section 3.9.6*. The magnification was 100X for panels (a)-(i). Panel (g) showed effects of *Leucaena leucocephala* water-aqueous partially purified at dose of 25 mg kg^{-1} on diabetic rats liver. Legends: Severe RBC infiltration and worse dilated sinusoid. Panel (h) showed effects of *Leucaena leucocephala* water-aqueous partially purified at dose of 50 mg kg^{-1} on diabetic rats liver. Legends: RBC infiltration and congestion, worsely dilated sinusoid.

Legends:
- Central vein
- Sinusoids
- Red blood cell infiltration
- Lymphocyte infiltration
- Kupffer cell

(g)



(h)

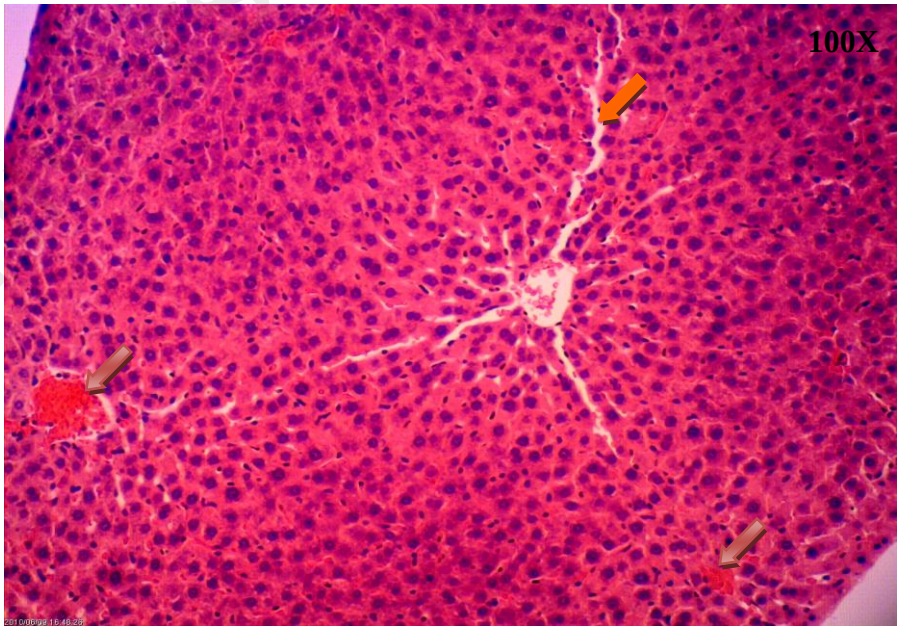
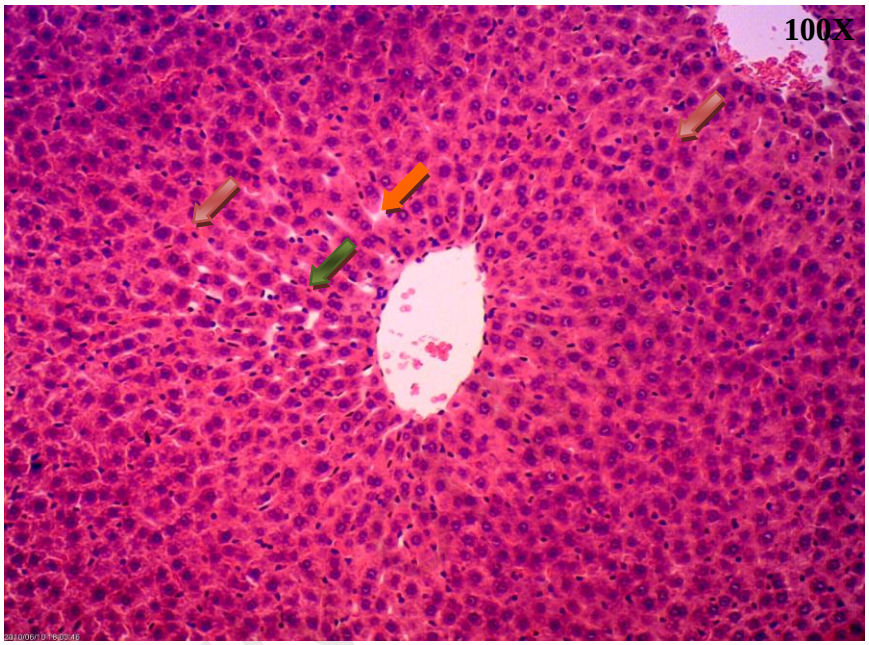


Figure 4.26: Effects of *Leucaena leucocephala* partially purified water-hexane, water-ethyl acetate and water-aqueous extract at dose of 25 mg kg^{-1} and 50 mg kg^{-1} on diabetic rats liver; sub-chronic study.

Figure 4.26 (a)-(i) showed normal and diabetic rats liver section stained with Hematoxylin and Eosin as in method *Section 3.9.6*. The magnification was 100X for panels (a)-(i). Panel (i) showed effects of Glibenclamide at dose of 1.25 mg kg^{-1} on diabetic rats liver. Legends: RBC infiltration (red arrow).

Legends:
- Central vein
- Sinusoids
- Red blood cell infiltration
- Lymphocyte infiltration
- Kupffer cell

(i)



4.4.10 Effect of *Leucaena leucocephala* water-hexane partially purified extract on diabetic damages in kidney pathology; sub-chronic study; histological examination.

The kidney function was details described in *Section 4.3.8* and the changes occur due to diabetes were briefly described in *Section 4.3.10*. Awad Allah, (2007), found that at day tenth after Streptozocin injection, there was a mild enlargement of glomeruli and glomerular capillaries. In this study, diabetic rats treated with Normal saline exhibited enlargement of glomerular capillaries (*Figure 4.27 b*) which was similarly found by Awad Allah, (2007) and also enlargement of capsular space (*Figure 4.27 b*). However, *Leucaena leucocephala* water-hexane partially purified extract at dose of 25 mg kg^{-1} exhibited protection from the initial changes on kidney damages in diabetic rats (*Figure 4.27 c*). Diabetic rats treated with *Leucaena leucocephala* water-hexane partially purified extract at dose of 50 mg kg^{-1} showed mesangial space becomes larger by deposition of extracellular matrix (*Figure 4.27 c*), an initial changes leading to glomerular sclerosis. Effects of *Leucaena leucocephala* water-ethyl acetate partially purified extract at dose of 25 mg kg^{-1} and 50 mg kg^{-1} exhibited increased amount of mesangial matrix in diabetic rats kidney (*Figure 4.28 e and f*). In addition, at dose of 50 mg kg^{-1} showed an enlargement of glomerular capillaries (*Figure 4.27 f*). *Leucaena leucocephala* water-aqueous partially purified extract at dose of 25 mg kg^{-1} exhibited increased amount of mesangial matrix and enlargement of capsular space on diabetic rats kidney meanwhile at dose of 50 mg kg^{-1} exhibited worsen. In Glibenclamide (1.25 mg kg^{-1}) treated group, there was enlargement of glomerular capillaries in diabetic rats kidney. Briefly it can be concluded that *Leucaena leucocephala* partially purified extract at dose of 25 mg kg^{-1} exhibited better protection in diabetes kidney, obviously in water-hexane treated group.

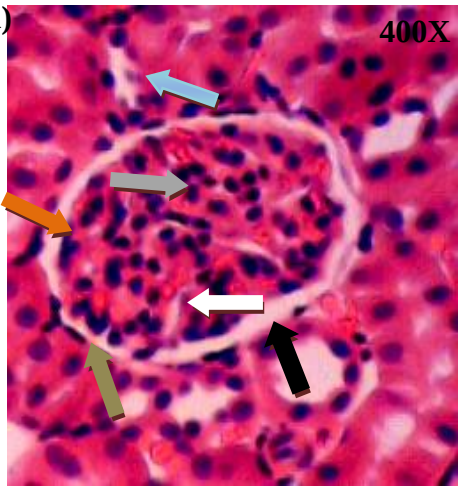
Figure 4.27: Effects of *Leucaena leucocephala* partially purified extract at 25 mg kg^{-1} and 50 mg kg^{-1} on diabetic rats' kidney in sub-chronic study

Figure 4.27 (a)-(i). showed normal and diabetic rats kidney section stained with Hematoxylin and Eosin as a method in *Section 3.9.6*. The magnification was 400 X for panels (a)-(h). Panel (a) showed normal rats kidney. Legends: Normal capillaries (white arrow), Normal capsular space (black arrow), Normal podocytes (orange arrow), Macula densa (blue arrow), Parietal blade of Bowman's capsule (green arrow), Mesangial cells (grey arrow). Panel (b) showed effects of untreated diabetic rats kidney. Legends: enlargement of glomerular capillaries (white arrow) and capsular space (black arrow). Panel (c) showed effects of *Leucaena leucocephala* water-hexane partially purified at 25 mg kg^{-1} on diabetic rats kidney. Legends: Glomerulus appear similar to panel (a). Panel (d) showed effects of *Leucaena leucocephala* water-hexane partially purified at 50 mg kg^{-1} on diabetic rats kidney. Legends: mesangial space becomes larger by deposition of extracellular matrix (grey arrow).

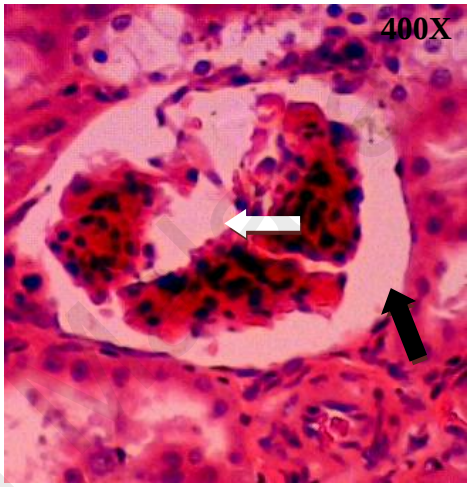
Legends:

- capillaries
- capsular space
- podocytes
- macula densa
- parietal blade of Bowman's capsule
- mesangial cells

(a)



(b)



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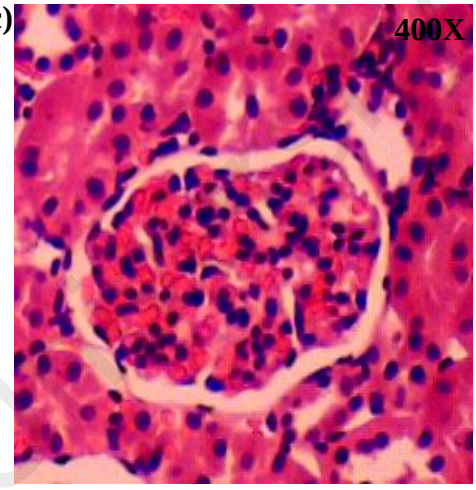

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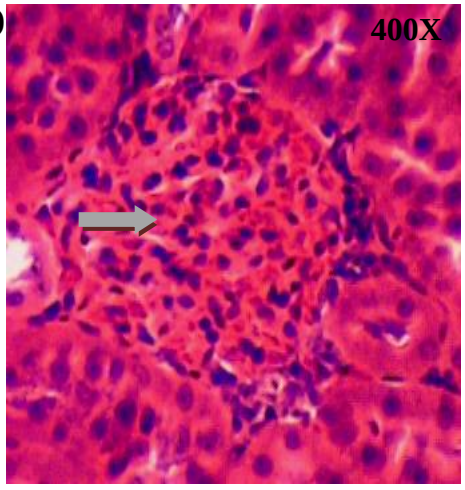


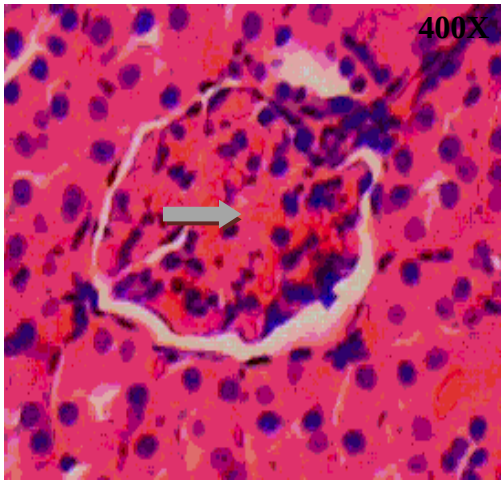
Figure 4.27: Effects of *Leucaena leucocephala* water-solvents partially purified, extracts at dose of 25 mg kg^{-1} and 50 mg kg^{-1} on diabetic rats kidney; sub-chronic study.

Figure 4.27 (a)-(i) showed normal and diabetic rats kidney section stained with Hematoxylin and Eosin as method in *Section 3.9.6*. The magnification was 400X for panels (a)-(i). Panel (e) showed effects of *Leucaena leucocephala* water-ethyl acetate partially purified extact at dose of 25 mg kg^{-1} on diabetic rats kidney. Legends: increased amount of mesangial matrix (grey arrow). Panel (f) showed effects of *Leucaena leucocephala* water-ethyl acetate partially purified extract at dose of 50 mg kg^{-1} on diabetic rats kidney. Legends: increased amount of mesangial matrix (grey arrow) and enlargement of glomerular capillaries (white arrow). Panel (g) showed effects of *Leucaena leucocephala* water-aqueous partially purified extract at dose of 25 mg kg^{-1} on diabetic rats kidney. Legends: increased amount of mesangial matrix (grey arrow) and enlargement of capsular space (black arrow). Panel (h) showed effects of *Leucaena leucocephala* hexane-aqueous partially purified extract at dose of 50 mg kg^{-1} on diabetic rats kidney. Panel (i) showed effects of Glibenclamide at dose of 1.25 mg kg^{-1} on diabetic rats kidney. Legends: enlargement of glomerular capillaries (white arrow).

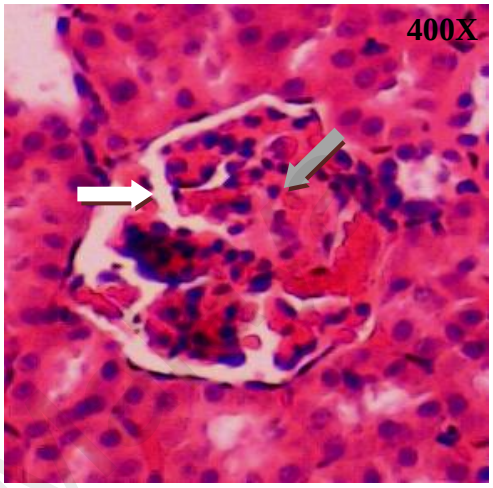
Legends:

- capillaries
- capsular space
- podocytes
- macula densa
- parietal blade of Bowman's capsule
- mesangial cells

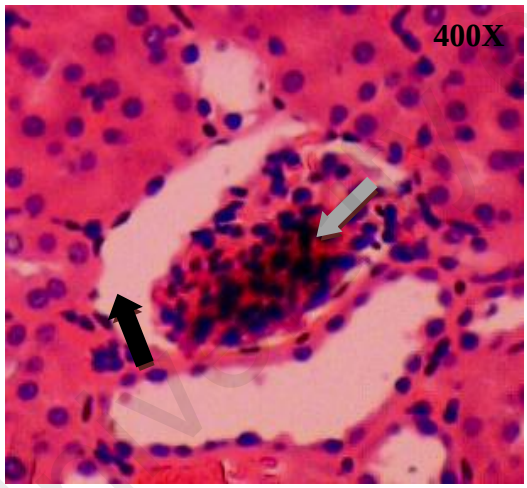
(e)



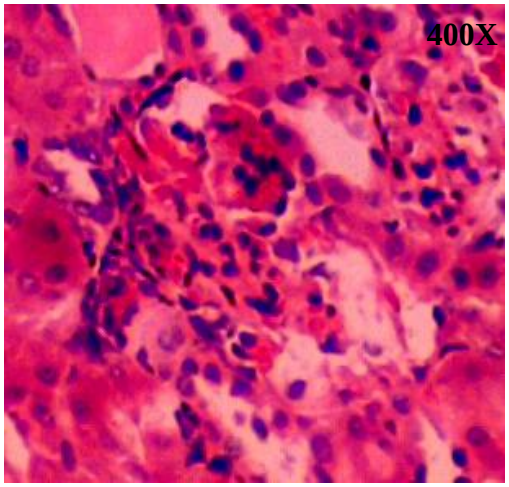
(f)



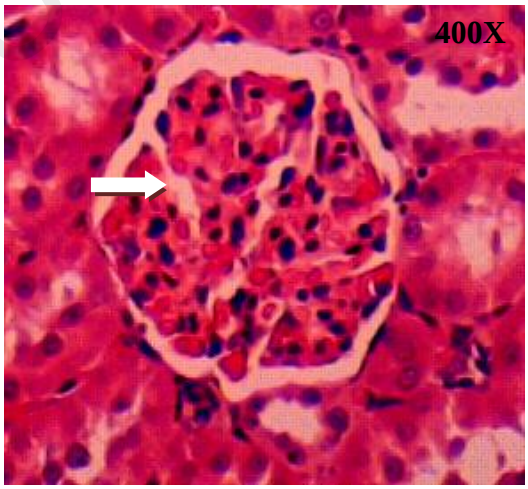
(g)



(h)



(i)



4.5 Identification of bioactives compound *via*

4.5.1 High Performance Liquid Chromatography (HPLC) fractionation and profiling of *Leucaena leucocephala* water-hexane partially purified extract.

In this study, 32 fractions were collected (*Table 4.2*). Fraction N_4 represent major weight (24.5 %) in the sample which expected to contained the most bioactive compound (*Table 4.2*). The N_4 fraction is basically polar compound. The N_3 and N_4 was further selected for LC-MS.

4.5.2 Liquid Chromatography Mass Spectrophotometry (LC-MS).

Results showed that at ~0.3 minutes is a peak of solvent (*Figure 4.29*) which functions as a blank. Results shows that fraction N_3 and N_4 appeared at all wavelengths tested (254 nm, 191 nm, 230 nm and 280 nm) and showed highest peak, as showed within retention time 2.0 to 4.0 minutes (*Figure 4.30 and 4.32*).

Both samples showed retention times at 1.05 minutes with a mass weight of 217.0937 g/mol (N_3) and 217.1948 g/mol (N_4) respectively. The maximum absorption for detection of the compound was 278 nm (N_3) and 207 nm (N_4). This might suggest that the active compound having molecular formula of $C_7H_{13}N_4O_4$ or $C_{11}H_{25}N_2O_2$. A cross reference were made using expected molecular weight with PubChem library for identification of compound. Molecular formula of $C_7H_{13}N_4O_4$ exhibited 42 hits and $C_{11}H_{25}N_2O_2$ showed 338 hits with molecular mass of ~216 g/mol.

Table 4.2: Fractions from *Leucaena leucocephala* water-hexane partially purified extract.

No	Code	Retention time (mins)	Weight of sample (mg)
1	N_1	0.0-0.1	19.9
2	N_2	1.0-2.0	28.2
3	N_3	2.0-3.0	28.5
4	N_4	3.0-4.0	511.4
5	N_5	4.0-5.0	101.6
6	N_6	5.0-6.0	430
7	N_7	6.0-7.0	39.7
8	N_8	7.0-8.0	25.2
9	N_9	8.0-9.0	23.8
10	N_{10}	9.0-10.0	23.8
11	N_{11}	10.0-11.0	56.4
12	N_{12}	11.0-12.0	33.2
13	N_{13}	12.0-13.0	38.2
14	N_{14}	13.0-14.0	40.7
15	N_{15}	14.0-15.0	41.5
16	N_{16}	15.0-16.0	46.1
17	N_{17}	16.0-17.0	19.2
18	N_{18}	17.0-18.0	36.8
19	N_{19}	18.0-19.0	36.7
20	N_{20}	19.0-20.0	17.9
21	N_{21}	20.0-21.0	20.1

No	Code	Retention time (mins)	Weight of sample (mg)
22	N_{22}	21.0-22.0	35.2
23	N_{23}	22.0-23.0	21.2
24	N_{24}	23.0-24.0	46.4
25	N_{25}	24.0-25.0	55.4
26	N_{26}	25.0-26.0	21.4
27	N_{27}	26.0-27.0	31.7
28	N_{28}	27.0-28.0	35.8
29	N_{29}	28.0-29.0	30.3
30	N_{30}	29.0-30.0	28.6
31	N_{31}	30.0-31.0	128.7
32	N_{32}	31.0-32.0	34.2

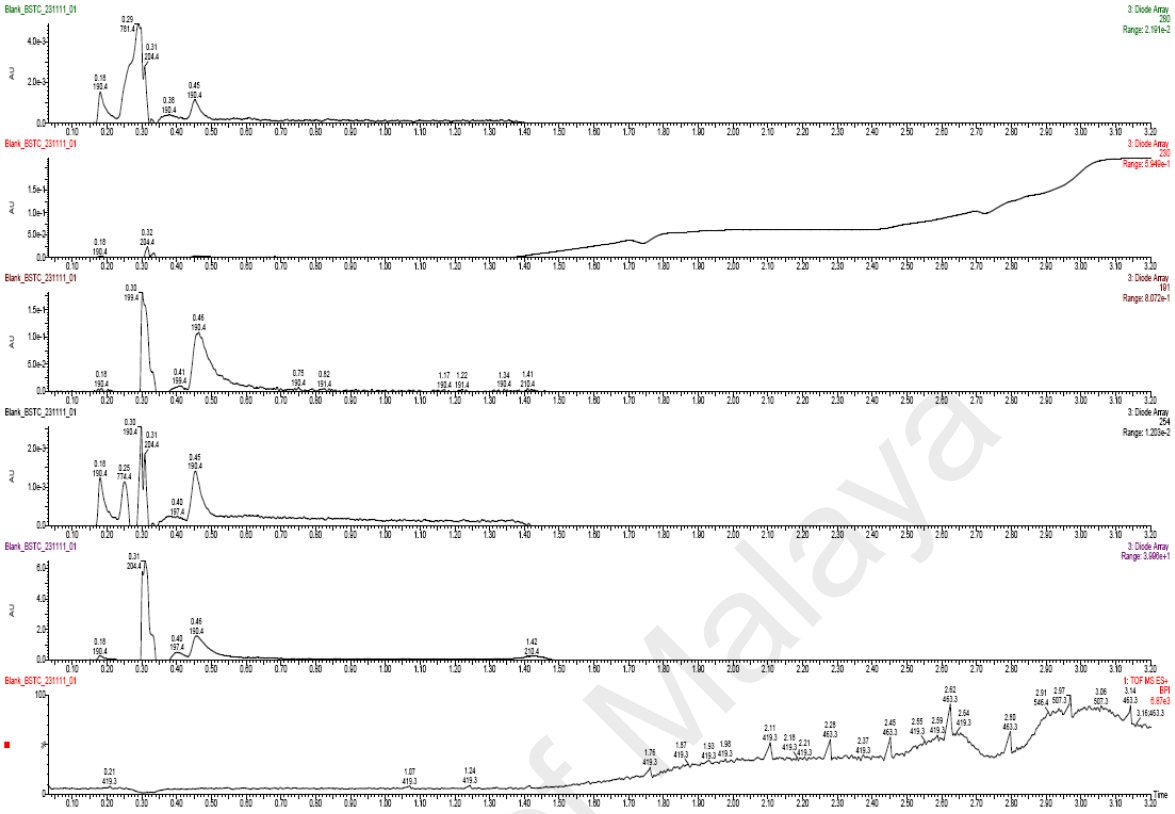


Figure 4.28 : Blank for HP-LC.

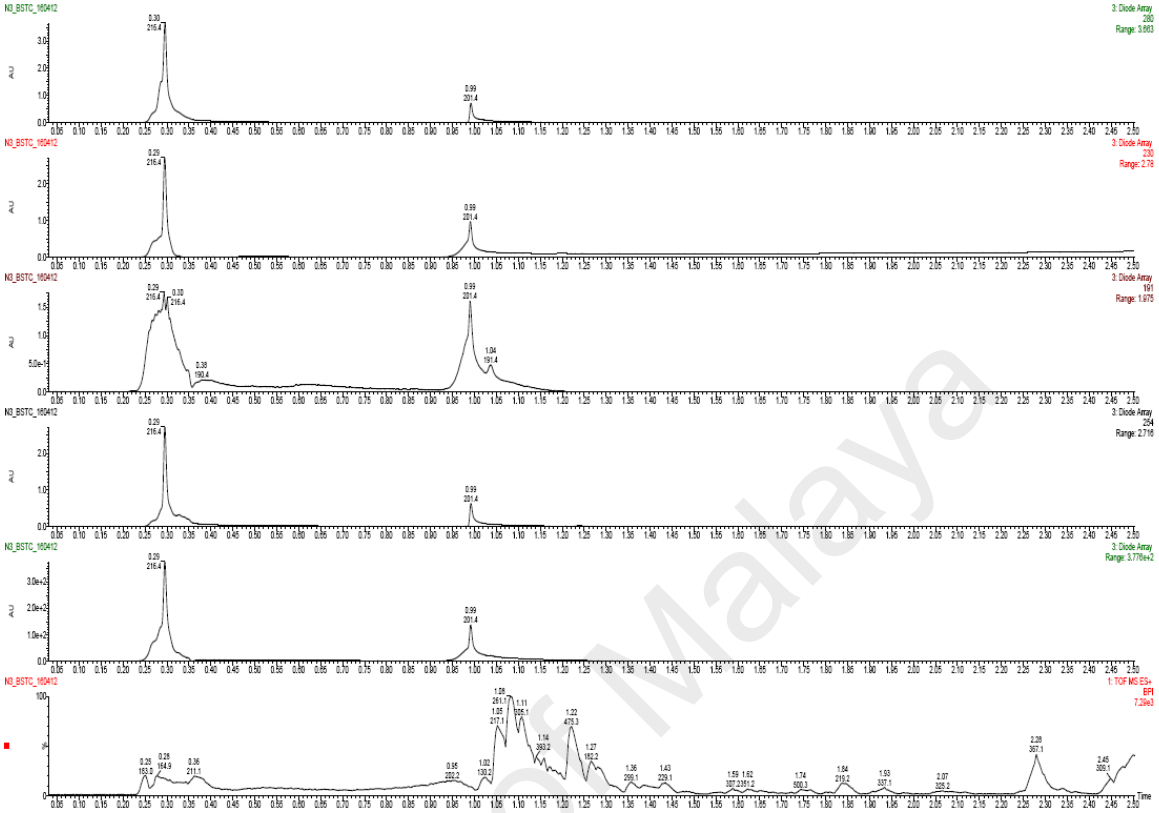


Figure 4.29 : HPLC chromatogram of N3 sample.

Single Mass Analysis

Tolerance = 15.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

271 formula(e) evaluated with 4 results within limits (up to 50 best isotopic matches for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O
217.0954	217.0937	1.7	7.8	3.5	C7 H13 N4 O4	20.3	0.987	37.26	7	13	4	4
	217.0950	0.4	1.8	8.5	C8 H9 N8	20.4	1.119	32.67	8	9	8	
	217.0977	-2.3	-10.6	7.5	C12 H13 N2 O2	21.0	1.686	18.52	12	13	2	2
	217.0923	3.1	14.3	-1.5	C6 H17 O8	21.5	2.158	11.55	6	17		8

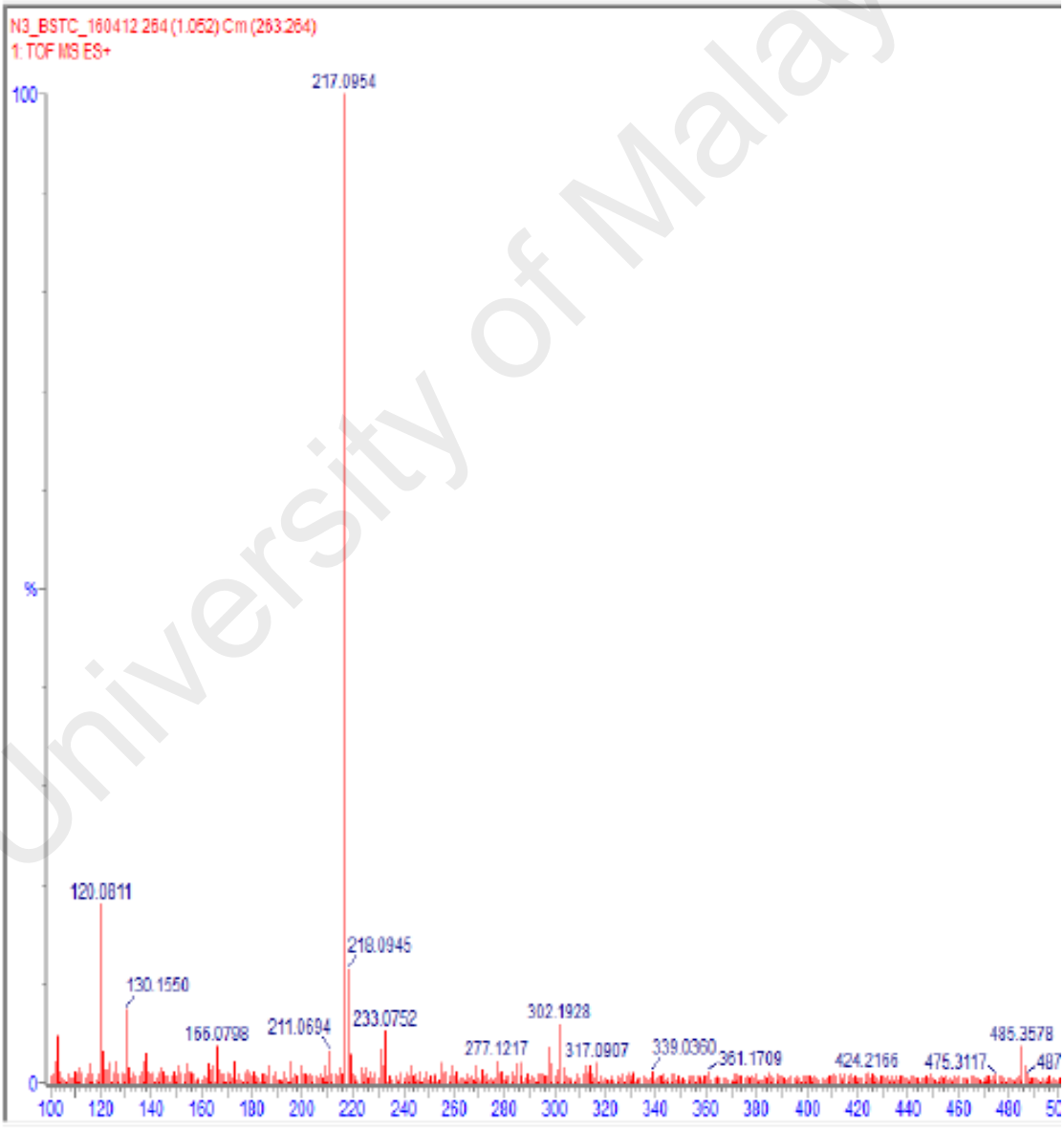


Figure 4.30 : Elemental Composition at R_t 1.052 minutes of N_3 sample.

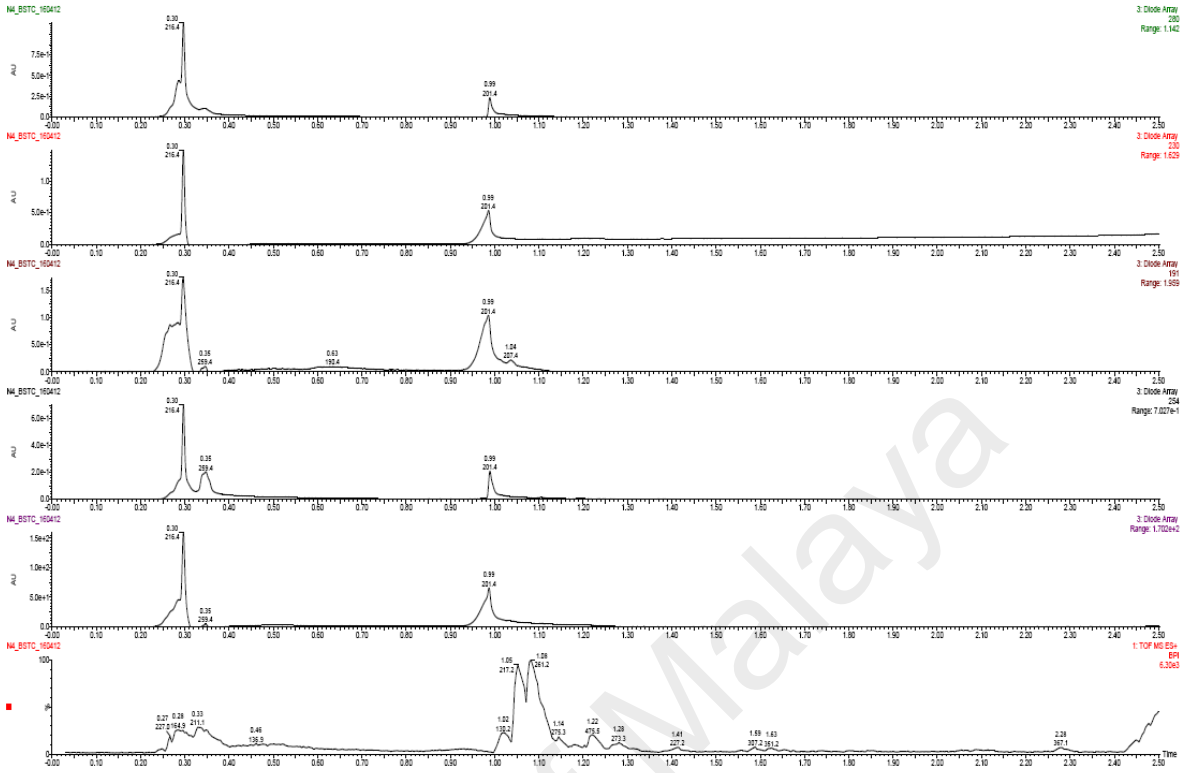


Figure 4.31 : HPLC chromatogram of N_4 sample.

Single Mass Analysis
Tolerance = 15.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
269 formula(e) evaluated with 2 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O
217.1948	217.1916	3.2	14.7	0.5	C11 H25 N2 O2	26.2	0.104	90.10	11	25	2	2
	217.1956	-0.8	-3.7	4.5	C16 H25	28.4	2.313	9.90	16	25		

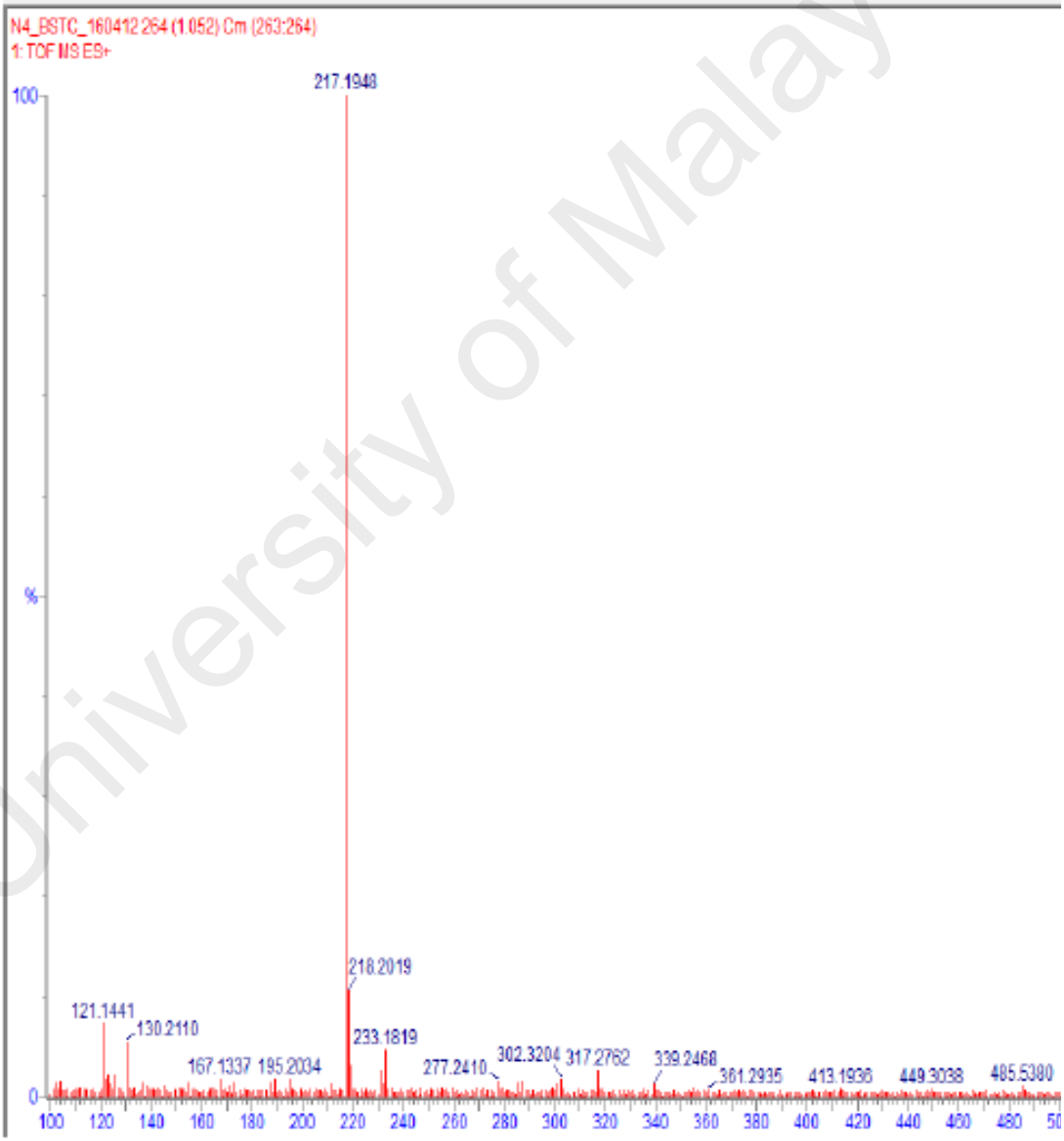


Figure 4.32 : Elemental Composition at R_t 1.052 minutes of N_4 sample.

CHAPTER FIVE : GENERAL DISCUSSION

5.1 General discussion

Preliminary assessment of *L. leucocephala* for potential anti-diabetic effect was carried out using an *in vitro* rat adipocyte model as well as *in vivo* OGTT in normal and diabetic rats. *L. leucocephala* up-regulated GLUT-4 gene expression twofold and HSL gene, reduced glucose absorption at 60 minutes in Oral Glucose Tolerance Test in normal rats and showed significant improvement at 150 minutes in Oral Glucose Tolerance Test in Streptozocin-induced diabetic rats.

In diabetes chronic study (30 day treatment), the dose used were 250 mg/kg and 500 mg/kg for *L. leucocephala* and 1.25 mg/kg for glibenclamide. Only a dose of 500 mg/kg and 5 mg/kg showed hypoglycemic activity within two hours after the glucose load in OGTT (*Figure 4.2* and *Figure 4.3*). A dose of 250 mg/kg of crude *L. leucocephala* did not show significant post-prandial hypoglycemia in the long-term control. In this study only two doses of extracts were used in order to minimize the use of animals (Griffin, 1998). The selected doses (250 mg/kg and 500 mg/kg) had been extensively used in many similar animal studies (Kamalakkanan, Rajadurai, & Prince, 2003; Kameswara, Kesavulu, Giri, & Appa, 1999). The toxicological evaluation were not carried out because the *L. leucocephala* leaves was used as crop food for goats and chicken (Anbarasu, et al., 2004; U ter Meulen, 1979). The nutritional value and quality of this plant has been extensively studied.

One of the components in the leave, mimosine was known to cause toxic effects in rare occasions and this could be solved with the presence of certain ruminal bacteria (DHP-degrading bacteria) which converts the dihydroxypyridines (DHP) into non-toxic compounds (Allison, Hammond, & Jones, 1990). There was only a case recorded on toxic effect in Brazilian goats (Peixoto, 2008) and the incident could be prevented by

rumen infusion (R. J. Jones & Lowry, 1984). Secondly, *L. leucocephala* young leaves and fruit were used as '*ulam*' in villagers in Malaysia, Indonesia, Thailand ("Leucaena," 1997) in which no toxic effect have been recorded in human. Although preliminary studies showed that 1.25 mg/kg managed to attenuate blood glucose level in OGTT, a dose of 5 mg/kg was selected for this study as it showed more significant hypoglycemic effect. The common dose prescribed to early diabetic patients is only 1.25 mg/kg and the dose will be increased if it fail to control the blood glucose, and the increase was limited to 2.5 mg/day (Leonard, Charles, & Morton, 2004). Therefore, the same dose was used for 30 days treatment in the animal study. The Glibenclamide was used as positive control due to its stimulatory effect on insulin secretion from the viable β-cells in the pancreas, since the dose of streptozocin in this study did not damage all the pancreatic β-cells. Glibenclamide was mostly used as positive control in ethnopharmacology diabetes research (R. Gupta & Gupta, 2010; NoorShahida, et al., 2009; Osadebe, Okide, & Akabogu, 2004; Pandikumar, Babu, & Ignacimuthu, 2009; Sriplang, Adisakwattana, Rungsipipat, & Yibchok-anun, 2007), and most commonly used drugs in developing countries such as Malaysia in either as single therapy or in combination with other drugs if it failed to control blood glucose. The rat model used in this study did not signify the major form of diabetes which is Type II. Nevertheless, the streptozocin induced diabetes in rats chosen (*Sprague Dawley*) were normal, healthy and non-obese. Therefore STZ induction of diabetes did not show any changes in lipid profiles compared to Wistar rats or genetically Type II Diabetes rats (e.g., Goto Kakizaki) relatively. The effects on specific metabolic parameters that influence diabetes mellitus could be investigated in future studies and different rat model could be used. The reason for using male rats in the study were not just due to their tendency to develop greater hyperglycemia but the body weight parameters were better in male rats

as male rats show greater increase in body weight than female (Schemmel, Mickelsen, & Gill, 1970) and have no resistance to induction due to female hormonal protection effect (Louet, LeMay, & Mauvais-Jarvis, 2004). The streptozocin dose used (50 mg/kg) was optimized in this study to induce mild diabetes and less mortality due to Streptozocin-induced diabetes complications, e.g., hypoglycemia or oxidative stress-induced nephropathy. Water extract of *L. leucocephala* was chosen as it reflected the traditional method used and it had better solubility (Schreier, Malheiros, & de Paula, 2000).

Leucaena leucocephala dry fruit water crude extract prevented worsening of glucose level in Streptozocin-induced diabetic rats in chronic study and *L. leucocephala* water-hexane partially purified extract at dose of 25 mg kg^{-1} attenuated glucose level in Streptozocin-induced diabetic rats in sub-chronic study. In chronic study, we can see the pattern where at a dose of 250 mg kg^{-1} showed a reduction on day 7 and at a dose of 500 mg kg^{-1} showed a reduction on day 15 (*Figure 4.4*).

Leucaena leucocephala dry fruit water crude extract restored weight loss in Streptozocin-induced diabetic rats in chronic study and *L. leucocephala* partially purified extract promoted reduction in body weight dose dependently in Streptozocin-induced diabetic rats in sub-chronic study. Similarly, reduction of body weight was observed on day 7 (*Figure 4.5* and *Figure 4.15*). The *L. leucocephala* water-hexane partially purified extract group showed the most significant weight reducing effect at a dose of 25 mg kg^{-1}. However, in long term (30 days) treatment the body weight was restored. It is possible to speculate that the weight loss at the early stage of treatment was due to absence of insulin and/or stimulation lipolysis.

Leucaena leucocephala dry fruit water crude extract stimulated more insulin production in Streptozocin-induced diabetic rats in chronic study and *L. leucocephala*

water-hexane partially purified extract at a dose 25 mg kg^{-1} stimulated secretion of insulin; which exerted hypoglycemic effect in Streptozocin-induced diabetic rats in sub-chronic study. The total antioxidant level as measured using the FRAP assay was found to be inversely correlated with MDA level (indicator of lipid peroxidation) (*Appendix H*) ($p < 0.05$) ($r = -0.8998$, $R^2 = 0.8096$). A high total antioxidant in pancreas significantly protected against lipid peroxidation induced by free radicals attack. Meanwhile, total antioxidant (FRAP) and protein oxidation test (AOPP) of pancreas in sub-chronic assay was significant ($p < 0.05$) and inversely correlated ($r = -0.7350$, $R^2 = 0.5403$) (*Appendix K*) whereas AOPP and MDA level were significantly ($p < 0.05$) correlated ($r = 0.7958$, $R^2 = 0.6333$) (*Appendix L*). Based on both chronic and sub-chronic study, it is evident that *L. leucocephala* is a good source of antioxidant which significantly protected the pancreas against protein and lipid damages, thus preserve its function. Therefore, it can stimulate more insulin production (*Figure 4.6*).

Leucaena leucocephala dry fruit juice water crude extract at a dose of 250 mg kg^{-1} exerted protective effects against protein and lipid damages in the brain and the effect was as good as Glibenclamide at a dose of 1.25 mg kg^{-1}. This could be due to enzymatic protection conferred by glutathione peroxidase (*Figure 4.10 a*) and non-enzymatic protection by total antioxidant activity (*Figure 4.10 b*). The AOPP-MDA was significantly correlated (*Appendix I*) ($p < 0.01$) ($r = 0.9599$, $R^2 = 0.9213$) in chronic study which indicated if either one of the oxidative parameter increased, the other will also increase.

Leucaena leucocephala dry fruit water crude extract significantly reduced diabetic damages in rat liver as shown in the liver histology (*Figure 4.12 c and d*) and in antioxidant status (*Figure 4.9 a* and *b*) in *L. leucocephala* (250 mg kg^{-1}) treated group in chronic study. *L. leucocephala* (water-hexane partially purified extract) significantly

reduced liver damage (*Figure 4.26 c* and *d*) and in antioxidant status (*Figure 4.21 a* and *b*) in sub-chronic study. Histological assessment showed absence or minimal fatty infiltration in *L. leucocephala* treated groups. This is due to the use of non-obese rat model. In *L. leucocephala* treated groups, no toxicity was found evident in the liver and this might be because *L. leucocephala* is rich in vitamin B_2. Generally, riboflavin plays a role in metabolism of carbohydrates, amino acids, lipids and cellular antioxidant (Combs Jr, 2012). In diabetic rats, riboflavin was found to be deficient but treatment with riboflavin returned the glutathione reductase enzyme activity to normal (A. S. Reddi, 1978). Its deficiency can cause fatty degeneration of the kidney and liver (Foraker, Khantwal, & Swaan, 2003). This might explain why *L. leucocephala* treated groups have no or less fatty infiltration. There is no clear correlation between glutathione peroxidase activity and total antioxidant level (based on FRAP assay) with protein oxidation and lipid peroxidation in the liver. However, there might be an indirect contribution by vitamin B_2 and magnesium contained in this extract in maintaining normal function of a liver.

The total antioxidant (FRAP) and lipid peroxidation (MDA) level of kidney in sub-chronic assay was significant ($p < 0.05$) and inversely correlated ($r = -0.7327$, $R^2 = 0.5368$) (*Appendix M*) and among AOPP-MDA was significant ($p < 0.01$) and positively correlated ($r = 0.8188$, $R^2 = 0.6705$) (*Appendix N*). It indicates that high total antioxidant could prevent or reduce protein oxidation and lipid peroxidation in the kidney. Despite glycemic control, genetic and environmental factors contribute to the risk of developing hypoglycemia-induced nephropathy in Type I diabetes.

Early damages in diabetic nephropathy are due to hyperglycemia-induced hemodynamic and metabolic changes. The hemodynamic changes are an increase in systemic and intraglomerular pressure and activation of various vasoactive pathways

which include renin-angiotensin system. That explains why patients with diabetes and hypertension would progress to nephropathy in the later phase of the disease progression. The histological alterations due to diabetes has been described in *Section 4.3.10.* The capillary surface area is important for glomerular filtration. However, the capillary surface was reduced due to the accumulation of matrix in mesangial area thus contributing to gradual loss of renal function. Other changes are podocyte and nephrin (podocyte protein) loss. Metabolic changes that occurred are changes in growth factors and cytokine levels e.g., VEGF , TGF-β, insulin-like growth factor 1, MCP-1 and IL-6. Podocytes produce VEGF and the increased accumulation of inflammatory cells and mesangial cells produce TGF-β which is involved in expansion of mesangial matrix. In these studies, levels of these parameters were not evaluated, therefore the mechanisms links to the protection effect of *L. leucocephala* on glomerulus cannot be hypothesized. Whether *L. leucocephala* bioactive compounds could inhibit the expression of VEGF and neutralizing TGF-β remains unknown (Wild, Roglic, Green, Sicree, & King, 2004).

Kalousova, Skrha and Zima., (2002) found that there was a correlation between AOPP and AGEs in both types of diabetes. In this study, low levels of protein oxidation (AOPP) as shown in *Figure 4.11 c* and *Figure 4.25 c* might imply low levels of AGEs products. A study by Witko-Sarsat, Friedlander, Nguyen Khoa, Capeillere- Blandin, Nguyen, Canteloup, Dayer, Jungers, Drueke, Descamps-Latscha (1996) in uremic patients, showed that plasma concentration of AOPP increased with progression of chronic renal failure and were closely related to AGEs. In addition, they concluded that AOPP can act as a mediator of oxidative stress, inflammation and monocyte activation in chronic renal failure. Thus, the reduction in AOPP level indicates attenuation of renal damage.

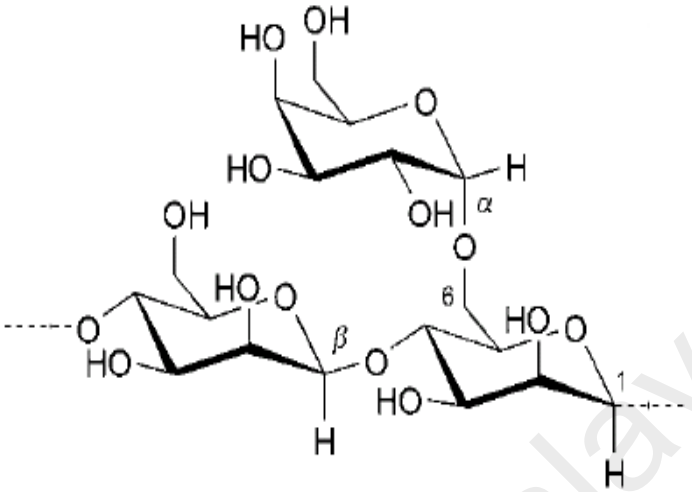


Figure 4.33: Chemical structure of galactomannan glycoside.

Syamsudin, Ros Sumarny and Partomuan Simanjuntak, (2010) first described that solvent fractions of *L. leucocephala* seeds had anti-diabetic effect on alloxan-induced diabetic rats. They claimed that the bioactive compounds namely glycosidic compounds with monosaccharide galactose clusters and many other saccharides that are responsible for reducing the hyperglycemic state. They also found that all the *L. leucocephala* seed fractions restored the body weight at 14th day with no gain in the rats' baseline body weight which is similar to the finding in this study. Syamsudin *et. al.*, (2010) also found that *L. leucocephala* were best extracted with polar solvents such as methanol and water for better yield or recovery from graded extraction compared to non-polar solvents (n-hexane) and semi-polar solvents (ethyl acetate). Lesniak and Liu (1981), showed that *L. leucocephala* seeds contain a galactomannan which is commonly found in plants of *Leguminosae* family. The chemical structure of galactomannan is in *Figure 4.33*. This hypothesis was supported by a study on a plant belonging to *Leguminosae* family *(Trigonela foenum graecum)* which also reduced hyperglycemia in Streptozocin-induced diabetic rats (Ali et al., 1995).

Galactomannan is a neutral water-soluble polysaccharide consisting of a mannose backbone with galactose side groups. These polysacharides (gums) is a soluble fibre that could act like a sponge and absorb water in the intestine, mixes the food into gel, thus slows down the rate of digestion and absorption. *L. leucocephala* delayed carbohydrate absorption as demonstrated *via* OGTT in normal rats (*Figure 4.2*). It showed that dietary fibres facilitate delayed absorption of glucose. Polysacharides have been used as fat substitutes in various low-calorie food products commonly used as stabilizing and thickening agents in capsules, tablets and in food products and they have been reported to reduce cholesterol (LDL and total cholesterol) and postprandial blood glucose. Chilkunda, Kari and Paramahans (2003) found that soluble fibres (5 % guar

gum) was more efficient in bringing about glycemic control than insoluble fibre (10 % wheat bran) but both were effective in controlling renal enlargement during early stages of diabetes-induced renal disease.

Based on physical characteristics of *L. leucocephala* extract which is white in colour and soapy/colloidal when mixed in water (*Figure 3.2*), it is speculated to be a type of saponin. Basic structure of saponin is glycosides (polar) attached to either steroid or triterpene backbone (non-polar) (Lappas, et al., 2005). Combination of polar and non-polar structural elements in their molecules makes them soapy in aqueous solutions. *L. leucocephala* partially purified water-solvents extracts were prepared by dissolving in 0.9 % normal saline because the extract is a medium-polar (combination of polar and non-polar) compound (Misra et al., 2011).

Saponins widely exist in leguminous plants. Few years ago, several steroidal compounds were detected in the whole plant of *L. leucocephala* (Chen & Wang, 2010; A. S. Reddi, 1978). Interestingly, there are few reports which showed that saponins from plant extracts posess antidiabetic (Wilson & Davis, 1983), hypocholesterolaemic (Al‐Habori & Raman, 1998) and anti-hyperglycemic properties (Bates, 1991; Farvid, Homayouni, Amiri, & Adelmanesh, 2011). *Momordica charantia* or bitter gourd contains saponin (butanol-fraction) which prevent increment of glucose level (Alluru S. Reddi, 1986).

In this study of *L. leucocephala*, the hypoglycemic activity was speculated to be mainly due to saponin. It is clear that the improvement in glucose-uptake activity was due to the increase in GLUT-4 gene expression and up-regulation of HSL gene and increased secretion of adiponectin which ultimately increase insulin sensitivity. *L. leucocephala* might have segretagous function on existing β-cells or significant antioxidant protection on pancreas that make it possible to stimulate secretion of insulin.

The saponin compound which most probably responsible for the hypoglycemic activity in water-hexane partially purified extract posessed a molecular weight of 217.0937 g/mol. The suggested molecular formula is $C_7H_{13}N_4O_4$.. However, the exact identification of the substance was not carried out in this study. More extensive work is required to identify the structure of the active compound and it is beyond the scope of this study.

5.2 Suggestions for future study

1. Further identification of *Leucaena leucocephala* bioactive compounds using TLC chromatography method before proceeding with NMR analysis.
2. Study effects of *Leucaena leucocephala* bioactive compounds on specific metabolic parameters e.g. lipid profiles that influence diabetes mellitus could be investigated in future studies using different rat model eg. C57BL/65 ob/ob.
3. Examine effects of *Leucaena leucocephala* bioactive compounds on levels of cytokines such as RAGE, VEGF and TGF-β in nephropathy.
4. Investigate effects of *Leucaena leucocephala* bioactive compounds on liver marker enzymes.
5. Inspect effects of *Leucaena leucocephala* bioactive compounds on glucose uptake in skeletal muscle.

CHAPTER SIX : CONCLUSION

The anti-diabetic and antioxidant effects of *Leucaena leucocephala* extract and its partially purified water-solvent extract were investigated in this study. The objective of this study as listed in *Section 1.3* has been achieved, in which *L. leucocephala* was demonstrated to have anti-diabetic and antioxidant properties based on chronic study and sub-chronic studies. The biochemical findings and the possible mechanism involved was discussed. *L. leucocephala* managed to reduce hyperglycemia and in long- term treatment and restored body weight loss. The increased production of adiponectin and insulin was possibly due to the protection of pancreas against free radical attacks; (FRAP-MDA inversely correlated ($p < 0.05$) in chronic and subchronic study, AOPP-MDA was significantly correlated ($p < 0.01$) in sub-chronic study. The insulinic effect were clearly observed in *L. leucocephala* water-hexane partially purified extract (dose 25 mg kg $^{-1}$) which showed normoglycemia in Streptozocin-induced diabetic rats in sub-chronic study. The protective effect on the liver might be indirectly contributed by vitamin B complex and magnesium (present in the extract) that may be involved in liver detoxification process and this concurred with FRAP-MDA inverse correlation in sub-chronic study. The protection of liver by the *L. leucocephala* extract was evident in histological assessment. *L. leucocephala* also prevented early damages of glomerulus in the diabetic kidney and this concurred with AOPP-MDA positive correlation. In the brain, *L. leucocephala* showed high glutathione peroxidase activity, moderate total antioxidant and protection against protein oxidation and lipid peroxidation. *L.leucocephala* water-hexane partially purified extract at a dose of 25 mg kg $^{-1}$ showed the most significant *in vivo* anti-diabetic and antioxidant effects.

NOVEL FINDINGS ARISING FROM THIS STUDY AND IMPLICATIONS FOR FUTURE RESEARCH

At present, this is among pioneer studies about *Leucaena leucocephala* and its medicinal properties. Novel findings derived from these studies are summarized as below :

Anti-diabetic activity

- *Leucaena leucocephala* up-regulates GLUT-4 gene expression twofold difference and HSL gene in adipocytes cell culture.
- *Leucaena leucocephala* dry fruit water crude extract prevent glucose worsening, restored from body weight loss, stimulates more insulin production, stimulated more production of adiponectin in Streptozocin-induced diabetic rats in chronic study.
- *Leucaena leucocephala* water-hexane partially purified extract at dose of 25 mg kg^{-1} stimulates secretion of more insulin; which exerts hypoglycemic effect in Streptozocin-induced diabetic rats in sub-chronic study.
- *Leucaena leucocephala* water-hexane partially purified extract promotes reduction in body weight dose dependently in Streptozocin-induced diabetic rats in sub-chronic study.
- Adiponectin concentration were positively correlated with percentages of changes in body weight in sub-chronic study.

Antioxidant activity

- *Leucaena leucocephala* dry fruit water crude extract has high phenolic content; test *via in vitro* and rich in vitamin B_2, calcium and magnesium sources.

- *Leucaena leucocephala* dry fruit water crude extract at dose of 250 mg kg^{-1} and 500 mg kg^{-1} exerts protection in pancreas where FRAP assay was found to be inversely correlated with MDA assay ($p < 0.05$) in Streptozocin-induced diabetic rats in chronic study.
- *Leucaena leucocephala* dry fruit water crude extract significantly reduced diabetic damages in liver pathology and at dose 250 mg kg^{-1} exerts protection in liver in Streptozocin-induced diabetic rats in chronic study which possibly due to benefit of vitamin B complex and magnesium that helps in phase II detoxification thus maintaining normal function of a liver.
- *Leucaena leucocephala* dry fruit water crude extract at dose of 250 mg kg^{-1} exerts protective effects in protein and lipid of brain as good as diabetes positive control where AOPP-MDA was significantly correlated ($p < 0.01$) in Streptozocin-induced diabetic rats in chronic study.
- *Leucaena leucocephala* dry fruit water crude extract significantly reduced diabetic damages in kidney pathology, prominently at dose of 250 mg kg^{-1} and at dose of 500 mg kg^{-1} exerts protective effects against lipid peroxidation in kidney in Streptozocin-induced diabetic rats in chronic study.
- *Leucaena leucocephala* water-hexane partially purified extract at dose of 25 mg kg^{-1} treated group posess the highest total antioxidant, prevent lipid peroxidation and secondly better in protein protection in pancreas where total antioxidant (FRAP) and protein oxidation test (AOPP) of pancreas in sub-chronic assay was significant ($p < 0.05$) inversely correlated and among AOPP-MDA was significantly ($p < 0.05$) correlated in Streptozocin-induced diabetic rats in sub-chronic study.

- *Leucaena leucocephala* water-hexane partially purified extract significantly reduced diabetic damages in liver pathology and at dose of 50 mg kg^{-1} has high glutathione peroxidase activity, high total antioxidant and protect against protein oxidation in the liver in Streptozocin-induced diabetic rats in sub-chronic study.
- *Leucaena leucocephala* water-hexane partially purified extract at dose of 25 mg kg^{-1} showed high glutathione peroxidase activity, moderately total antioxidant, protection against protein oxidation and lipid peroxidation in brain in Streptozocin-induced diabetic rats in sub-chronic study.
- *Leucaena leucocephala* water-hexane partially purified extract at dose of 25 mg kg^{-1} significantly reduced diabetic damages in kidney pathology at dose of 50 mg kg^{-1} exerts best protection effect in kidney.where total antioxidant (FRAP) and lipid peroxidation test (MDA) of kidney in sub-chronic assay was significant ($p < 0.05$) inversely correlated and among AOPP-MDA was significantly ($p < 0.01$) correlated in Streptozocin-induced diabetic rats in sub-chronic study.

Therefore, *Leucaena leucocephala* have more potentials to discover as future anti-diabetic drug.

LIMITATION OF THIS STUDY

There are several limitations in these studies, firstly is the time-framed and lack of funding for further research whereas *Leucaena leucocephala* needs details investigations as listed in *Suggestions for further study (page 213)* before an attempt into clinical study. Secondly, some rats develop resistance to Streptozocin-induced diabetes and therefore the rats' blood glucose level was checked again on day 7^{th} before deciding for another injection at the same dosage. The reason for doing so was because the lack of rats meanwhile the demands of *Sprague Dawley* rats among researcher in Animal House, Faculty of Medicine was high and to maintain the quality of rats used throughout the study. However, if the rats had failed to become hyperglycemia after two times injection, that rats excluded from being a sample. Thirdly, the extraction process used was limited, as we did not boil (100°C) the crude extract to preserve most of the protein and natural chemical structure exist in *Leucaena leucocephala* fruit. The direct solvent crude extraction was not performed and tested on normal and Streptozocin-induced diabetic rats. The effect of *Leucaena leucocephala* water-solvent partially purified extract were not observed in normal rats for chronic and sub-chronic studies. The LD_{50} was not done.

LIST OF AWARDS, PUBLICATIONS AND PROCEEDINGS

Poster

Nooriza. A., Kuppusamy. U.R., Mahmood. A. A. Assessment of local plants for glucose tolerance in rats. *Proceedings of 7th COSTAM/SFRR (ASIA/MALAYSIA) International Workshop 2009 and 4th Biennial Meeting of SFRR ASIA*. 9th -12th July 2009.

Nooriza. A., Kuppusamy. U.R., Mahmood. A. A. *Leguminosae* fruit (*LL*) extract protects against liver and kidney damage in Streptozocin-induced diabetic Rats. *Innovation and Creativity Expo 2010 University of Malaya* .1-3rd April 2010.

Article paper

Kuppusamy. U.R., Bavani. A, **Nooriza A.**, and Chai J.W. *Leucaena leucocephala* fruit aqueous extract stimulates adipogenesis, lipolysis and glucose uptake in primary rat adipocytes [Submitted paper]

Nooriza. A., Kuppusamy.U.R., and Mahmood A.A. Effects of *Leucaena leucocephala* fruit extract in Streptozotocin diabetic rats. [Manuscript in preparation]

(Adeneye, 1991)

(Allison, et al., 1992)

(Awad Allah R S, Dkhil M A, & Danfour M A, 2008)

(Brenner, Meyer, & Hostetter, 1982)

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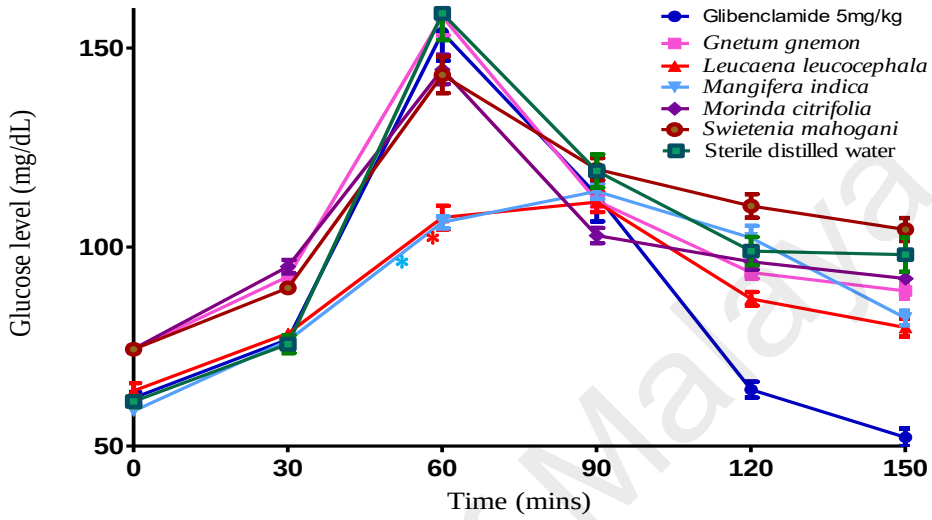
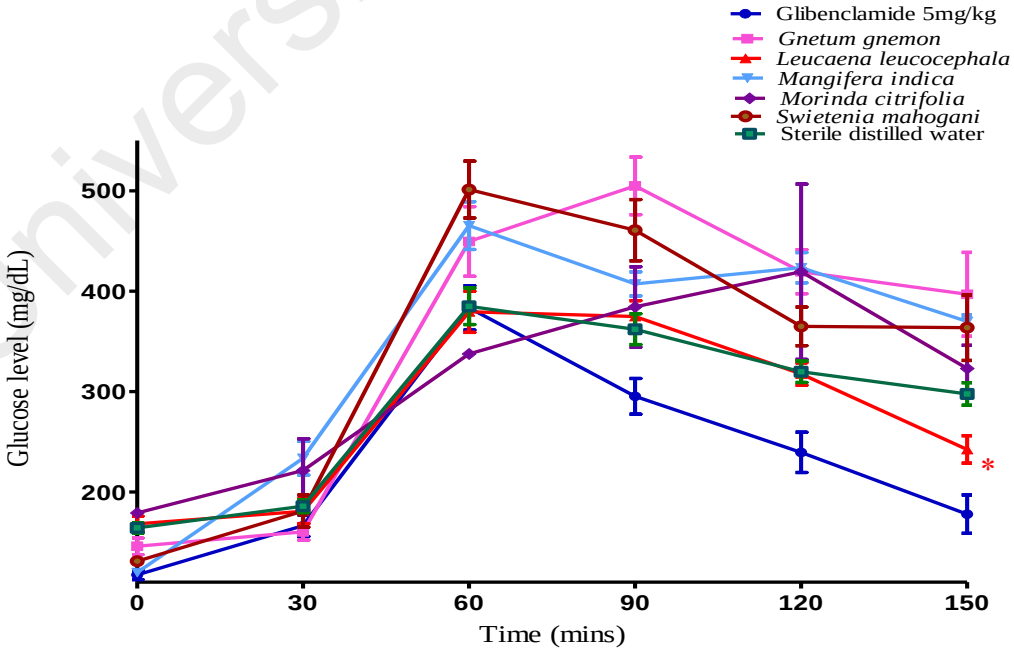
Appendix A

(i) OGTT of normal rats pretreated with 500 mg kg^{-1} *Leucaena leucocephala* and others water crude extracts.

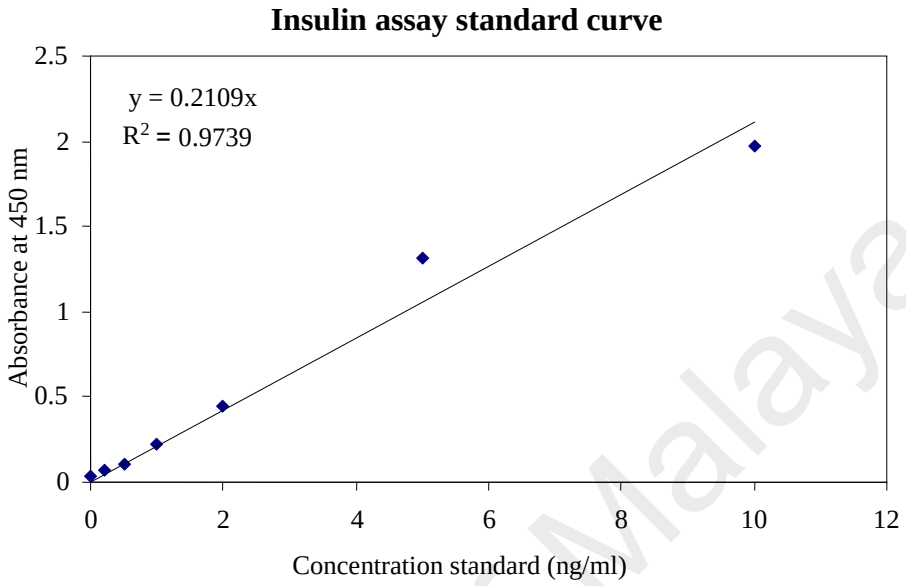


(ii) OGTT of diabetic rats pretreated with 500 mg kg^{-1} *Leucaena leucocephala* and others water crude extracts.

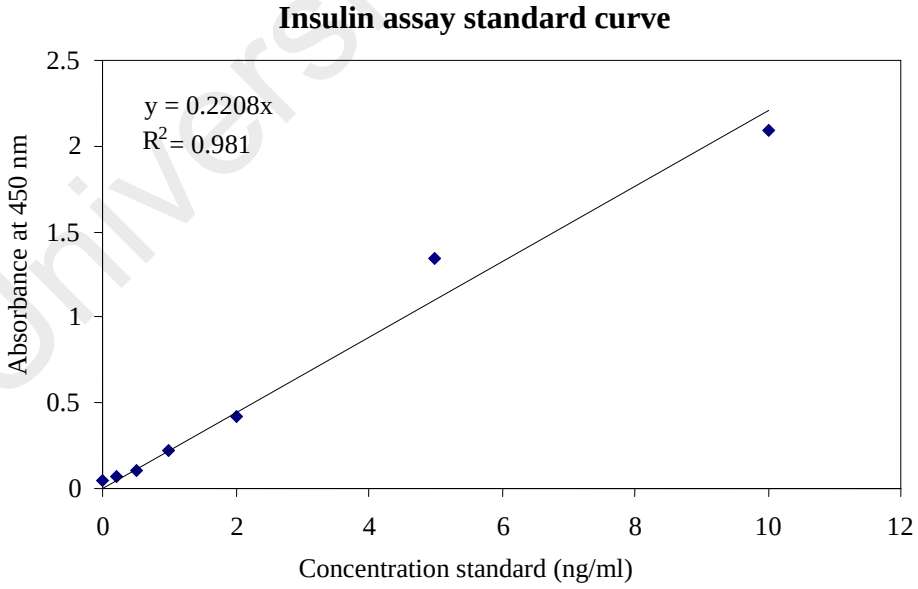


Appendix B

(i) Insulin assay standard curve for chronic study

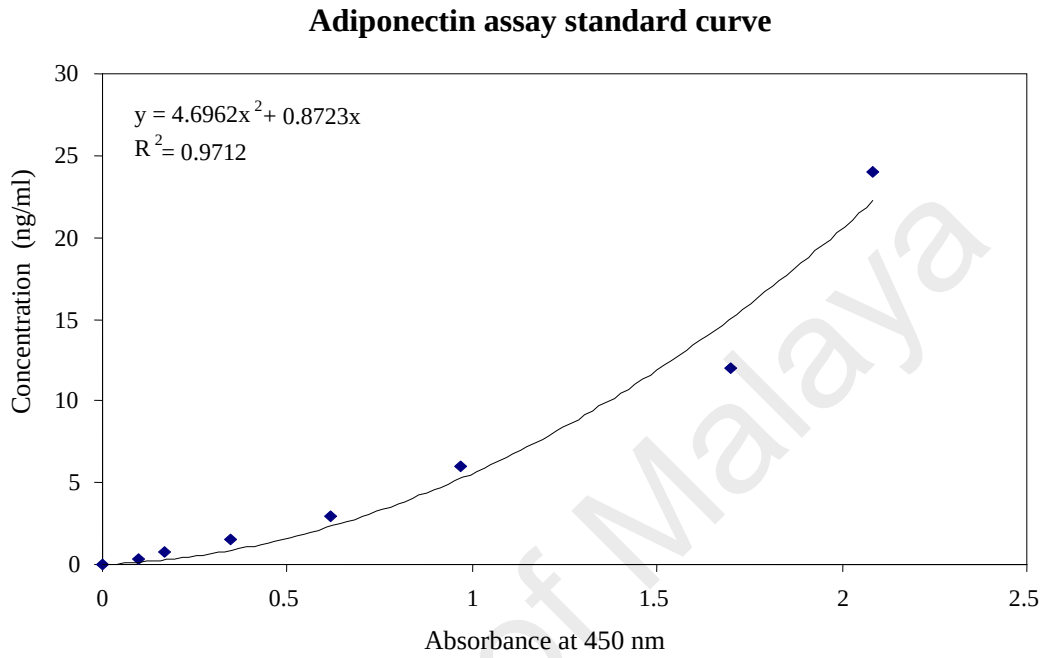


(ii) Insulin assay standard curve for sub-chronic study

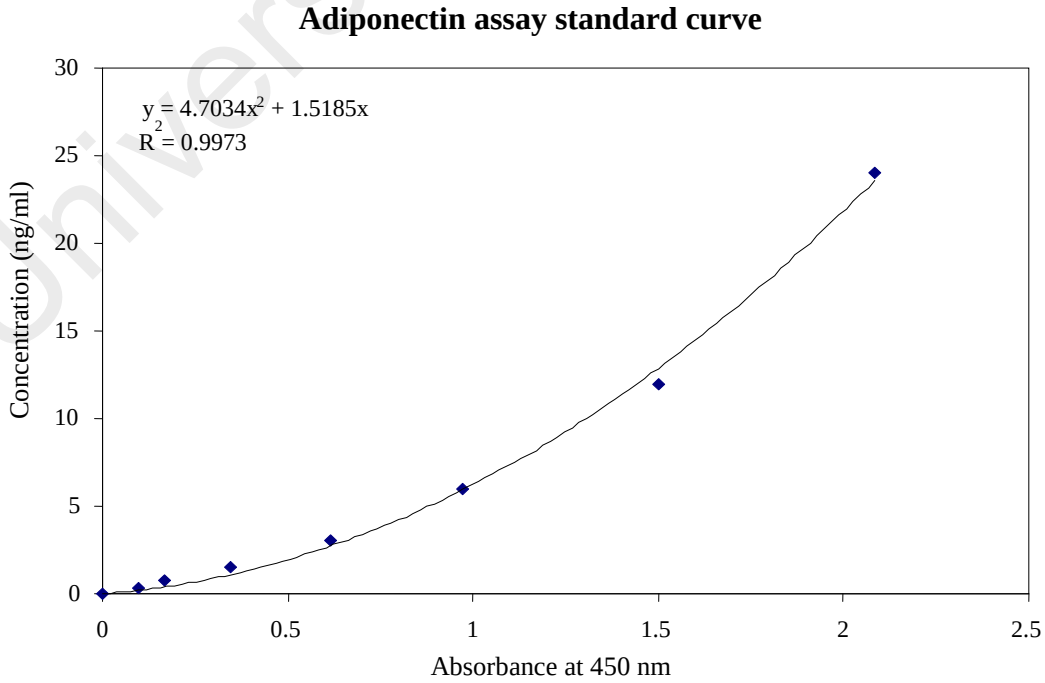


Appendix C

(i) Adiponectin assay standard curve for chronic study

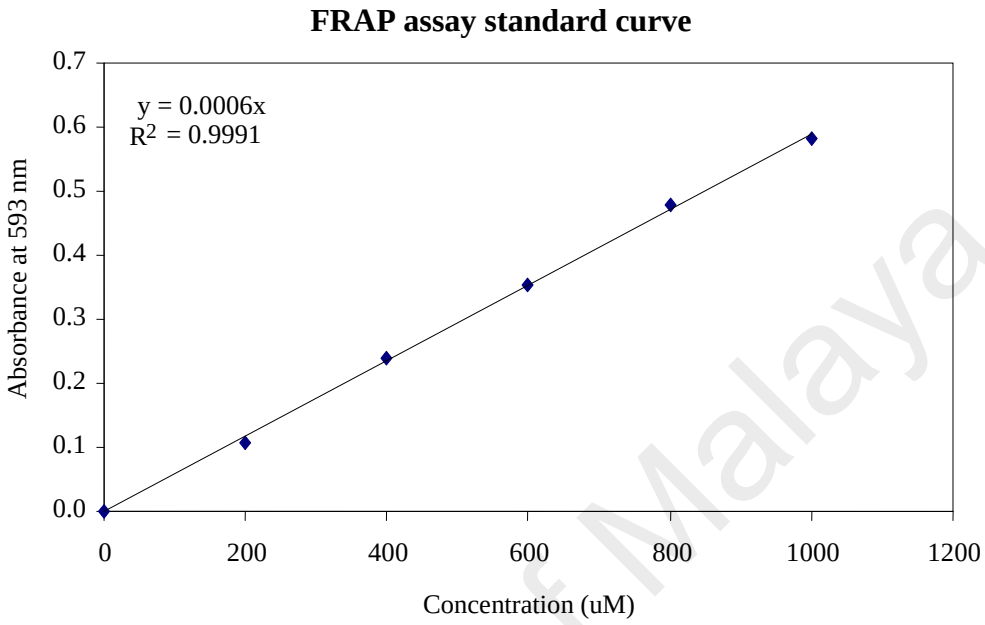


(ii) Adiponectin assay standard curve for sub-chronic study

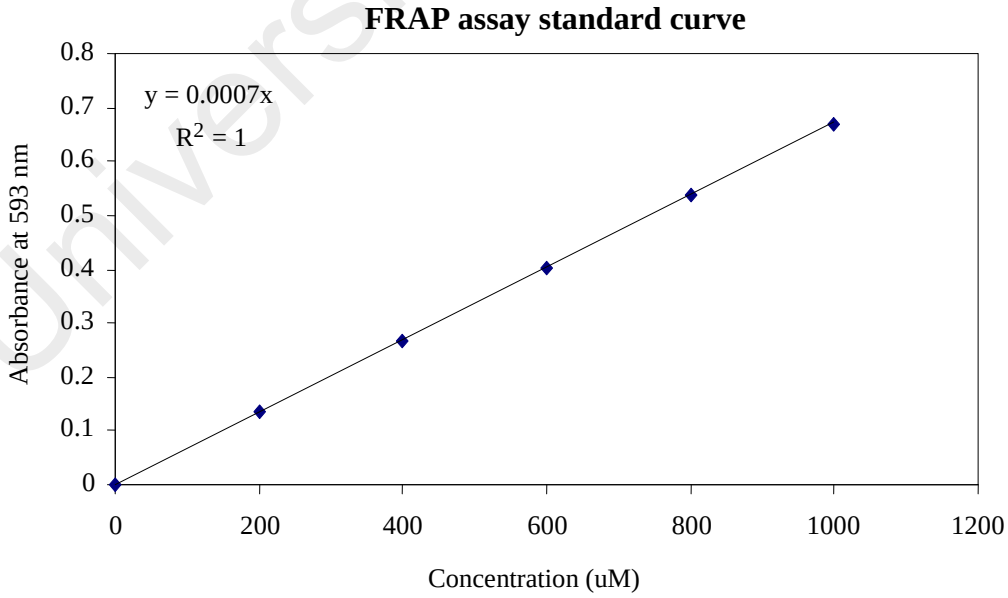


Appendix D

(i) FRAP assay standard curve for homogenates organ in chronic study

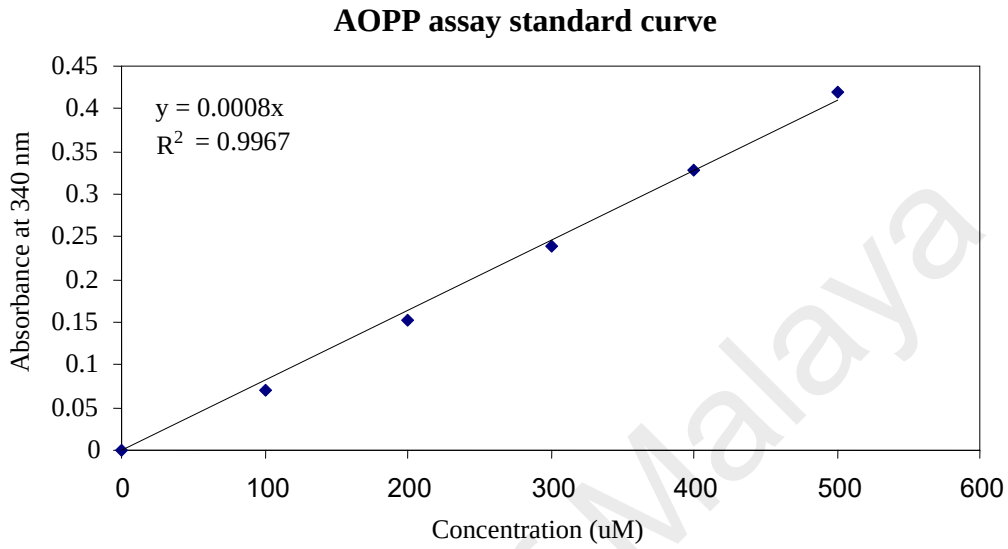


(iii) FRAP assay standard curve for homogenates organ in sub-chronic study

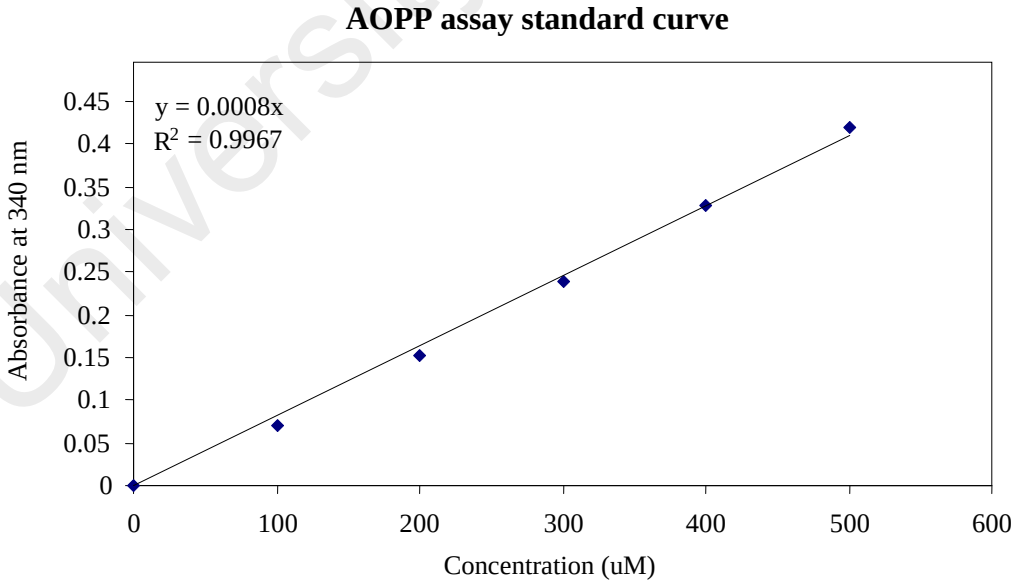


Appendix E

(i) AOPP assay standard curve for homogenates organ in chronic study

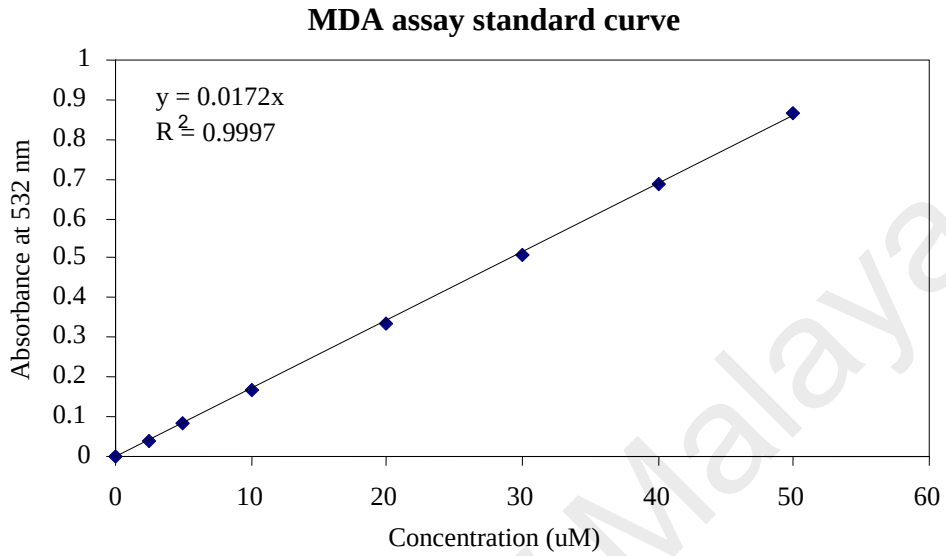


(ii) AOPP assay standard curve for homogenates organ in sub-chronic study

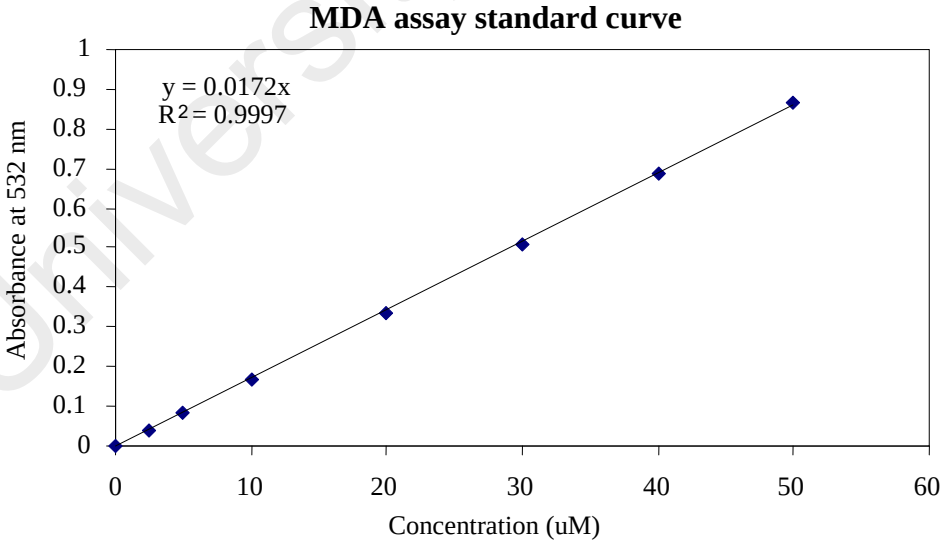


Appendix F

(i) MDA assay standard curve for homogenates organ in chronic study

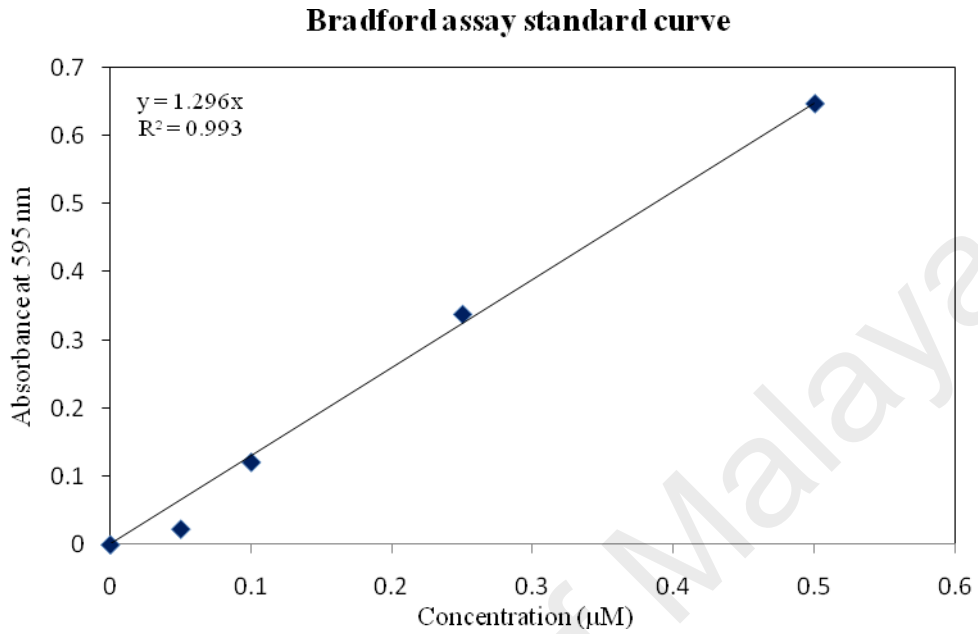


(ii) MDA assay standard curve for homogenates organ in sub-chronic study

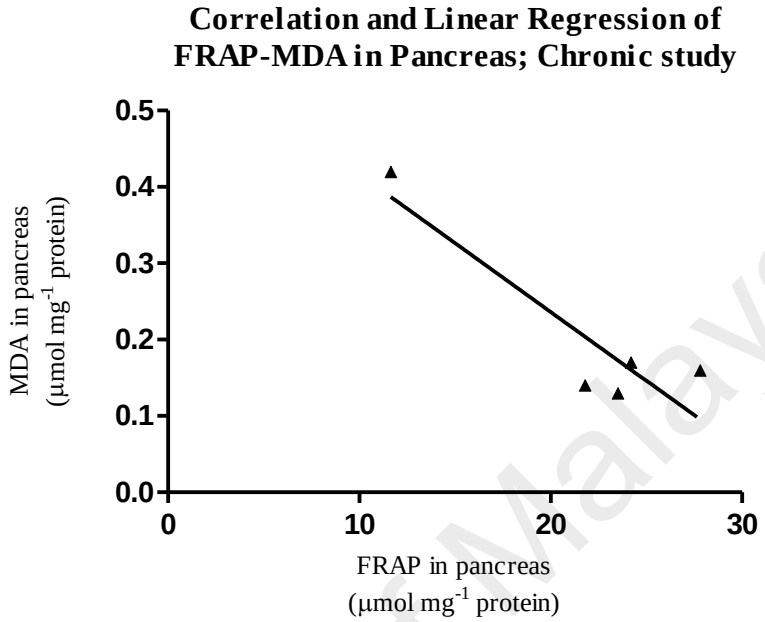


Appendix G

Bradford assay standard curve

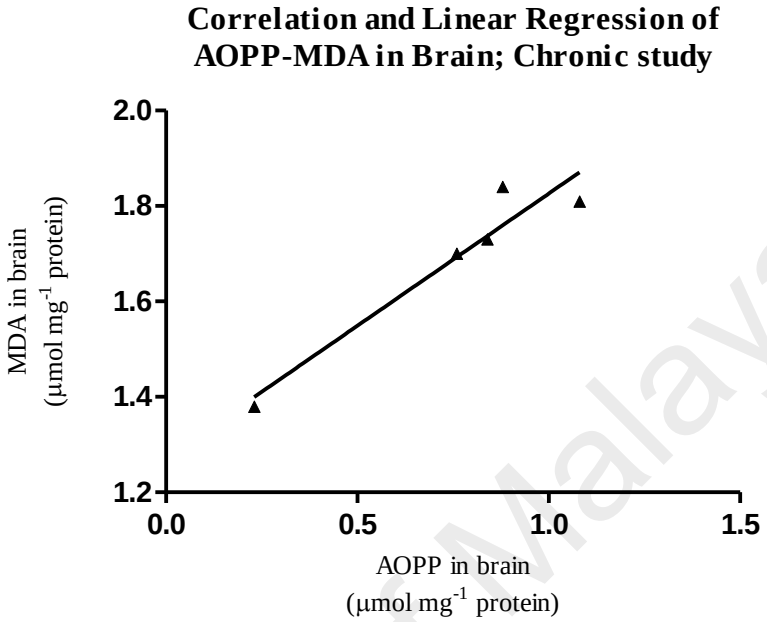


Appendix H



Number of XY Pairs	5
Pearson r	-0.8998
95% confidence interval	-0.9934 to -0.08453
P value (two-tailed)	0.0375
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.8096

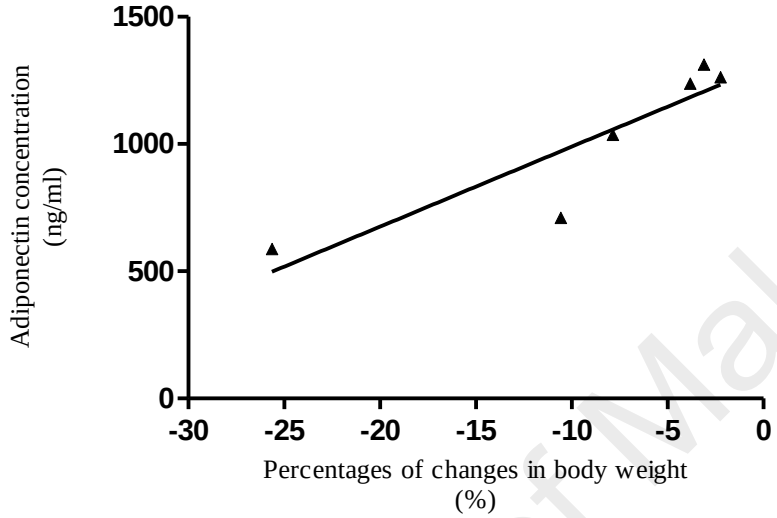
Appendix I



Number of XY Pairs	5
Pearson r	0.9599
95% confidence interval	0.5064 to 0.9974
P value (two-tailed)	0.0096
P value summary	**
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.9213

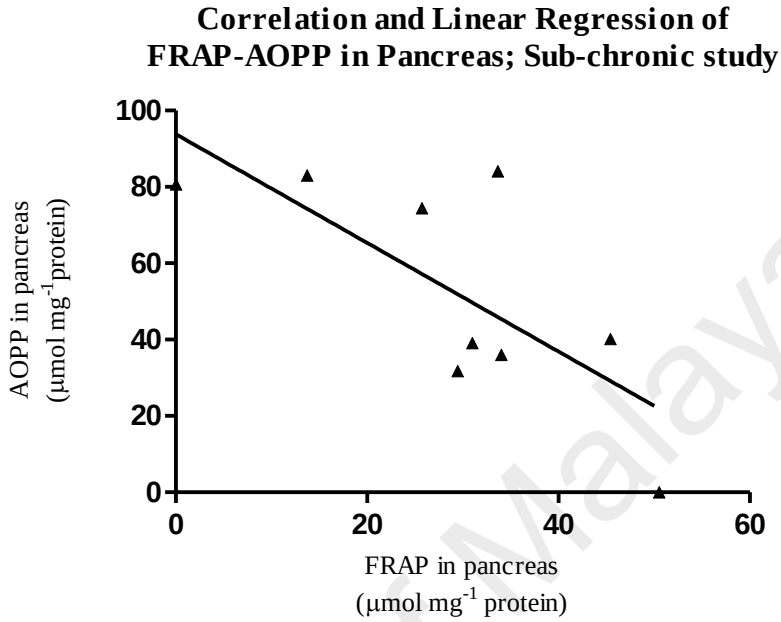
Appendix J

Correlation and Linear Regression of Percentage change in body weight - Adiponectin concentration; Sub-chronic study



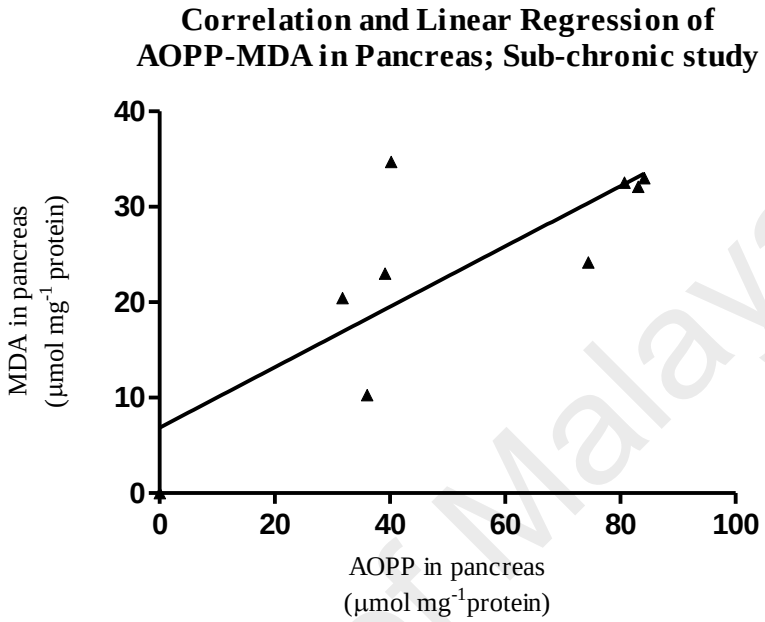
Number of XY Pairs	6
95% Confidence Intervals	10.02 to 52.82
P value	0.0151
P value summary	*
Is the correlation significant (alpha=0.05)	Yes
R squared	0.8060

Appendix K



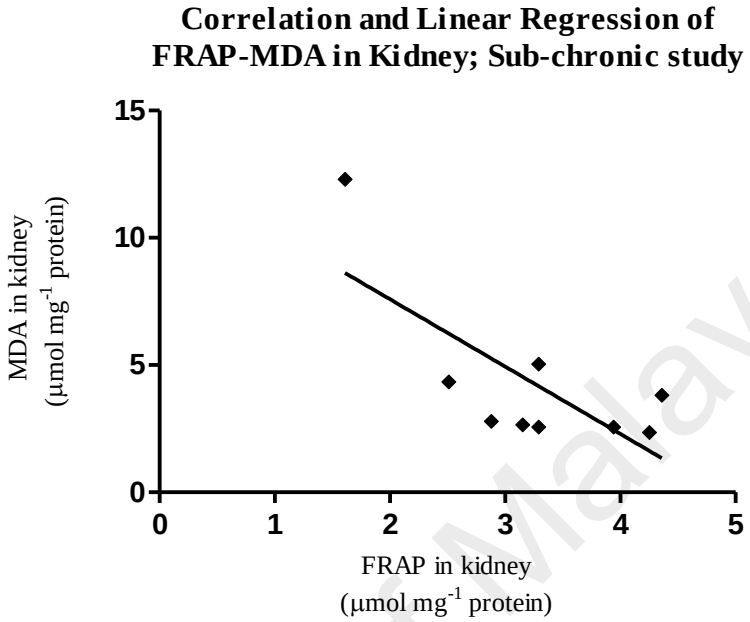
Number of XY Pairs	9
Pearson r	-0.7350
95% confidence interval	-0.9402 to -0.1383
P value (two-tailed)	0.0241
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.5403

Appendix L



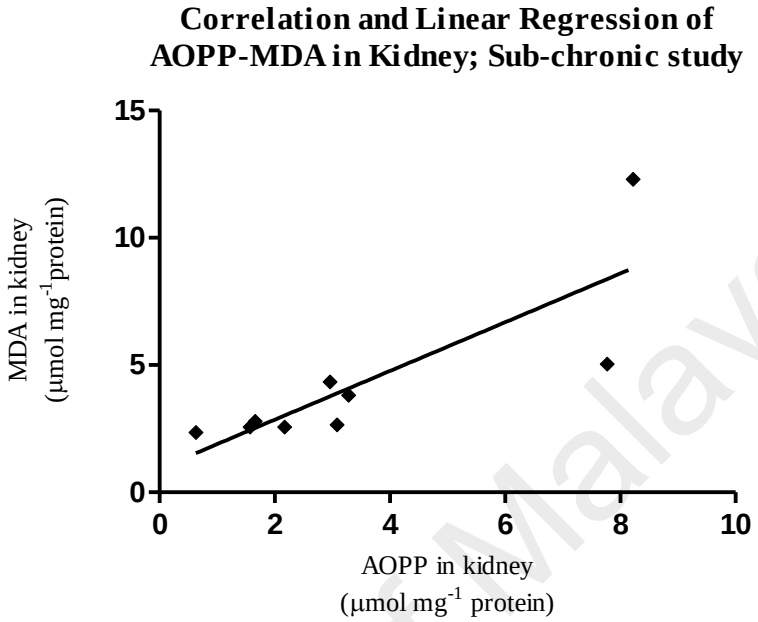
Number of XY Pairs	9
Pearson r	0.7958
95% confidence interval	0.2791 to 0.9551
P value (two-tailed)	0.0103
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.6333

Appendix M



Number of XY Pairs	9
Pearson r	-0.7327
95% confidence interval	-0.9396 to -0.1333
P value (two-tailed)	0.0247
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.5368

Appendix N



Number of XY Pairs	9
Pearson r	0.8188
95% confidence interval	0.3390 to 0.9606
P value (two-tailed)	0.0069
P value summary	**
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.6705