EVALUATION OF THE ANTI-DIABETIC PROPERTIES OF *AVERRHOA BILIMBI* IN ANIMALS WITH EXPERIMENTAL DIABETES MELLITUS

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LIST OF ABBREVIATIONS

AAI: Anti-atherogenic index

AAS: Atomic absorption spectroscopy

ABe: Ethanolic leaf extract of *Averrhoa bilimbi*

AF: Aqueous fraction of ABe

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

BuF: Butanol fraction

C57BL/6J: Non-albino mouse strain

CH_3^+: Carbonium ions

CNS: Central nervous system

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

EF: Ethyl acetate fraction

ELISA: Enzyme linked immunosorbent assay

FBG: Fasting blood glucose

FPG: Fasting plasma glucose

Fru-1, 6-P2ase: Fructose-1, 6-bisphosphatase

Glc-6-P: Glucose-6-phosphate

Glc-6-Pase: Glucose-6-phosphatase

GPx: Glutathione peroxidase

G6PDH: Glucose-6-phosphate dehydrogenase

GS: Glycogen synthase

GSI: Activated glycogen synthase

GSSG: Oxidized glutathione

GST: Glutathione S-transferase

HDL-C: High density lipoprotein cholesterol

H_2O_2: Hydrogen peroxide

HF: Hexane fraction of ABe

HFD: High fat diet

HGP: Hepatic glucose production

HPLC: High performance liquid chromatography

IDDM: Insulin dependent diabetes mellitus

IPGTT: Intraperitoneal glucose tolerance test

IRTK: Insulin receptor tyrosine kinase

LC-MS: Liquid chromatography coupled with mass spectrometry

LC-NMR: Liquid chromatography coupled with nuclear magnetic resonance spectroscopy

LC-UV: Liquid chromatography coupled with ultra-violet spectroscopy

LC-IR: Liquid chromatography coupled with infrared spectroscopy

LDL-C: Low density lipoprotein cholesterol

mRNA: Messenger ribonucleic acid

Mg: Magnesium

$MgCl_2$: Magnesium chloride

$MgSO_4$: Magnesium sulfate

MgV: Magnesium vanadate

MDA: Malonaldehyde

NA: Nicotinic acid or Niacin

NaV: Sodium vanadate

NAD^+: Nicotinamide adenine dinucleotide

NADP: Nicotinamide adenine dinucleotide phosphate

NAM: Niacinamide

NEFA: Non-esterified fatty acids

$NiCl_2$: Nickel chloride

NIDDM: Non-insulin dependent diabetes mellitus

NPY: Neuropeptide Y

NOD mice: Non-obese diabetic mice

$O_2^{\bullet-}$: Superoxide

$OH^{\bullet}$: Hydroxyl radicals

OFRs: Oxygen free radicals

OGTT: Oral glucose tolerance test

O.D: Optical density

PG: Plasma glucose

PBS: Phosphate-buffered solution

PKC: Protein kinase C

PPAR: Peroxisome proliferator activated receptor

RP-HPLC: Reverse phase high performance liquid chromatography

SD: Sprague-Dawley

SEM: Standard error of mean

SDS: Sodium dodecyl sulphate

STZ: Streptozotocin

TBARS: Thiobarbituric acid reactive substances

TC: Total cholesterol

TG: Triglycerides

TxA_2: Thromboxane A_2

TxB_2: Thromboxane B_2

TZDs: Thiazolidinediones

UCP: Uncoupling protein

VLDL-C: Very low density lipoprotein cholesterol

WHO: World Health Organization

LIST OF PUBLICATIONS

I. Publications in International Journals

1. Pushparaj P, Tan CH, Tan BKH. Effects of *Averrhoa bilimbi* leaf extract on blood glucose and lipids in streptozotocin-diabetic rats. Journal of Ethnopharmacology (IRELAND). 2000; 72 (1-2): 69-76.

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II. Book Chapter

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III. International Conference Papers

1. Pushparaj P, Tan CH, Tan BKH. Effects of *Averrhoa bilimbi* leaf extract on blood glucose and lipids in streptozotocin-diabetic Sprague Dawley rats. Diabetologia (GERMANY). 1 August 1999; 42: 871 (Suppl. 1).

2. Pushparaj P, Tan CH, Tan BKH. Mechanism of hypoglycaemic action of *Averrhoa bilimbi* in streptozotocin-diabetic rats. Diabetologia (GERMANY). 1 August 2001; 44: 873 (Suppl. 1).

3. Pushparaj PN, Tan CH, Tan BKH. Influence of metal ions on carbohydrate metabolism in vivo and in vitro. Abstracts of Papers of the American Chemical Society CARB Part 1 Aug 2001; 222: 118.

4. Pushparaj P, Tan CH, Tan BKH. Pancreatic β-cell protective action of *Averrhoa bilimbi* leaf extract against streptozotocin in Sprague-Dawley rats. Proceedings, International Symposium on the Utilization of Natural Products in Developing Countries: Trends and Needs, Kingston, Jamaica, West Indies. 2002; 138-144.

IV. Poster Presentations in International Conferences

1. Pushparaj P, Tan CH, Tan BKH. Effects of *Averrhoa bilimbi* leaf extract on blood glucose and lipids in streptozotocin-diabetic Sprague Dawley rats. 35th Annual Meeting of the European Association for the Study of Diabetes, 28th September-2nd October, 1999, Brussels, Belgium.

2. Pushparaj P, Tan CH, Tan BKH. Mechanism of hypoglycaemic action of *Averrhoa bilimbi* in streptozotocin-diabetic rats. 37th Annual Meeting of the European Association for the Study of Diabetes, 9th- 13th September, 2001, Glasgow, United Kingdom.

3. Pushparaj P, Tan BKH, Tan CH. Effects of *Averrhoa bilimbi* on blood glucose and lipids in type 2 rat model of diabetes mellitus. 2nd International Conference on Natural Products, 1st-4th July, 2002, Singapore.

V. Oral Presentations in International Conferences

1. Pushparaj P, Tan CH, Tan BKH. Pancreatic β-cell protective action of *Averrhoa bilimbi* leaf extract against streptozotocin in Sprague-Dawley rats. International Symposium on the Utilization of Natural Products in Developing Countries: Trends and Needs, 9th – 14th July, 2000, Kingston, Jamaica, West Indies.

2. Pushparaj P, Tan CH, Tan BKH. Semi-purified fractions of *Averrhoa bilimbi* exert both hypoglycaemic as well as hypolipidaemic activities in streptozotocin-diabetic rats. National Symposium on Medicinal Plants, 5th – 6th February, 2001, Tiruchirappalli, Tamil Nadu, India.

3. Pushparaj P, Tan CH, Tan BKH. Antidiabetic effects of *Averrhoa bilimbi* in experimental animals with Type I and Type 2 diabetes. 3rd International Conference on Natural Products, 23rd – 25th October 2004, Nanjing, China.

VI. Oral Presentation in Local Conference

1. Pushparaj P, Tan CH, Tan BKH. Evaluation of natural products for anti-diabetic properties. 2nd GSS-FOM Scientific Conference, 22nd March, 2002, Faculty of Medicine, National University of Singapore, Singapore.

VII. Poster Presentations in Local/Regional Conferences

1. Pushparaj P, Tan CH, Tan BKH. Effects of semi-purified fractions of *Averrhoa bilimbi* leaves on blood lipids in streptozotocin-diabetic rats. 4th NUH-NUS Faculty of Medicine, Annual Scientific Meeting, 30th June – 1st July, 2000, Singapore.

2. Pushparaj P, Tan CH, Tan BKH. Anti-diabetic properties of semi-purified fractions of *Averrhoa bilimbi* in streptozotocin-diabetic rats. 2nd Combined Annual Scientific Meeting, 8th – 9th September, 2000, Singapore.

3. Pushparaj P, Tan CH, Tan BKH. On the mechanism of hypoglycemic action of *Averrhoa bilimbi* in type 1 animal model of diabetes mellitus. The 1st Bilateral Symposium on Advances in Molecular Biotechnology and Biomedicine between the NUS and University of Sydney, 23rd – 24th May, 2002, Singapore.

SUMMARY

The present work aims to investigate the anti-diabetic effects of *Averrhoa bilimbi* leaves in animals with experimental diabetes mellitus. The ethanolic leaf extract of *A.bilimbi* (ABe) was evaluated for its antidiabetic activity in streptozotocin (STZ) induced diabetic Sprague-Dawley (SD) rats. At a dose of 125 mg/kg body weight, ABe increased glucose tolerance in an oral glucose tolerance test (OGTT) in these rats. Moreover, it showed potent hypoglycemic, hypotriglyceridemic, anti-lipid peroxidative and anti-atherogenic activities when administered twice a day for 2 weeks.

ABe was partitioned with organic solvents - butanol, ethyl acetate and hexane - to obtain aqueous (AF), butanol (BuF), ethyl acetate (EF) and hexane (HF) soluble fractions. The hypoglycemic property of each fraction was assessed by OGTT at a dose of 125-mg/kg-body weight in both STZ and high fat diet fed (HFD)-STZ diabetic rats. AF and BuF produced significant improvement in glucose tolerance. In the long-term study, twice a day administration of AF and BuF also at a dose of 125 mg/kg for 14 days in both STZ/HFD-STZ diabetic rats showed a significant blood glucose lowering action.

Moreover, AF was found to be more potent than BuF and increased serum insulin level in STZ-diabetic rats as well as lowered hepatic glucose-6-phosphatase activity significantly in both STZ/HFD-STZ diabetic rats and these results indicated that AF is more potent than BuF in the amelioration of hyperglycemia in both STZ and HFD-STZ-diabetic rats. AF was more potent in the amelioration of diabetes and β-cell protection against streptozotocin toxicity than BuF.

Reverse-phase high performance liquid chromatography (RP-HPLC) of AF and BuF revealed the presence of nicotinic acid (NA) in these fractions. In addition, the atomic absorption spectrophotometric analysis (AAS) showed the presence of magnesium (Mg) in higher concentration in AF than BuF. Hence, the effects of both Mg and NA on glucose tolerance were tested in four different animal models of diabetes viz., STZ-diabetic SD rats and STZ-diabetic C57BL/6J; both represent the type 1 diabetic model while HFD-STZ-diabetic SD rats and HFD-fed C57BL/6J represent type 2 diabetic model. The administration of both NA and Mg together improved glucose tolerance more than either Mg or NA alone. This synergistic interaction of NA and Mg in *A.bilimbi* extract could be one of the reasons for the amelioration of diabetes in animals with experimental diabetes mellitus.

CHAPTER 1

GENERAL INTRODUCTION

1. Diabetes mellitus and blood glucose homeostasis

1.1 Diabetes mellitus and its diagnosis

Diabetes mellitus is a principal cause of morbidity and mortality in human populations (Steppan et al., 2001). It is a syndrome characterized by hyperglycemia, polydipsia and polyuria and causes complications to the eyes, kidneys, and nerves. It is also associated with an increased incidence of cardiovascular disease (Pickup and Williams, 1991). The clinical manifestations and development of diabetes often differ significantly between countries and also between racial groups within a country. For example, diabetes currently affects an estimated 15.1 million people in North America, 18.5 million in Europe, 51.4 million in Asia, and just under 1 million in Oceania (Kuhlmann, 1996). It is estimated that globally, the number of people will rise from 151 million in the year 2000 (Amos et al., 1997), to 221 million by the year 2010, and to 300 million by 2025 (King et al., 1998).

Diabetes mellitus is becoming increasingly common in Singapore population. The prevalence of type 2 diabetes doubled between 1984 and 1992 in Singaporean Chinese (Chen et al., 1999). This increase can be attributed to many factors, including a stressful lifestyle as well as improper dietary habits. This is of economic concern as the disease requires life-long treatment and is also associated with high morbidity from the resulting complications.

The clinical diagnosis of diabetes is often suggested by the presence of hyperglycemic symptoms and glycosuria, sometimes with drowsiness or coma. The World Health Organization (WHO) criteria define diabetes by fasting plasma glucose (FPG) level of 140mg/dL (7 mmol/L) or greater, or post-prandial 2-h plasma glucose (PG) level of 200mg/dL (11.1 mmol/L) or greater during an oral glucose tolerance test (WHO, 1985).

The National Diabetes Data Group of the National Institutes of Health recommends the following criteria for diagnosing diabetes:

a. Fasting (overnight) venous plasma glucose concentration greater than or equal to 140 mg/dL on at least two separate occasions.
b. Venous plasma glucose concentration greater than or equal to 200 mg/dL at 2-h post-ingestion of 75 g of glucose and at least one other sample during the 2-h test.

1.2 The classification of diabetes mellitus

Diabetes mellitus represents a heterogeneous group of disorders. Some distinct diabetic phenotypes can be characterized in terms of specific aetiology and/or pathogenesis, but in many cases overlapping phenotypes make etiological and pathogenetic classification difficult (Leslie, 1997). In general, diabetes mellitus can be classified into two major types: insulin-dependent diabetes mellitus (IDDM, Type 1 diabetes) and non-insulin-dependent diabetes mellitus (NIDDM, Type 2 diabetes), based principally upon clinical symptoms and, when possible, on more specific etiologic characterization. In IDDM, there is destruction of the β-cells of

the pancreas, with consequent insulin deficiency. At clinical presentation, IDDM is often associated with marked hyperglycemia and its attendant symptoms and signs: polyuria, polydipsia, and unexplained weight loss. The cause of NIDDM is often a combination of resistance to insulin action and inadequate compensatory insulin secretion. Although patients with this type of diabetes may have insulin levels that appear normal, insulin levels always are low relative to the elevated plasma glucose levels (Ward et al., 1984). In NIDDM, hyperglycemia sufficient to cause functional and pathologic changes in target organs may be present without clinical symptoms. The incidence of each type of diabetes varies widely throughout the world. There are genetic and environmental components in the causation of both IDDM and NIDDM (Zimmet et al., 1989).

1.3 Incidence and epidemiology

The incidence of diabetes mellitus in the United States is estimated at approximately 4.5%, of which 85-90% are NIDDM and the rest IDDM. In 1992, while diabetics accounted for only 4.5% of the US population, their care required roughly 14.6% of the total US health care expenditures ($105 billion). Annually, between 500,000 and 600, 000 Americans are detected with NIDDM. More than 75% of the individuals with diabetes will develop neurological, microvascular, or macrovascular complications (Mazze, 1994).

The prevalence of diabetes mellitus is rising and it is now the seventh leading cause of death in USA. At the current rate of increase (6%/year), the numbers of diabetics will double every 15 years. Epidemiologically, diabetes mellitus has been

linked to the western lifestyle and is uncommon in cultures consuming a more "primitive" diet. As cultures switch from their native diets to the "foods of commerce", their rate of diabetes mellitus increases, eventually reaching the same proportions seen in Western societies.

1.4 Regulation of glucose metabolism by insulin and pathophysiology of diabetes

Plasma glucose concentrations are effectively maintained within a fairly narrow range despite wide fluctuations in the body's supply (e.g. meals) and demand (e.g. exercise) for nutrients (Gerich, 1993). Changes in plasma blood glucose levels are moderated by the actions of the liver primarily under the control of insulin and glucagon (Unger and Orci, 1981). Insulin, secreted by the β-cells of the pancreas, lowers the concentration of glucose in blood by inhibiting hepatic glucose production and stimulating the uptake and metabolism of glucose by muscle and adipose tissue (Davis and Granner, 1996).

All forms of diabetes mellitus are due to a decrease in the circulating concentration of insulin (insulin deficiency) and a decrease in the response of peripheral tissue to insulin (insulin resistance). These abnormalities lead to alterations in the metabolism of carbohydrates, lipids, ketones, amino acids; the central feature of the syndrome is hyperglycemia. Insulin plays a role in regulating both glycogenolysis and gluconeogenesis in liver (Cherrington et al., 1987). The absence or deficiency of insulin's effects not only engenders an increased hepatic net extraction of glucogenic amino acids, lactate, glycerol and their conversion to

glucose, but also stimulates both the quantity and activity of gluconeogenesis enzymes, such as glucose-6-phosphatase (Glc-6-Pase), fructose-1,6-bisphosphatase and pyruvate carboxylase (Weber, 1964; Taunton et al., 1974). The enzyme, Glc-6-Pase, catalyzes the terminal step in both gluconeogenic and glycogenolytic pathways, so it is a key determinant in the production of glucose by the liver. Both mRNA levels and activity of Glc-6-Pase are low in the fed and refed states, where insulin levels are elevated. Both mRNA levels and activity of Glc-6-Pase are elevated in diabetic rats and administration of insulin to diabetic rats results in the reduction of the mRNA and activity of this enzyme (Argaud et al., 1996; Massillon et al., 1996).

Insulin has many actions within the central nervous system (CNS), including reducing food intake and body weight and interacting in predictable ways with other controllers of meal size (McGowan et al., 1990). On the other hand, its anabolic effects in peripheral tissue would promote weight gain. These two major actions of insulin tend to counterbalance one another, as the peripheral anabolic effect of insulin would cause weight gain yet appetite would be suppressed via insulin's central catabolic action (Schwartz et al., 1994). It is believed that insulin and leptin (Zhang et al., 1994), an adipose tissue hormone, modulate energy homeostasis, such as causing change in food intake and body weight at the brain level (Woods et al., 1998).

Hypoinsulinemia and low circulating leptin concentrations may contribute to hyperphagia via upregulation of hypothalamic neuropeptide Y (NPY) system in uncontrolled type I diabetes (Havel et al., 1998). However in this kind of diabetes, the extreme hypoinsulinemia causes a wasting of peripheral tissue and consequent weight loss due to the lack of a peripheral insulin anabolic effect, even though there is also a concomitant enhanced appetite in this situation (The DCCT study group, 1988).

Insulin stimulates lipoprotein lipase activity and promotes fat and muscle storage of both exogenously derived triglycerides as well as that produced endogenously (Eckel and Yost, 1987). It also inhibits the hormone-sensitive lipase in adipose tissue and thus inhibits the hydrolysis of triglycerides stored in the adipocytes. Elevations in plasma triglycerides and cholesterol are evident in diabetic animals. This is related to decreases in activity of insulin-dependent lipoprotein lipase and in the apoprotein content of lipoproteins (Tavangar et al., 1992; Sparks et al., 1992), necessary for the recognition and efficient lipolysis of the triglyceride-rich particles at the sites of their uptake.

Steady-state levels for insulin mRNA appears to be important for regulation of insulin production. Insulin mRNA levels varied with the change in demand for insulin in several experimental conditions and correlated directly with rates of insulin biosynthesis when both were measured *in vivo* (Permutt et al., 1984; Giddings et al., 1985).

In a rat model for diabetes, maintenance of glucose homeostasis correlated with maintenance of pancreatic insulin mRNA content. When prediabetic or mildly glucose intolerant rats were challenged with a diabetogenic agent, maintenance of normal glucose levels correlated with increases in insulin mRNA content. When this adaptive response failed, hyperglycemia worsened (Giddings et al., 1985). After administration of STZ and alloxan, a marked reduction in insulin mRNA level was observed (Mulder et al., 1995).

The insulin gene is present as a single copy in most species. However, in rats, two nonallelic insulin genes (Insulin I gene and Insulin II gene) are expressed (Clark and Steiner, 1969; Lomedico et al., 1979). Their mRNAs are quite similar, being approximately 93% homologous in the coding regions with only 34 of 439 nucleotides different (Ullrich et al., 1977). Insulin I gene has been observed to be expressed in pancreas, but insulin II, the ancestral gene, is expressed not only by pancreas but also by extra pancreatic tissue, including yolk sac and fetal liver (Giddings and Carnaghi, 1989). The two rat insulin genes may function independently. The conversion products, insulin I and II, are usually stored in unequal amounts. The ratio of the cellular contents of insulin I over insulin II fluctuates between 1 and 2 in a basal fed or fasting state, but increases 2- to 4-fold during pregnancy or chronic hyperglycemia. Glucose is an important modulator of the rate of insulin biosynthesis, through changes in mRNA levels. (Kakita et al., 1982). Rat β-cells exhibit a differential regulation of biosynthesis of the two insulin isoforms at the level of both transcription and translation. This leads to an

increase in the ratio of insulin I over insulin II in terms of both their respective mRNA content as well as their peptide content (Ling et al., 1998).

1.5 Free radicals and the complications of diabetes

The causes of death in the diabetic population changed drastically after the advent of insulin therapy by Banting and Best in 1922. While insulin and other medical treatments can control many aspects of diabetes, numerous complications are not uncommon. The microvascular, neuropathic and macrovascular complications are a major health problem for patients with either IDDM or NIDDM (Herman and Crofford, 1998). Oxidative damage appears to be involved in the pathogenesis of long-term complications in diabetes, based on the increased concentration of lipid peroxidation products and the accumulation of advanced glycosylation end products and glycoxidation products in tissue proteins of diabetic patients with complications. Enzymatic and non-enzymatic oxidation of lipids and carbohydrates yield reactive carbonyl compounds, including aldehydes derived from lipid peroxidation and dicarbonyl sugars derived from glucose, which are key intermediates in the chemical modification and cross-linking of proteins in diabetes (Baynes, 1995).

Oxygen free radicals (OFRs), such as superoxide ($O_2^{\bullet -}$), hydrogen peroxides (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$), are implicated in the pathophysiology of ischemia/reperfusion injury and atherosclerosis (McCord, 1985; Mantha et al., 1993). Oxidation of lipids in plasma lipoproteins and in cellular membranes is

associated with the development of vascular disease in diabetes (Morel et al., 1983). Much of the experimental evidence suggests that diabetes and hyperlipidemia alone are not sufficient to provoke vascular disease but oxidative stress may be an important and independent risk factor in the development of vascular disease (Hunt et al., 1990). Although antioxidant therapy has not been adequately tested, it may provide an important defense against oxidative damage and the development of complications in diabetes.

1.6 Animal models of diabetes and prevention of diabetes

1.6.1 Chemically-induced diabetes in animals

Chemically induced type I diabetes is the most commonly used animal model of diabetes. Alloxan (2, 4, 5, 6-tetraoxo hexahydro pyrimidine) was the first agent that was reported to produce permanent diabetes in laboratory animals (Dunn, 1943). Streptozotocin (STZ) has replaced alloxan as the principal agent used to produce experimental diabetes. This is due to the greater selectivity of β-cells for STZ (Junod et al., 1969) and lower mortality rate seen in STZ-diabetic animals (effective diabetogenic dose of STZ is four or five times less than its lethal dose) (Hoftiezer and Carpenter, 1973).

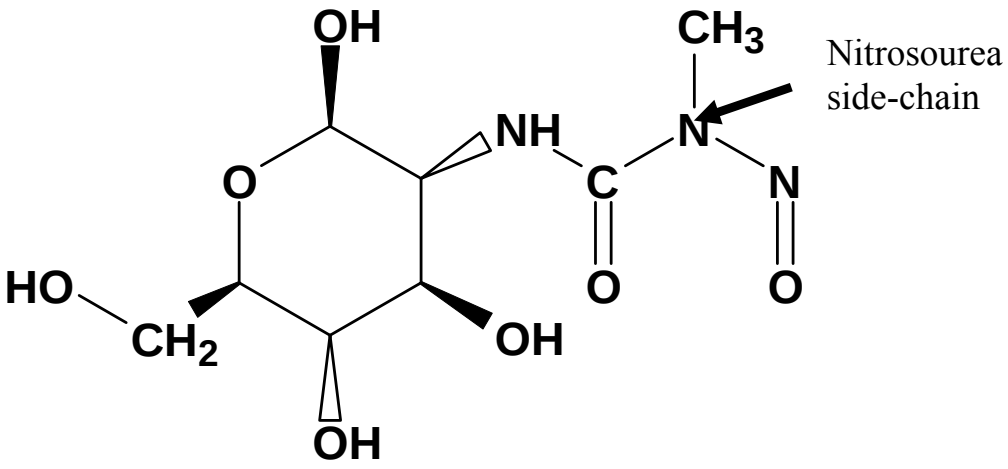

Figure 1. Molecular structure of streptozotocin (STZ).

The chemical structure of STZ (Figure 1) comprises a glucose molecule with a highly reactive nitrosourea side chain that is thought to initiate its cytotoxic action. As previously reported, diabetes was consistently produced at doses of 50-70 mg/kg of STZ (Ar'Rajab and Ahren, 1993). The absence of ketosis in animals having received intravenous STZ at doses of 65 mg/kg or less is adequately explained by incomplete, although marked, insulin depletion (Junod et al., 1969).

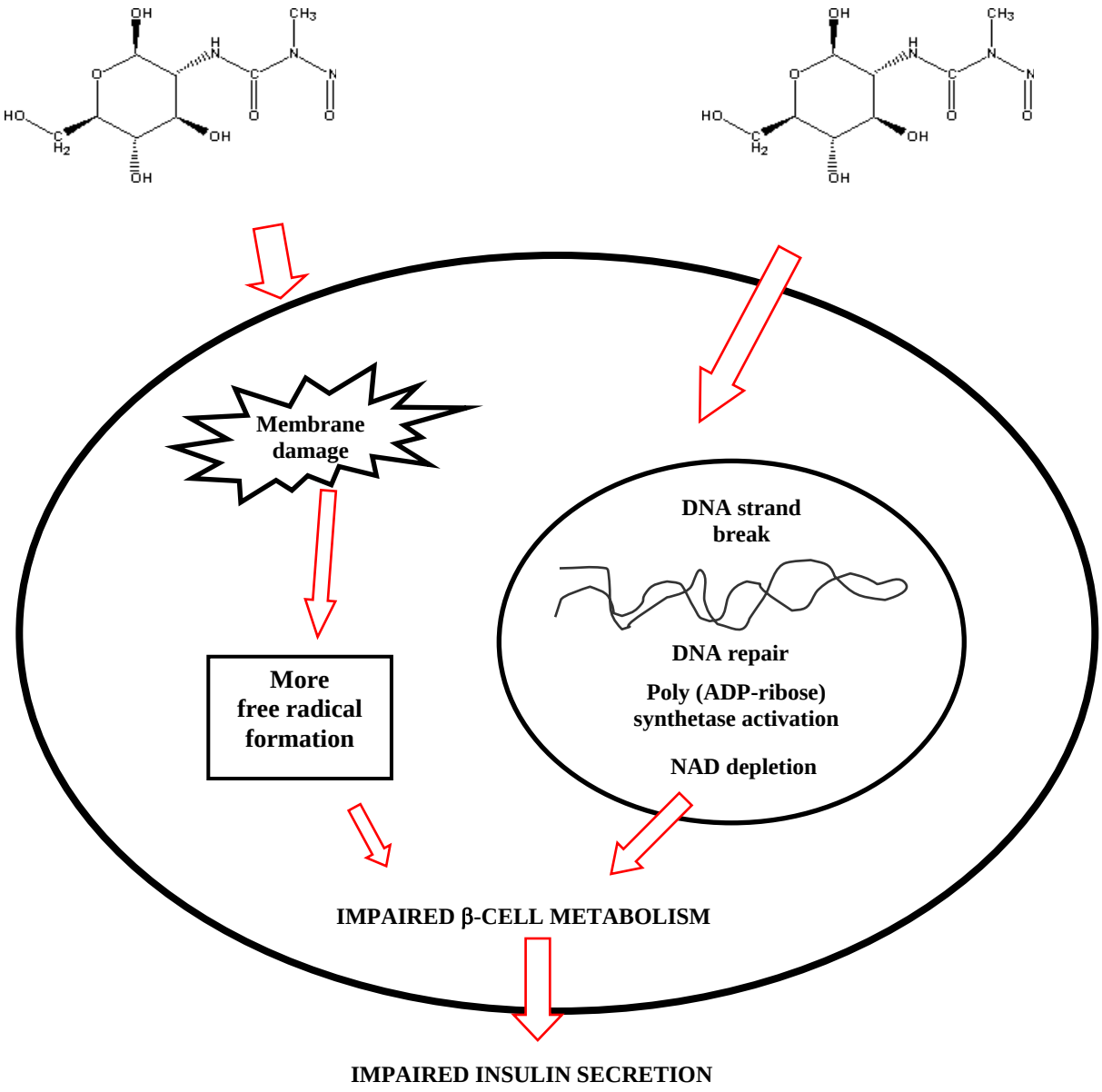

Figure 2. Schematic representation of the mechanism of pancreatic β-cell destruction by streptozotocin.

As shown in Figure 2, the glucose moiety directs this agent to the pancreatic β-cells, where it binds to a membrane receptor to cause structural damage (Johansson and Tjalve, 1978). The deleterious effect of STZ results from the generation of highly reactive carbonium ions (CH_3^+) that cause DNA breaks by alkylating DNA bases at various positions, resulting in activation of the nuclear enzyme, poly(ADP-ribose) synthetase, thereby depleting the cellular enzyme substrate (NAD^+), leading to cessation of NAD^+-dependent energy and protein metabolism. This in turn leads to reduced insulin secretion (Yamamoto et al., 1981). It has been suggested that free radical stress occurred during β-cell destruction mediated by mononuclear phagocytes and cytokines (Pitkanen et al., 1992; Nagy et al., 1989). Since free radical scavengers have been demonstrated to protect against the diabetogenic properties of STZ (Robbins et al., 1980), it is likely that oxidative stress may play a role in determining STZ toxicity.

Some poly (ADP-ribose) synthetase inhibitors, such as nicotinamide and 3-aminobenzamide, could prevent the onset of diabetes (Uchigata et al., 1983). It was also reported that metallothionein, a free radical scavenger, could provide some protection against the diabetogenic properties of STZ (Yang and Cherian, 1994). Cytoprotective components, such as zinc (Yang and Cherian, 1994) and lipid components from the soybean (Lee and Park, 2000) may prevent β-cell death by stabilizing membrane integrity and normalizing membrane biochemical alterations.

1.6.2 Diabesity-prone C57BL/6J mice

The non-obese, non-diabetic BL/6J mice, the genomic host of the ob/ob mutation, are susceptible to diabesity when placed on an affluent fat and sucrose-rich diet. They become hypertensive and exhibit an insulin-resistant syndrome: increased outflow from the sympathetic nervous system, deranged β-cell function and adipocyte metabolism, hyperleptinemia but without hyperphagia or elevation of corticosterone secretion (Martin-Dixton et al., 2002).

Genetic mapping has identified differences in the expression of uncoupling protein (UCP2) which may have a role in the development of diabesity (Petro and Surwit, 2000). Thus, inbred laboratory mice, without overt metabolic disturbance, were demonstrated to be vulnerable to metabolic abnormalities on high fat diet (HFD). The hyperinsulinemia most probably interferes with the action of catecholamines on β1 and β3 adrenergic receptors, thereby affecting the uptake of glucose by adipocytes and increasing the sympathetic outflow. Thus, the C57BL/6J mice present an attractive model for the study of multiple endocrine abnormalities induced by dietary hyperinsulinemia (Shafrir, 2003).

1.7 Oral hypoglycemic agents

Oral hypoglycemic agents that could effectively control the abnormalities of carbohydrate, lipid, and protein metabolism that occur in patients with diabetes have been used for over half a century. There are two major structurally and functionally different oral antidiabetic drug classes, the sulfonylureas and the

biguanides, that are widely used in the world. The sulfonylureas include chlorpropamide, glibenclamide and tolbutamide. Both pancreatic and extra-pancreatic effects have been suggested to contribute to the therapeutic benefit of sulfonylureas for type II diabetic patients. Sulfonylureas directly stimulate insulin release from the β-cells in the islets of Langerhans, and this effect do not require the presence of glucose or other secretagogues (Gorus et al., 1988).

Among biguanides, metformin and phenformin have been employed for oral diabetic therapy since 1960s (Bailey, 1992). Only metformin was approved for use in the United State in early 1995. Phenformin was withdrawn in many countries during 1970s because of its association with lactic acidosis. In contrast to sulfonylureas, metformin has blood glucose reducing effect only in diabetes; and it does not produce hypoglycemia in normal subjects. Metformin also exerts little or no effect on basal insulin release by the pancreas or isolated islets of nondiabetic animals (Schatz et al., 1972; Gregorio et al., 1989).

Alpha-glucosidase inhibitors, such as Acarbose, found in the mid-1990, has rationalized and simplified the treatment of diabetes. It is a competitive inhibitor of the major α-glucosidase enzymes in the brush border of the mucosal cell of the small intestine. It inhibits the digestion of the complex carbohydrates in the upper jejunum so that they are digested throughout the length of the small intestine. The major effect of this drug is to reduce the postprandial rise in plasma glucose (Bailey, 1992).

Thiazolidinedione (TZDs) analogues (Glitazones or TZDs), a new class of antidiabetic drugs represented by ciglitazone, have been shown to be effective antihyperglycemic compounds in animal models of non-insulin dependent diabetes mellitus (NIDDM) (Fujita et al., 1983). The two analogues in this chemical series, pioglitazone (Sugiyama et al 1990b) and rosiglitazone (Fujiwara et al, 1988) are now available for therapeutic use (Lebovitz, 1997).

1.8 Botanical medicines

Before the advent of insulin, diabetes was treated with plant medicines. The World Health Organization (WHO) urged researchers to examine whether traditional medicines produced any beneficial clinical results (WHO, 1980). The plant kingdom represents a largely unexplored reservoir of biologically active compounds not only as drugs, but also as unique templates that could serve as a starting point for synthetic analogs and an interesting tool that can be applied for a better understanding of biological processes. Folkloric uses are supported by a long history of human experience. Numerous biologically active plants are discovered by evaluation of ethnopharmacological data, and these plants may offer the local population immediately accessible therapeutic products (Aquino et al., 1995).

The earliest known documentation of plant-derived treatments for diabetes is found in the Ebers Papyrus of about 1550 BC. Since then, multitudes of herbs, spices, and other plant materials have been described for the treatment of diabetes throughout the world (Bailey and Day, 1989). Traditional anti-diabetic plants

might provide a useful source of new oral hypoglycemic compounds for development as pharmaceutical entities, or as simple dietary adjuncts to existing therapies. However, since the availability of insulin, folklore medicines for diabetes have almost disappeared from occidental societies, although they continue to be the cornerstone of therapy in underdeveloped regions.

Renewed attention to alternative medicines and natural therapies has stimulated a new wave of research interest in traditional practices. In the last 20 years, scientific investigation has confirmed the efficacy of many of these preparations, some of which are remarkably effective. Mentioned hereafter are those plants that appear most effective, are least toxic, and have substantial documentation of efficacy.

More than 400 different plants and plant extracts have been described for the diabetic patient. From these, various molecular species with hypoglycemic activity have been identified, including alkaloids, flavonoids, glycosides, and polysaccharides (Day, 1990). For example, castanospermine, an alkaloid isolated from seeds of *Castanospermum australe*; epicatechin, a flavonoid isolated from the heartwood of *Pterocarpus marsupium*; and neomyrtillin, a glycoside isolated from *Vaccinium myrtillus*, were claimed to exert hypoglycaemic effect (Day, 1990). Like the sulfonylureas, some plants act by increasing the release of insulin and require a minimum of β-cells to exert their action. These plants include *Momordica foetida* (Marquis et al., 1977), *Euphorbia prostrata* and *Fumaria parviflora* (Akhtar et al., 1984), *Taraxacum officinale* (Akhtar and Ali, 1985) and Eribotry *japonica* (Noreen et al., 1987). Like metformin, other plant extracts, such

as *Aloe* (Al-Awadi et al., 1985), *Momordica charantia* (Sarkar et al., 1996), *Cecropia obtusifolia*, *Coccinia indica* and *Hammada salicornia* (Ivorra et al., 1989) act by modifying glucose metabolism rather than altering insulin levels. Some are shown to correct complications of diabetes, for example, masoprocol, a pure compound isolated from *Larrea tridentata*, which decreases the elevated levels of serum cholesterol, free fatty acids and triglycerides in fat-fed/diabetic rats (Reed et al., 1999). Even with the use of these herbs, which possess blood glucose lowering effects, proper and effective natural treatment of diabetics require careful integration of diet, nutritional supplements, lifestyle, and botanical medicine.

Figure 3. Picture of *Averrhoa bilimbi* leaves and fruits.

1.9 *Averrhoa bilimbi* Linn.

Averrhoa bilimbi Linn (Oxalidaceae) (Figure 3) is a small-sized tree growing up to 15 m tall and 30 cm in diameter. Young parts are covered with long persistent, yellowish to rusty, velvety hairs. Leaves are compound, often crowded at the ends of the branches with 7-19 leaflets each measuring up to 12 cm by 4 cm, variable in shape with 6-14 pairs of lateral veins. Flowers are borne in dense, fascicled, pendulous clusters on bare branches and on knobby protuberances along the tree trunk; calyx is yellowish green, and petals red to purple. Fruits are rounded and angular in cross section, up to 10 cm by 5 cm, fleshy and juicy but acidic when ripe. Though widely cultivated in the low lands of Southeast Asia, its country of origin is unknown but tropical America has been suggested. The plant flowers and fruits intermittently throughout the year. The other names are viz., *Averrhoa obtusangula* Stokes; Belimbing asam, Belimbing buluh, Belimbing wuluh (Malay, Javanese); Kamias, kalamias, Iba, Kolonanas (Tag); Ta-ling-pring (Thai).

1.9.1 Chemical constituents of *A.bilimbi*

The chemical compounds that have been identified in *A.bilimbi* include amino acids, citric acid, cyanidin-3-O-β-D-glucoside, phenolics, potassium ion, sugars and vitamin A.

1.9.2 Ethnopharmacological uses of *A.bilimbi*

The fruits of *A.bilimbi* possess antibacterial, antiscorbutic, astringent and post-partum protective properties. The decoction of the leaves is being used as medicine for treating fever, inflammation of the rectum, diabetes, mumps and pimples. The

paste of leaves is being used for the treatment of itches, boils, rheumatism, cough and syphilis. The juice of preserved fruits is being used for the treatment of scurvy, stomach ache, bilious colic, whooping cough, and hypertension. Moreover, the syrup of flowers is being given to treat children's cough (Wee, 1992; Goh et al., 1995).

1.10 Aims of the thesis

A preliminary study in our laboratory showed decreases in blood glucose levels and food intake in STZ-diabetic rats given aqueous or ethanolic extracts of *A. bilimbi* fruits and leaves intraperitoneally (Tan et al., 1996). However no reports are available about the constituent(s) of *A. bilimbi* which is responsible for the anti-diabetic activity and the mechanism (s) of its anti-diabetic action. Hence the aims of this thesis are to:

a) investigate the effects of ethanol-extract of *A.bilimbi* (ABe) leaves on blood glucose and lipid levels in STZ- diabetic rats
b) identify the bioactive semi-purified fraction(s) of ABe by bioassay guided fractionation
c) evaluate the β-cell protective effect of ABe and its bioactive fractions against STZ in rats
d) investigate the anti-diabetic effects of the bioactive fractions of *A.bilimbi* in rat model of type 2 diabetes
e) isolate and characterize the anti-diabetic principle (s) of *A.bilimbi*
f) evaluate the effect of the anti-diabetic principle (s) of *A.bilimbi* on glucose tolerance in animals with type 1 and type 2 experimental diabetes mellitus

It is expected that this study could provide a scientific basis for the use of this plant in folk medicine to ameliorate the complications of diabetes mellitus.

CHAPTER 2

MATERIALS AND METHODS

Section 1. Materials

1.1 Chemicals and reagents

BDH Laboratory Supplies (England)

Sodium citrate

Bio-Rad Laboratories (Hercules, CA, USA)

Bio-Rad protein assay reagent

Merck KGaA (Darmstadt, Germany)

Acetone; Methanol; nButanol; Ethanol; Hexane; Chloroform; HCl; H_2SO_4; Dodecyl sulphate sodium (SDS); Isopropyl alcohol; Potassium dihydrogen phosphate (KH_2PO_4); Phosphoric acid; Potassium chloride; Sucrose; Trichloroacetic acid (TCA)

Sigma-Aldrich (St Louis, MO, USA)

All other chemicals and reagents used in the present study

1.2 Kits

Sigma Diagnostics, INC. (St Louis, MO, USA)

Glucose assay kit (Trinder method)

Boehringer Mannheim (GmbH, Mannheim, Germany)

Cholesterol (TG) reagent; Peridochrom® Triglyceride (TC) reagent

HDL Cholesterol kit (CHOD-PAP method)

R&D systems (MN, USA)

Rat leptin ELISA kit

Mercodia (Uppsala, Sweden)

Rat insulin ELISA kit

I-STAT portable glucose analyzer (i-STAT Corporation, East Windsor, NJ, USA)

1.3 Facilities

Buchi rotary evaporator R-144 (Buchi Labortechnik AG, Switzerland)

Buchi water bath B-480 (Buchi Labortechnik AG, Switzerland)

Chemical hood (Pacific Vinitex Pte Ltd, Singapore)

-86°C Freezer (Forma Scientific)

-20°C & 4°C ACMA Refrigerator

Balance (Precisa 40SM-200A, Swiss)

Beckman Avanti™ J-25I Centrifuge (Fullerton, CA, USA)

Beckman JA 25-25 Rotor (Fullerton, CA, USA)

Beckman Optima™ L-90K Ultracentrifuge (Fullerton, CA, USA)

Beckman Type 70 Ti Rotor (Fullerton, CA, USA)

ELX 800 Microplate Reader (Bio-Tek Instruments Inc., USA)

Jouan Centrifuge (Everbloom Medical & Scientific Pte. Ltd., Singapore)

Kubota KR-20000T Centrifuge (Kubota Seisakusho Co., Ltd., Japan)

Kubota RA-1M Micro Tube × 16 Rotor (Kubota Seisakusho Co., Ltd., Japan)

Perkin-Elmer 1100B Atomic Absorption Spectrophotometer

Polytron Homogenizer (Polytron, Switzerland)

Table 1. Composition of the basic rodent diet, AIN-93G

Ingredient	Content (g/kg food)
Casein	178.6
Sucrose	100
Starch	367.5
Dextrinished starch	132
Methionine	3
Choline chloride (50%, w/w)	2.5
Natural oil	100
AIN-93M minerals	10
AIN-93M vitamins	35
Total	1000

SYNERGI 4 u hydro- RP 80A (Part No: 00G-4375-E0) column (Phenomenex)

Shimadzu Class LC-10 (version 1.64) with PC control

UV-1601 Spectrophotometer (Shimadzu, Japan)

Waring blender (Waring Laboratory, Torrington, CT, USA)

Water Incubator (Everbloom Medical & Scientific Pte, Ltd., Singapore)

1.4 Animals

Male Sprague-Dawley (SD) rats, aged 10 weeks (220-260 g), were obtained from The Laboratory Animal Center, National University of Singapore. The rats were housed in individual cages in an animal room with lighting from 0600 to 1800 h and maintained on standard pelleted diet, AIN-93G, (Table 1) (Glen Forrest, WA, Australia) with water *ad libitum.*

Male C57BL/6J (Animal Holding Unit, National University of Singapore, Singapore), 6 weeks of age, were used for all studies. They were housed about 6 per cage in a room with a 12-h light and 12-h dark and an ambient temperature of 22-25º C.

2. Methods

2.1 Preparation and partitioning of plant extract

2.1.1 Preparation

The plant was collected from a private garden and identified as *Averrhoa bilimbi* by Dr. Ruth Kiew, Keeper of Herbarium and Library, Singapore Botanic Gardens. A dried specimen was deposited in the herbarium (Voucher specimen No. BT 2). The fresh leaves of *A. bilimbi* (1 kg) were blended and extracted with 80% ethanol

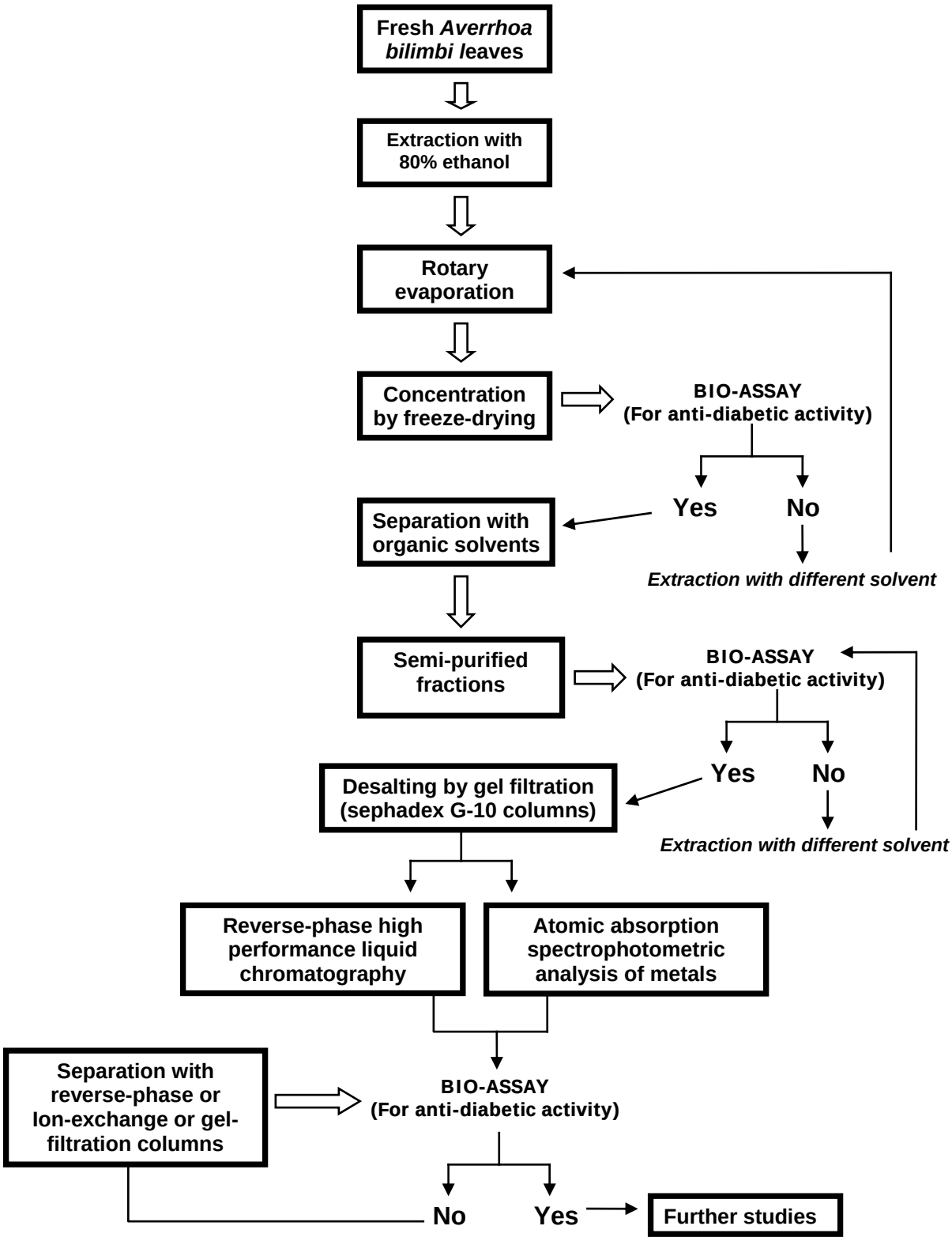

Figure 4. Schematic representation of the bio-assay guided fractionation procedure for the isolation of anti-diabetic principle (s) from *A.bilimbi* leaves.

(10 L) until exhaustion. The mixture was filtered with Whatman No 1 filter paper (Whatman International Ltd., England). The filtrate was centrifuged for 10 minutes at 10,000g to remove particulate substances. The clear supernatant was concentrated at 40°C, using a rotavapor (Buchi Labortechnik AG, Switzerland) to 1 L. The concentrate was freeze-dried and suspended in distilled water before use.

2.1.2 Partitioning

The clear supernatant of 80% ethanolic extract (prepared according to Method 2.1.1) was partitioned between butanol and water to obtain the aqueous fraction (AF) and the butanol fraction (BuF). The AF was further partitioned by ethyl acetate and hexane to obtain the ethyl acetate (EF) and hexane soluble fractions (HF). Each fraction was concentrated at 40°C, using a rotavapor (Buchi Labortechnik AG, Switzerland) and freeze-dried, to yield about 40 g of AF, 25g of BuF, 15 g of EF and 12 g of HF. The extract was suspended in distilled water before use.

2.2 Streptozotocin (STZ)-induced diabetic rats

After fasting for 18-h, the rats were intraperitoneally injected with a single dose of 60 mg/kg STZ, freshly dissolved in citrate buffer (0.01 M, pH 4.5). Diabetes in the rats was identified by polydipsia, polyuria and by measuring non-fasting serum glucose concentration 48-h after injection of STZ. Rats with a serum glucose level above 300 mg/dL were selected for experiments.

Table 2. Composition of the high fat rodent diet, SF-01-14

Ingredient	Content (g/kg food)
Casein	200
Sucrose	528.4
Corn oil	10
Cocoa butter	150
Methionine	3
Choline chloride (50%, w/w)	10
DL-α-tocopherol	10
AIN-93M minerals	10
AIN-93M vitamins	35
Cellulose	51
Total	1000

2.3 High fat diet (HFD)-fed -STZ-induced diabetic rats

Male SD rats were fed HFD, SF-01-14 (Table 2), consisting of 20% fat, 46% carbohydrate and 20% protein (w/w) (Glen Forrest Stock Feeders, WA, Australia). After 2 weeks on HFD, animals were administered streptozotocin (STZ, 50 mg/kg) intraperitoneally. Diabetes in the rats was identified by measuring fasting serum glucose concentration 72-h after injection of STZ. Rats with a serum glucose level above 300 mg/dL were selected for experiments.

2.4 STZ-induced diabetic C57BL/6J mice

Male C57BL/6J mice were administered with streptozotocin (STZ, 100 mg/kg) intraperitoneally. Animals had free access to food and water after STZ injection. Diabetes in the mice was identified by polydipsia, polyuria and by measuring fasting serum glucose concentration 72-h after injection of STZ. Mice with a serum glucose level above 300 mg/dL were selected for experiments.

2.5 HFD-induced diabetic C57BL/6J mice

Male C57BL/6J mice were fed with HFD, SF-01-14, for 8-10 weeks to develop diabetes similar to NIDDM in humans (Shafrir, 2003). Diabetes in the mice was identified by measuring fasting glucose concentration after 10 weeks of feeding HFD. Mice with a serum glucose level above 250 mg/dL were selected for experiments.

2.6 Determination of blood glucose by the glucose assay kit

Glucose is first oxidized to gluconic acid and hydrogen peroxide. This reaction is catalyzed by glucose oxidase. The hydrogen peroxide formed reacts in the presence of peroxidase with 4-aminoantipyrine and p-hydroxybenzene sulfonate to form quinoneimine dye, with an absorbance maximum at 505 nm. The intensity of the color produced is directly proportional to the glucose concentration in the sample (Trinder, 1969). The serum glucose concentration was expressed as mg/dL.

2.7 Determination of total cholesterol by the Cholesterol (TG) reagent kit

Cholesterol esterase in the reagent solution cleaves cholesterol esters in the serum to release cholesterol and free fatty acids. The free cholesterol and the enzyme-liberated cholesterol are then oxidized by cholesterol oxidase to form cholestenone and hydrogen peroxide (H_2O_2). The peroxidase catalyses the reaction between the H_2O_2, 4-aminophenazone and phenol to form a pink complex [4-(p-benzo-quinone-monoimino)-phenazone]. To estimate TC concentration, 1 ml reagent solution was added to 10 µl sample in 10 mm X 75 mm disposable tubes, mixed and then incubated at ambient temperature (25^{o} C) for 10 min. The absorbance was read at 500 nm within 1 hour. The cholesterol concentration was calculated according to the formula:

TC concentration (mg/100 mL) = 575 X (OD_{500}) sample

The serum TC concentration was expressed as mg/dL.

2.8 Determination of serum high density lipoprotein cholesterol (HDL-C) by the HDL-cholesterol kit (CHOD-PAP method)

To 100 μL of serum was added 250 μL of precipitating solution (0.55 mmol/L, phosphotungstic acid and 25 mmol/L, $MgCl_2$) in order to precipitate chylomicrons, very low density lipoprotein cholesterol (VLDL-C) and low density lipoprotein cholesterol (LDL-C). The mixture was vortex-mixed and left to stand for 10 min at ambient temperature before centrifugation for 15 min at 1500 g. The HDL-C in the supernatant was determined for its cholesterol content as previously described for TC. The serum HDL-C concentration was calculated using the formula:

HDL-C concentration (mg/100 mL) = 219.2 X (OD_{500}) sample

The serum HDL-C concentration was expressed as mg/dL.

2.9 Determination of serum triglycerides by the Peridochrom® Triglyceride (TC) reagent

An aliquot (1.0 mL) of reagent solution was added to 10 μL of sample, vortex-mixed and incubated at 25 ^{o}C for 10 min before reading the absorbance at 500 nm. The TG in the sample was hydrolysed by lipase to liberate free fatty acid and glycerol. Glycerol kinase in the reagent converted glycerol to glycerol-3-phosphate, which in turn was oxidized by glycerol phosphate oxidase to form dihydroxyacetone phosphate and H_2O_2. Peroxidase would then catalyse the enzymatic reaction of H2O2 with 4-aminophenazone and 4-chlorophenol to form the pink 4-(p-bezoquinone-mono-imino)-phenazone, which absorbs at 500 nm.

The following was used to calculate TG concentration:

TG concentration (mg/100 mL) = 760 X (OD_{500}) Sample

The serum TG concentration was expressed as mg/dL.

2.10 Determination of low density lipoprotein cholesterol (LDL-C)

The LDL-C was determined by the Friedewald's formula (Friedewald et al., 1972).

LDL-C = TC– HDL-C – TG/5

The LDL-C concentration was expressed as mg/dL.

2.11 Determination of anti-atherogenic index (AAI)

A low level of circulating HDL-C is a surrogate marker for an atherogenic metabolic situation which is commonly known as the metabolic syndrome and several strands of evidence indicate that at a low level of circulating high density lipoprotein may be causally related to the development of atherosclerosis (Fogelberg et al., 1990; Brunzell et al., 2003). Hence, the AAI was calculated by the following formula;

AAI = HDL-C/TC- (HDL-C) X 100

2.12 Serum insulin assay by ELISA kit

Serum insulin level was measured by an enzyme-liked immunosorbent assay (ELISA) procedure using Mercodia rat insulin ELISA kit. Briefly, the solid phase two-site enzyme immunoassay is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants

(epitopes) on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtitration well. After washing three times, unbound enzyme labeled antibody was removed. The bound conjugated insulin was detected by reacting with 3, 3', 5, 5'-tetramethylbenzidine. The reaction was stopped by adding acid to give a colorimetric end-point and optical density was measured with a microplate autoreader (Bio-tek Instrument Inc., USA) at a wavelength of 450 nm. The serum insulin was expressed as μg/L.

2.13 Serum leptin assay by ELISA kit

Leptin level was also detected by an ELISA assay using a kit. Optical density was measured with the same microplate autoreader as above and at a wavelength of 450 nm. The serum leptin was expressed as pg/L.

2.14 Pancreatic insulin assay

According to the method of Portha et al. (1979), the pancreas was homogenized for 1 min by ultrasonic disintegration at 4°C in acid-alcohol solution (75% ethanol, 1.5% 12 mol/L HCl, 23.5% distill water). The homogenate was left to stand overnight at -20°C and then centrifuged the next morning at 6000 g for 15 min. The insulin concentration of the supernatants was determined by the method previously described (Method 2.12.). The results were expressed as ng/g wet weight of tissue.

2.15 Estimation of liver glucose-6-phosphatase (Glc-6-Pase) activity

Glc-6-Pase activity was assayed according to Baginsky et al. (1974) by estimation of inorganic phosphate liberated from glucose-6-phosphate (Glc-6-P). For this assay, 1 g of frozen liver tissue was homogenized in ice-cold sucrose solution with a Polytron homogenizer. The homogenate was centrifuged sequentially at 11,000 g for 30 min, then at 105,000 g for 1 h using an ultracentrifuge (Beckman L8-70, Beckman Instruments, Inc., CA, USA). The solid pellet was resuspended in ice-cold sucrose/EDTA solution and used as the source of the enzyme. Tubes were divided into samples, blanks and standard. To each were added 0.1 mL of sucrose/EDTA buffer (0.25 M/L mM, pH 7.0), 0.1 mL of Glc-6-P (100 mM), and cacodylate buffer solution. This was followed by the addition of 0.1 mL of sample to the sample tube, 0.1 mL of sucrose/EDTA solution to the blank and 0.1 mL of different concentration of K_2HPO_4 (0.5 mM, 1 mM, 1.5 mM and 2 mM) to the standard tube. All tubes were incubated at 37°C for 15 min and the enzyme activity was then terminated by adding 2 ml TCA/ascorbate (10%/2%). The tubes were centrifuged at 3000 g for 10 min. To 1.0 mL of this clean supernatant were added 0.5 ml ammonium molybdate (1%) and 1 mL of Na-arsenite/Na-citrate (2%/2%). The tubes were then allowed to stand for 15 min at room temperature and absorbance was read at 840 nm. The mount of inorganic phosphate liberated by the enzyme was calculated by comparing with absorbance values of the standard. Enzyme activity was expressed in mmol of P_i liberated/min/mg of protein.

2.16 Liver glycogen assay

Liver glycogen content was measured according to the method of Murat and Serfaty (1974). Weighed frozen tissue was placed in chilled citrate buffer (0.1 M, pH 4.5) and homogenized with a polytron homogenizer (Kinematica, GmbH, Switzerland). After measuring free glucose in the homogenate, amyloglucosidase was added to the homogenate at a concentration of 1 mg enzyme/mL of homogenate and incubated overnight (16 h) at room temperature. The glycogen content of the liver samples was estimated by comparing glucose liberated from the tissue with a standard curve obtained by treating known amounts of glycogen with amyloglucosidase enzyme. The liver glycogen content was expressed as mg/g wet tissue.

2.17 Measurements of malondialdehyde (MDA) levels in liver, kidney and pancreas by the thiobarbituric acid (TBA) method

Malondialdehyde (MDA), an end product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored substance. Measurement of MDA by TBA reactivity is the most widely used method for assessing lipid peroxidation. Kidney, liver and pancreas samples were minced finely and homogenized by a Polytron homogenizer in ice-cold 1.15% KCl to make a 25% (w/v) (kidney and liver) or 20% (w/v) (pancreas) homogenate. The determination of thiobarbituric acid reactive substance (TBARS) values was performed by the method of Uchiyama and Mihara (1978). To 0.1 mL of the homogenate, 0.2 mL of 8.1% dodecyl sulfate sodium salt (SDS), 1.5 mL of 1% phosphoric acid, 0.2 mL distilled water and 1.0 mL of 0.6% 2-TBA were added. The mixture was heated in a boiling

water-bath for 45 min. After the reaction, the mixture was cooled in an ice-bath; the cold TBA reactants were extracted with 4.0 mL of n-butanol. After centrifugation at 1000 g for 5 min, the optical density of the n-butanol layer was determined at 535 nm. The TBARS values in kidney and liver were expressed as nmol of MDA per 25 mg wet tissue while the values of pancreas were expressed as nmol of MDA per mg wet tissue.

2.18 Protein determination

The protein concentration was initially determined using Lowry's method. Later it was replaced by Bradford method.

2.18.1. Lowry's method

This is the most widely used method for quantitative determination of protein concentration and is sensitive to the level of 10 μg of protein per mL. Reaction of the phenolic moiety of tyrosine in protein occurs with Folin-Ciocalteau reagent, which contains phosphomolybdic/tungstic acid mixture produces a blue/purple colour with absorption maximum around 660 nm. Additionally, the use of a copper reagent enhances the colour formation by chelating with the peptide bonds and allowing for efficient electron transfer to the chromophore formed (Lowry et al., 1951). The protein content of the sample was expressed as mg/dL.

2.18.2 Bradford's method

The protein content in the sample was determined by the method of Bradford (1976), using the Bio-Rad protein assay reagent. The protein content in the samples (mg protein/mL sample) was determined by spectrophotometry according to the method of Bradford (1976) and using the Bio-Rad protein assay reagent. This method is a dye (Coomassie blue)-binding assay in which differential color changes occur in dye in the presence of various concentrations of protein. The dye binds primarily to basic and aromatic amino acid residues, especially arginine. The maximum absorbance wavelength for an acidic solution of Coomassie® Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. The absorbance of the samples was determined by spectrophotometry at 595 nm and the protein content was expressed as mg/dL.

2.19 Determination of the microsomal cytochrome P_{450} content

2.19.1 Preparation of liver microsomes

Liver microsomes were prepared by the ultra-centrifugation method (El Defrawy et al., 1974). Rat liver (2g) was homogenized in ice cold 1.15% KCl (20 mL) using a glass Potter-Elvejhem homogenizer. The homogenate obtained was centrifuged at 10,000g for 10 min at 4^oC. The resulting supernatant obtained was recentrifuged at 100,000g for further 45 min at 4^oC. The microsomal pellet was resuspended in a volume of glycerol buffer [200 mM potassium phosphate buffer, pH 7.4; 50% (v/v) and 1.15% (w/v) potassium chloride (5:8:7)] equivalent to twice the liver weight. The pellet was further homogenized with 7 strokes of the pestle after which aliquots of the microsomal suspension were stored at -70^oC.

2.19.2 Assay of liver microsomal cytochrome P_{450} content

An aliquot of microsomal preparation of 1 mg protein/mL was obtained by adding 0.5 mL of 1M potassium phosphate buffer and the required volume of 1.15% KCl. A modified technique (Omura and Sato, 1964)) was adopted in this assay to eliminate the absorption peak at 420 nm due to contamination by hemoglobin in the sample. The microsomal preparation was placed in two cuvettes and initially saturated with carbon monoxide. A small amount of sodium dithionite (not more than 2 mg) was added to the sample cuvette only. The microsomal P_{450} content was then determined from the difference in absorbance values between the dithionite reduced and control microsomal preparations using a Shimadzu UV-dual-beam spectrophotometer. The molar extinction co-efficient of microsomal P_{450} at the λ max of 450 nm was 91 mM^{-1} cm^{-1}. The liver cytochrome P_{450} content was expressed as nmol/mg of protein.

2.20 High performance liquid chromatography

High performance liquid chromatography (HPLC) analysis of ABe, AF, BuF standard niacin (98% pure), was performed on Shimadzu Class LC-10 (version 1.64) with computer control. The system consists of a dual reciprocating plunger solvent delivery module connected to a degasser, an automatic injector with 1 μL sample loop. A 250 mm x 4.6 mm stainless steel SYNERGI 4 u hydro- RP 80A (Part No: 00G-4375-E0) column (Phenomenex) was used. Detection was made using photodiode array as the photo-detector element. The eluting solvents, HPLC grade acetonitrile and deionized water, were filtered through 0.45 μm filters,

mixed then placed into screw capped reagent bottles. Separation of samples was achieved using a linear gradient of acetonitrile (0 to 100%). The separation temperature was kept constant at 50 ^{o}C, flow rate and sample volume were set to 1.0 mL/min and 10 μL, respectively. The separations were monitored simultaneously with ultraviolet detection in the wavelength range of 215 nm to 280 nm. The retention time of the main peak of standard niacin was compared to those of ABe, AF, and BuF.

2.21 Metal analysis by atomic absorption spectrophotometer

Perkin-Elmer 1100B Atomic Absorption Spectrophotometer, a microcomputer-controlled atomic absorption/emission spectrometer, was used for ppm level detection of metal elements of ABe, AF and BuF by the flame technique. The sample was heated to a high temperature in a flame. After evaporation of the solvent, the flame dissociates chemical bonds and releases free metal atoms which absorb light that is characteristic for individual elements. The band wavelengths at which each element absorbs is narrow and almost unique. The unexcited atoms absorb light, which raises the valence electron (s) to an excited state; as a result of this absorption, the intensity of the original light is reduced. The amount of light absorbed is proportional to the concentration of the element present (Sawyer et al. 1984).

2.22 Statistical analysis

The results are presented as means ± SEM. The statistical methods used to analyse the data in this study were unpaired Student's t-test (two-tailed) and two-way analysis of variance (ANOVA) using MS-Excel software program. Comparisons with P values < 0.05 were considered to be statistically significant.

CHAPTER 3

RESULTS AND DISCUSSION OF SIX EXPERIMENTS

Experiment 1: Effects of *A.bilimbi* leaf extract on blood glucose and lipids in STZ-diabetic rats

1.1 Aims

In the present study, we evaluated the antidiabetic effects of an ethanolic extract of *A.bilimbi* leaves (prepared according to Method 2.1.1) on glucose tolerance in normal and STZ-induced SD diabetic rats and fasting blood glucose and serum lipid profile in STZ-diabetic SD rats, and compared its effects with those of metformin, a biguanide used as an antidiabetic agent.

1.2 Experimental procedure

1.2.1 The OGTT in normal and STZ - diabetic SD rats

Prior to OGTT, male SD rats were fasted for 16 h. Distilled water (control), a reference drug metformin (500 mg/kg), or each of three different doses of ABe (125 mg, 250 mg, and 500 mg/kg) was then orally administered to groups of 5 rats each. Thirty minutes later, glucose (3 g/kg) was orally administered to each rat with a feeding syringe (Al-awadi et al., 1985). Blood samples were collected from the tail vein by tail milking at - 30 min (just before the ABe and metformin administration), 0 (just before the oral administration of glucose), 60, 120, and 180 min after glucose load for the assay of glucose. The OGTTs were performed in STZ-diabetic rats using the same procedure as described for the normal rats. Six animals were used for distilled water (control), metformin (positive control) and ABe-treated groups.

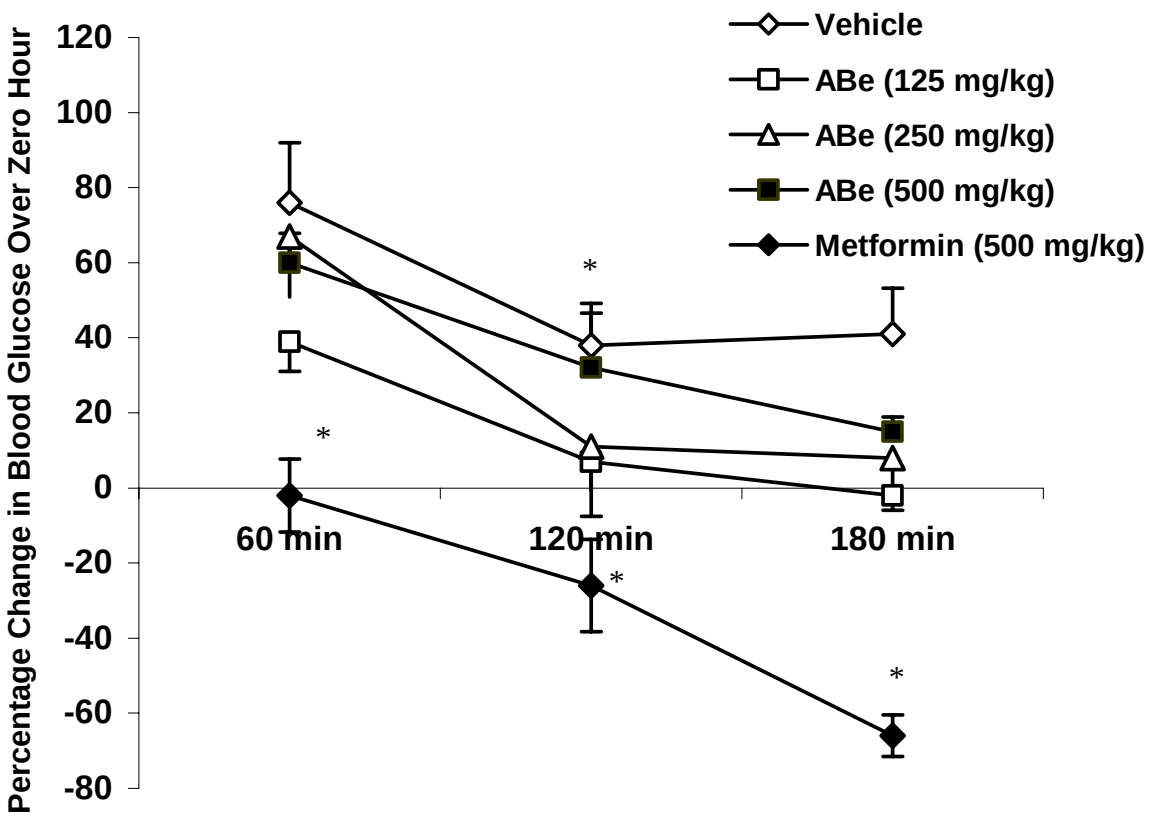

Figure 5. OGTT in normal SD rats.
Values are expressed as the mean ± SEM for 5 rats in each group. The % change in blood glucose at 60, 120, and 180 min was calculated from the corresponding 0-h value (just before the oral administration of glucose) in each group.

*P< 0.05 compared with control (Student's t-test).

1.2.2 Repeated administration of ABe in STZ-diabetic SD rats

One week after STZ induction of diabetes in male SD rats, the fasting blood glucose levels were measured. The hyperglycemic rats (blood glucose >350 mg/dl) were divided on day zero into three groups (each with 6 rats). The fasting blood glucose level (FBG), total cholesterol (TC), triglycerides (TG), HDL-cholesterol (HDL-C), and LDL-cholesterol (LDL-C) concentrations were also measured on day zero. Distilled water, metformin (500 mg/kg) and ABe (125 mg/kg) were then administered orally twice a day to control, positive control and the treatment groups respectively for 2 weeks. Body weight, food and water intakes were monitored daily for 2 weeks. On day 15, after 16 h fasting, the rats were decapitated and the blood was collected for estimation of the FBG, TC, TG, HDL-C and LDL-C. The organs such as liver and kidney were isolated, weighed and stored at -70°C for the assay of hepatic cytochrome P_{450} and thiobarbituric acid reactive substances (TBARS) in both liver and kidney

1.3 Results and discussion

1.3.1 Dose response effect of ABe on glucose tolerance in normal and STZ – diabetic rats

The blood glucose levels of the normal rats reached a peak at 60 min after the oral administration of glucose and gradually decreased to pre-glucose load level (Figure 5). Of the three different doses viz., 125 mg, 250 mg, and 500 mg/kg, the lowest dose caused a significant attenuation in the blood glucose at 180 min compared to the vehicle-treated control group ($P<0.05$). Metformin (500 mg/kg)

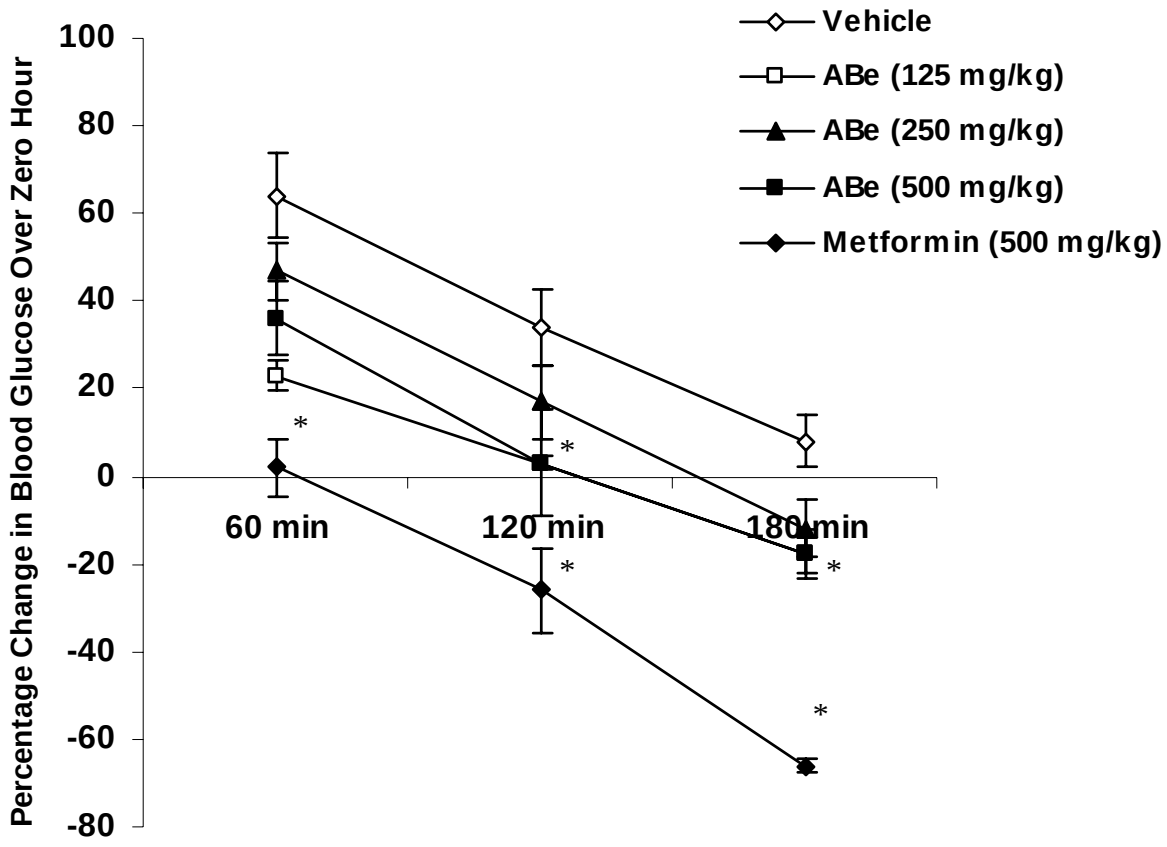

Figure 6. OGTT in diabetic SD rats.
Values are expressed as the mean ± SEM for 6 rats in each group. The % change in blood glucose at 60, 120, and 180 min was calculated from the corresponding 0-h value (just before the oral administration of glucose) in each group.
*P< 0.05 compared with control (Student's t-test).

also produced a significant decrease ($P<0.01$) in blood glucose level at 180 min after the administration of the oral glucose load.

In the diabetic rats, fasting blood glucose levels were 4-5 times higher than that of the normal SD rats. ABe at a dose of 125 mg/kg produced a significant attenuation in the blood glucose ($P<0.05$) at 120 min and 180 min ($P<0.01$) after the oral glucose load (Figure 6). No significant attenuation was observed in the rats administered 250 mg of ABe/kg, even at 180 minutes. However, ABe at a dose of 500 mg/kg caused a significant attenuation ($P<0.01$) in the blood glucose only at 180 min when compared to the vehicle-treated group. Metformin (500 mg/kg) caused significant attenuation at 60 min ($P<0.001$), 120 min ($P<0.01$) and 180 min ($P<0.01$) when compared to the vehicle-treated group. Of the three doses of ABe tested, the lowest dose (125 mg/kg) appeared to be most effective in improving glucose tolerance ($P<0.01$). Hence this dose was selected for the 2- week study.

1.3.2 Effects of 2-week administration of ABe (125 mg/kg) and metformin on blood glucose and lipids in STZ-diabetic rats

The body weights in the ABe and the metformin-treated group were increased significantly ($P<0.001$) on day 14 when compared with the vehicle-treated group. The food intake was significantly lowered in the ABe and metformin-treated group ($P<0.001$) when compared with the vehicle-treated group. Similarly, water intake was significantly reduced ($P<0.001$) in both ABe and metformin-treated groups (Table 3).

Table 3. Body weight, water and food intakes in STZ-diabetic rats before and after oral treatment with vehicle, ABe, and metformin twice a day for 2 weeks

Treatment Group (n = 6)	Body weight (g) (mean ± SEM)		Water intake (ml/100 g body weight of rat/day) (mean ± SEM)		Food intake (g/100 g body weight of rat/day) (mean ± SEM)	
	Before	After	Before	After	Before	After
Vehicle	222 ± 11	205 ± 14	67 ± 12	59 ± 10	20 ± 2.2	20 ± 2
ABe	259 ± 18	282 ± 31*	46 ± 11	44 ± 5*	13 ± 3	15 ± 2*
Metformin	258 ± 13	323 ± 47*	48 ± 10	40 ± 10*	12 ± 2.5	13 ± 0.4*

*P<0.001 compared with vehicle-treated rats (two-way ANOVA)

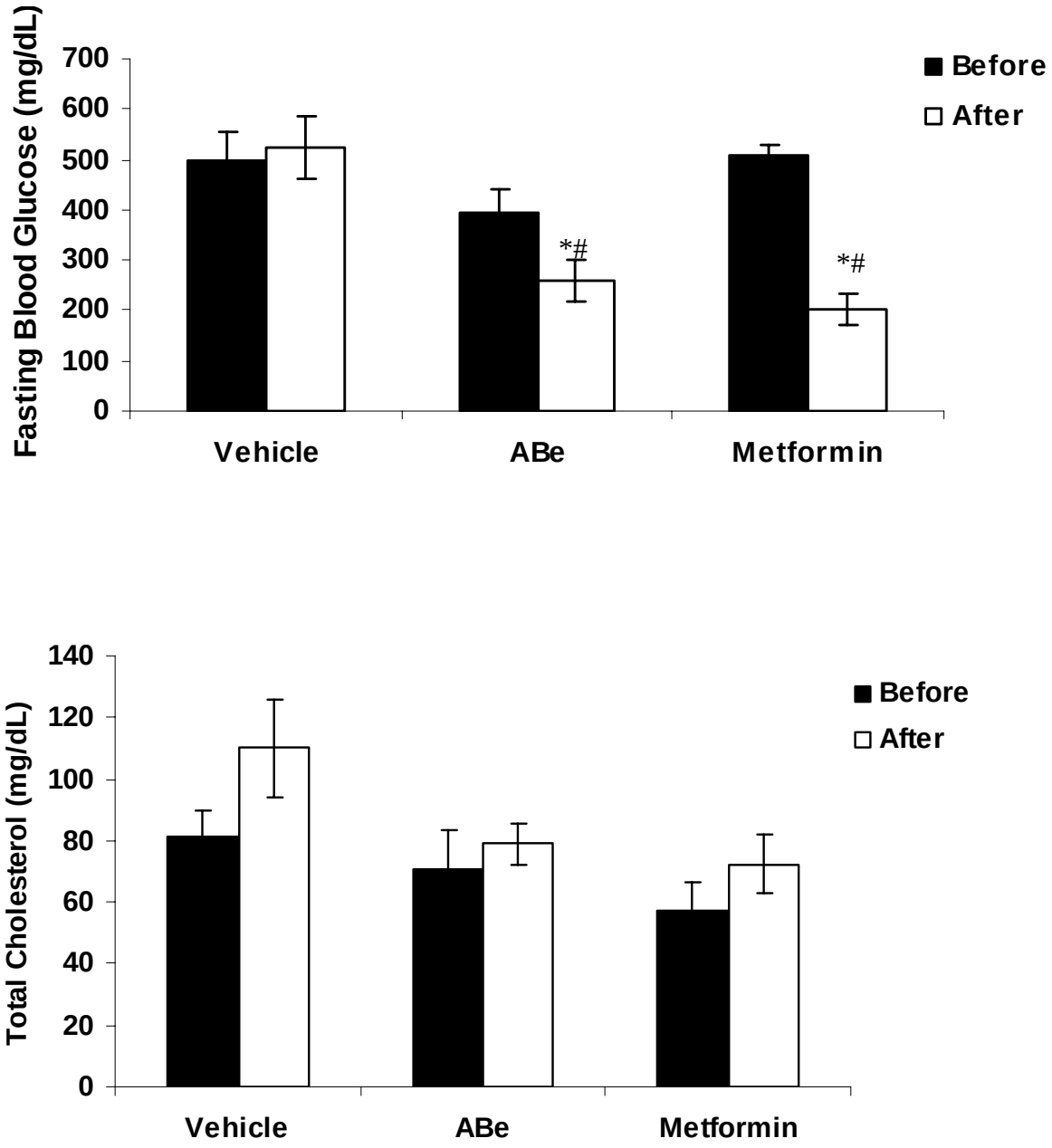

Figure 7. Effects of ABe treatment on FBG and TC levels in STZ-diabetic rats. FBG and TC levels in diabetic rats before and after daily oral treatment with vehicle (distilled water), ABe (125 mg/kg) and metformin (500 mg/kg) for 2 weeks. Columns represent the mean ± SEM (n= 6).

*P<0.05 compared against control (Student's t-test).
#P<0.05 compared with day zero value (Student's t-test).

As shown in Figure 7, the daily administration of ABe (125 mg/kg) twice a day for 14 days in STZ-diabetic SD rats caused a significant reduction in blood glucose level when compared with the vehicle-treated control ($P<0.01$) rats and day zero value ($P<0.05$). Similarly, repeated administration of metformin (500 mg/kg) twice a day for 14 days caused a significant reduction ($P<0.01$) in the blood glucose level in STZ-diabetic SD rats when compared to vehicle and day zero values. There was a significant decrease in serum TG ($P<0.05$) and a significant increase in HDL-C ($P<0.05$) in the ABe-treated SD rats (Figure 8) when compared to the vehicle-treated control SD rats. However, ABe did not decrease serum cholesterol and LDL-C concentrations significantly ($P>0.05$). The daily administration of metformin to STZ-diabetic SD rats caused a significant decrease in the serum TG ($P<0.01$) when compared to the vehicle-treated control rats. Metformin, however, did not decrease serum cholesterol and LDL-C concentrations (Figure 8). It also failed to increase serum HDL-C concentration. The AAI (refer to Method 2.11.) was significantly increased in the ABe-treated group ($P<0.001$) when compared to the vehicle-treated group of rats (Figure 9). However, there was no significant difference in the AAI of the Metformin-and the vehicle-treated groups ($P>0.05$).

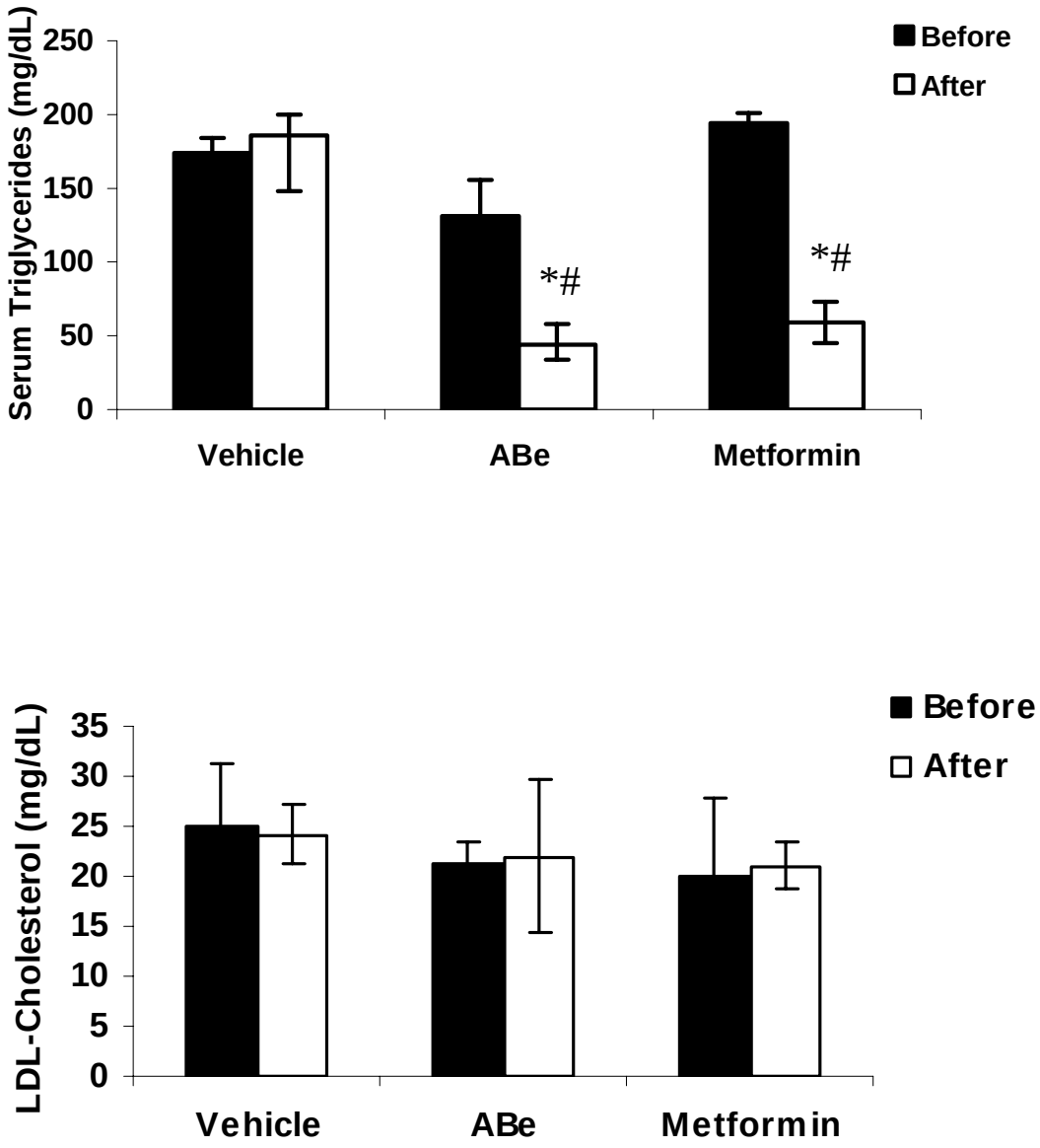

Figure 8. Effects of ABe treatment on serum TG and LDL-C in STZ-diabetic rats. Serum TG and LDL-C levels in diabetic rats before and after daily oral treatment with vehicle (distilled water), ABe (125 mg/kg) and metformin (500 mg/kg) for 2 weeks. Columns represent the mean ± SEM (n= 6).

*P<0.05 compared against control (Student's t-test).

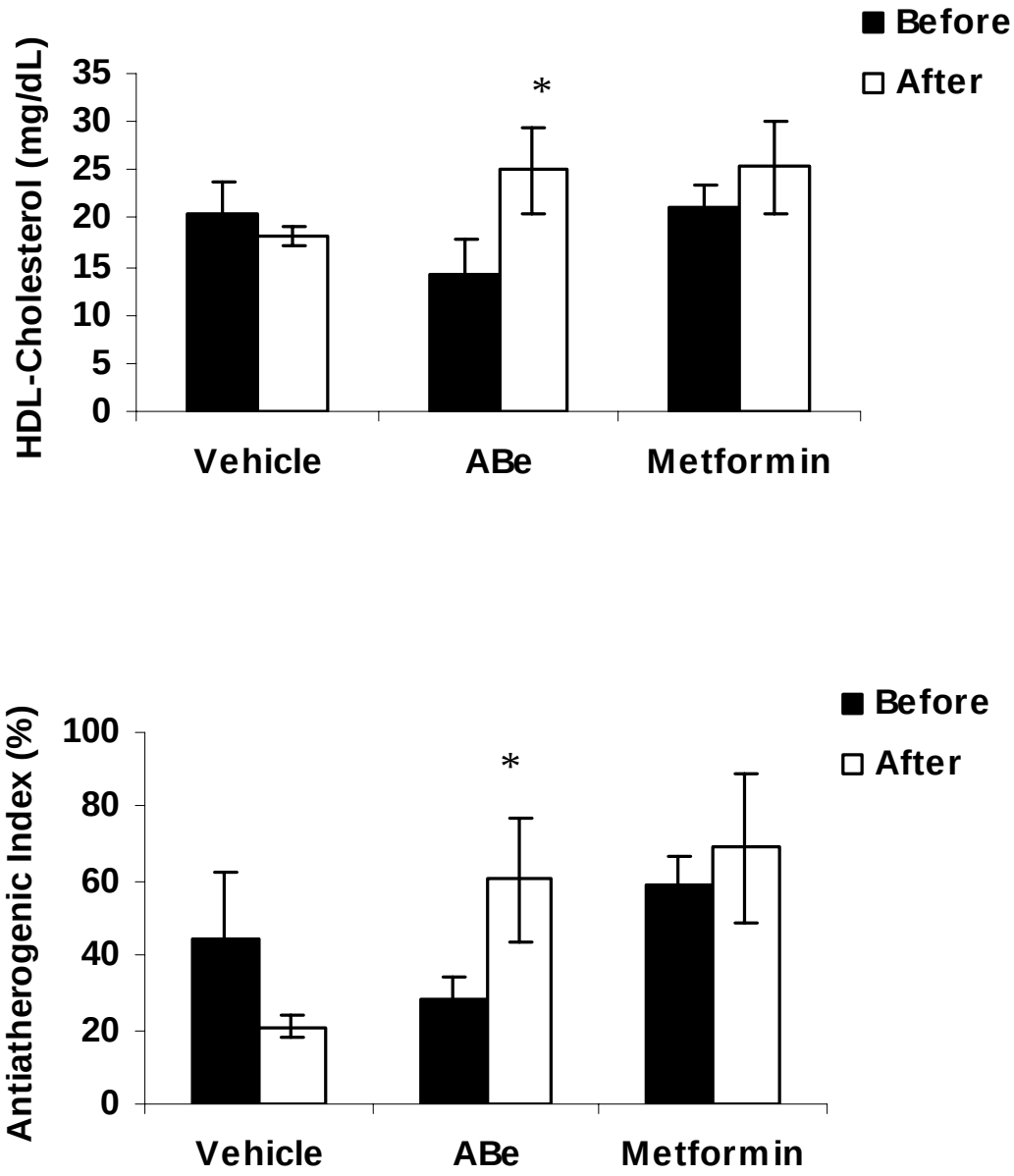

Figure 9. Effects of ABe treatment on HDL-C and AAI in STZ-diabetic rats. HDL-C level and AAI of diabetic rats before and after daily oral treatment with vehicle (distilled water), ABe (125 mg/kg), and metformin (500 mg/kg) for 2 weeks. Columns represent the mean ± SEM (n= 6).

*P<0.05 compared with control (Student's t-test).

Table 4. Liver cytochrome P_{450} content and lipid peroxidation level in the kidney and liver of STZ-diabetic rats after 2 weeks of oral treatment twice a day with vehicle, ABe, and metformin

Treatment group (n = 6)	Liver cytochrome P_{450} content (nmol/mg protein) (mean ± SEM)	TBARS (nmol of malonaldehyde per 25 mg of tissue) (mean ± SEM)	
		Liver	Kidney
Vehicle	1.2 ± 0.07	3.3 ± 0.25	4.8 ± 0.2
ABe	1.1 ± 0.08	2.9 ± 0.04	3.5 ± 0.17 **
Metformin	1.01 ± 0.03 *	2.8 ± 0.13	3.3 ± 0.25 **

*P<0.05 compared with vehicle-treated rats ((Student's t-test).
** P<0.01 compared with vehicle-treated rats (Student's t-test).

As shown in Table 4, there was no significant difference in the hepatic microsomal cytochrome P_{450} content between ABe- and vehicle-treated control STZ-diabetic SD rats ($P>0.05$). However there was a significant reduction in the hepatic microsomal cytochrome P_{450} content in the metformin-treated group when compared to the vehicle-treated group ($P<0.05$).

The TBARS levels were significantly reduced in the kidneys of both ABe- and metformin-treated STZ-diabetic SD rats ($P<0.01$). However there was no significant difference in TBARS levels in the livers of both ABe- and metformin-treated diabetic rats when compared to the vehicle-treated control rats.

The single high dose STZ-induced diabetic rat is one of the animal models of human IDDM or type I diabetes mellitus. In this model, diabetes arises from irreversible destruction of the β-islet cells of the pancreas, causing degranulation or reduction of insulin secretion (Junod et al., 1969). Our present studies show that ABe demonstrates a definite hypoglycemic, hypotriglyceridemic, anti-atherogenic, and anti-lipid peroxidative properties in STZ-diabetic rats after 2 weeks of treatment. The hypoglycemic activity of ABe was observed at the lowest dose (125 mg/kg) in normal as well as STZ-diabetic rats and was similar to the action of metformin. Metformin, a biguanide, does not induce the secretion of insulin from the β-islet cells of pancreas, but increases glucose utilisation in the extra-hepatic tissues, reduces hepatic gluconeogenesis (Bailey, 1992) and increases the expression of insulin receptors in the liver plasma membranes (Kanigur-Sultuybek et al., 1995). Since ABe reduced blood glucose potently in the STZ-diabetic SD

rats like metformin, it may have hypoglycemic principle(s) that are similar in action to metformin.

The daily administration of ABe (125 mg/kg) and metformin (500 mg/kg) to STZ-diabetic rats twice a day for two weeks caused a statistically significant reduction in food and water intakes, and an increase in the body weight of STZ- diabetic rats. This could be the result of improved glycemic control induced by the compounds. ABe might reduce TG by decreasing the serum non-esterified fatty acids (NEFA) in the STZ-diabetic rats similar to masoprocol (nordihydroguaiaretic acid), a pure compound isolated from *Larrea tridentata* (Reed et al., 1999). Since ABe increased HDL-C, it significantly increased the AAI. ABe thus has the potential to prevent the formation of atherosclerosis and coronary heart disease which are the secondary diabetic complications of severe diabetes mellitus (Fontbonne et al., 1989). In contrast, metformin failed to increase the HDL-C level and did not increase the AAI. However it has been reported that metformin can reduce blood lipid parameters in non-diabetic patients with coronary heart disease (Carlsen et al., 1996). Hence, ABe may contain a hypolipidemic principle(s), which could act in a way different from that of metformin. The cytochromes are the primary system (phase I detoxification enzymes) responsible for chemical defense in animals (Elizabeth Gillam, 1998). The cytochrome P_{450} content in the liver has been found to be increased in diabetic animals (Lucas et al., 1998). The reduction in insulin levels in the diabetic state also causes an increase in the level of cytochrome P_{450} enzymes (Woodcroft and Novak., 1997).

In this study, cytochrome P_{450} content of the ABe-treated group was similar to that of the vehicle-treated group. However, a significant reduction ($P<0.05$) was found in the metformin-treated group. As metformin is not known to reduce insulin levels, mechanism by which it reduces the cytochrome P_{450} content is not known.

The hyperglycemia in STZ-treated rats leads to the formation of hydrogen peroxide, which subsequently generates free radicals such as O^{2-} and OH°. These reactive compounds can cause peroxidation of lipids, resulting in the formation of hydroperoxy fatty acids and endoperoxides. This increases the formation of malonaldehyde (MDA) and thromboxane-B_2 (TxB_2). The accumulation of TxB_2 together with thromboxane-A_2 (TxA_2) can cause platelet aggregation and promote thrombosis (Sushil Jain et al., 1998). Since ABe has the ability to reduce the formation of TBARS, it could potentially prevent platelet aggregation and thrombosis.

Experiment 2: Evaluation of the anti-diabetic effects of semi-purified fractions of ABe in a rat model of type 1 diabetes

2.1 Aims

In the present study, the aim was to evaluate the antidiabetic effects of semi-purified fractions of ABe in STZ-induced diabetic rats compared with metformin.

2.2 Experimental procedure

2.2.1 The OGTT in STZ – diabetic rats using the semi-purified fractions of ABe

Prior to OGTT, rats were fasted for 16 h. Distilled water (control), four different fractions of ABe viz. AF, BuF, EF and HF each at a dose of 125 mg/kg body weight and the reference drug, metformin, at a dose of 500 mg/kg body weight were orally administered to groups of 5-6 rats each. Thirty minutes later, glucose (3 g/kg) was orally administered (Al-Awadi et al., 1985) to each rat with a feeding syringe. Blood samples were collected from the tail vein by tail milking at – 30 min (just before the administration of distilled water, fractions of ABe and metformin in respective groups), 0 (just before the oral administration of glucose), 30, 60, 120, and 180 min after glucose load for the assay of glucose.

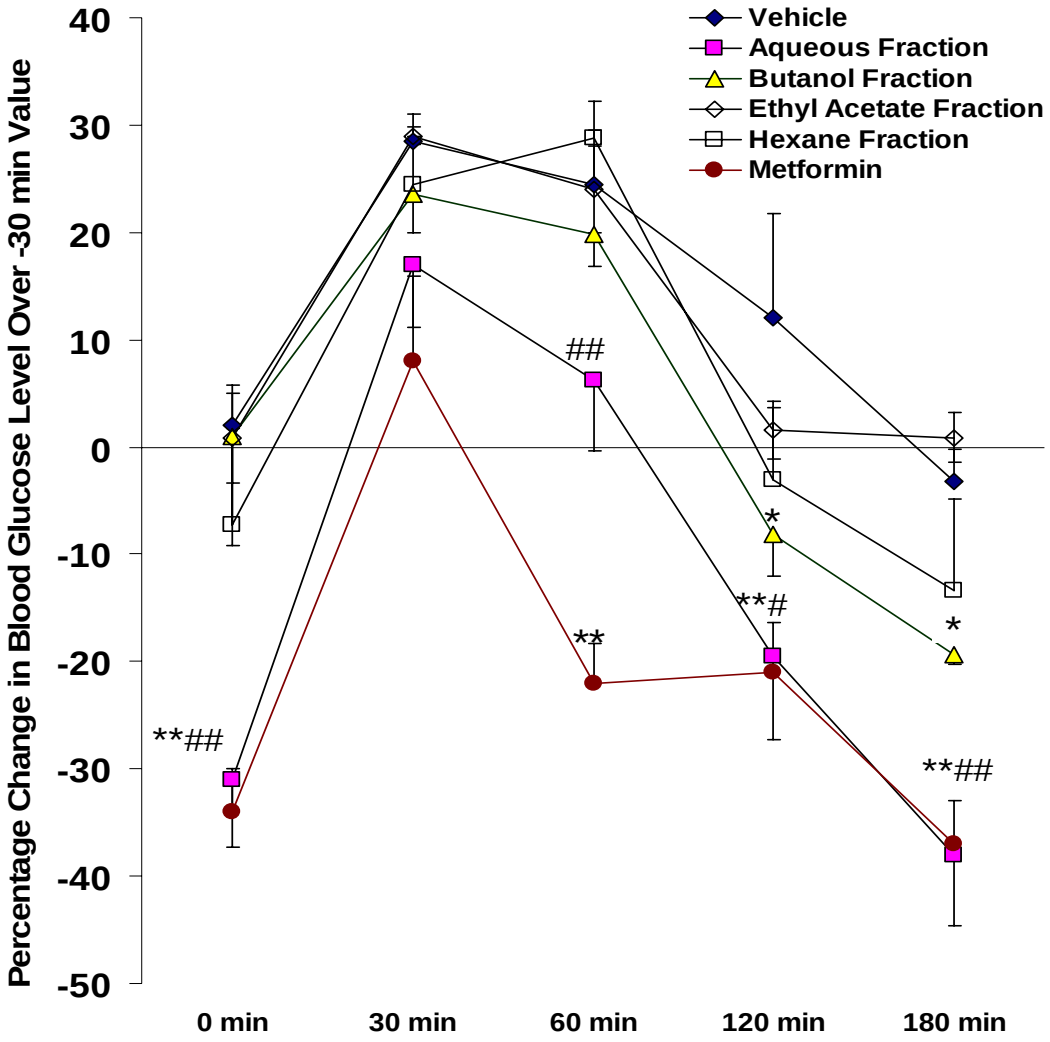

Figure 10. The OGTT in STZ-diabetic rats using the semi-purified fractions of ABe.

The graph represents the mean percentage changes in blood glucose concentration over – 30 min level in Vehicle (565 ± 28), Aqueous Fraction [AF] (608 ± 42), Butanol Fraction [BuF] (540 ± 51), Ethyl acetate Fraction [EF] (487 ± 11), and Hexane Fraction [HF] (448 ± 19) of ABe, each at a dose of 125 mg/kg and Metformin (603 ± 30) –treated (500 mg/kg) diabetic rats, while bars represent SEM (n = 6). The blood glucose concentration (mg/dL) of each group at –30 min is given in brackets.

*P<0.05 BuF-treated group vs vehicle-treated group (Student's t-test).
**P<0.01 Metformin-treated group vs vehicle-treated group (Student's t-test).
#P<0.05 AF-treated group vs vehicle-treated group (Student's t-test).
P<0.01 AF-treated group vs vehicle-treated group (Student's t-test).

2.2.2 Twice daily oral administration of AF (125 mg/kg) and BuF (125 mg/kg) for two weeks in STZ-diabetic rats

One week after STZ induction of diabetes in male SD rats, the fasting blood glucose levels were measured. The hyperglycemic rats (blood glucose >350 mg/dl) were divided on day zero into four groups (each with 6 rats). The fasting blood glucose level was measured on day zero at 9.00 am. Distilled water, AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg) were then administered orally twice a day at 9.00 am and 9.00 pm to diabetic control, treatment and positive control groups respectively for 2 weeks. Body weight, food and water intakes were monitored every day between 9.00 and 10.00 am for 2 weeks. On the 15^{th} day, after 16 h fasting, the rats were decapitated and blood was collected at 9.00 am for estimation of the fasting blood glucose. The organs, liver and kidney, were isolated, weighed and stored at -70°C for the assay of hepatic Glc-6-Pase, glycogen, cytochrome P_{450} and TBARS contents.

2.3 Results and discussion

In the OGTT (Figure 10), AF (125 mg/kg) caused a significant hypoglycemic effect within 30 minutes after oral administration to STZ-diabetic rats. AF also produced a significant attenuation ($P < 0.01$) in blood glucose level at 0 min, 120-min and 180 min when compared with vehicle control. BuF (125 mg/kg) had no significant effect on blood glucose at 0 min, but produced a significant attenuation ($P < 0.05$) at 120 and 180 minutes after oral administration. The other two-fractions, EF and HF did not cause any reduction in blood glucose level at any time point.

Table 5. Body weight, water and food intakes in STZ-diabetic rats before and after oral treatment with vehicle, AF, BuF, and metformin twice a day for 2 weeks

Treatment group (n = 6)	Body weight (g) (mean ± SEM)		Water (mL/rat/day) (mean ± SEM)		Food (g/rat/day) (mean ± SEM)	
	Before	After	Before	After	Before	After
Control	223 ± 3	221 ± 11	166 ± 10	195 ± 10	32 ± 5	50 ± 6
AF	225 ± 5	243 ± 19	144 ± 19	158 ± 19*	33 ± 3	43 ± 4*
BuF	221 ± 9	251 ± 30	145 ± 10	168 ± 5*	41 ± 3	46 ± 1*
Metformin	236 ± 6	241 ± 9	153 ± 7	145 ± 6*	40 ± 2	43 ± 2*

*P<0.001 compared with the vehicle-treated rats (two-way ANOVA).

In this type I model of diabetes, insulin is markedly depleted, but not completely absent (Junod et al., 1969). Although insulin has become one of the most important therapeutic agents known to medicine, there is a continuing effort to find insulin substitutes, secretagogues, or sensitizers from synthetic or plant sources for the treatment of diabetes. Over 150 plant extracts and some of their active principles including flavonoids are known to be used for the treatment of diabetes mellitus (Olajide et al., 1999). The present study revealed that the semi-purified fractions, AF and BuF, of ABe have potent hypoglycemic property when given for 2 weeks to STZ-diabetic rats. Tan et al (1996) reported the hypoglycemic properties of both aqueous and ethanolic extracts of the leaves of *A.bilimbi* in STZ-diabetic Wistar rats when these were administered intraperitoneally at doses of 100 mg/kg and 300 mg/kg respectively.

Although the body weight of the rats did not differ significantly (Table 5), there was about 8% increase in the AF treated group and 13% increase in the BuF-treated group when compared to the vehicle-treated control group. However, the food and water intakes of AF- and BuF-treated diabetic rats were significantly different as in the metformin-treated group when compared to the vehicle-treated group. Similar effects were reported for other hypoglycemic agents such as tungstate and vanadate (Gil et al., 1988; Barbera et al., 1994).

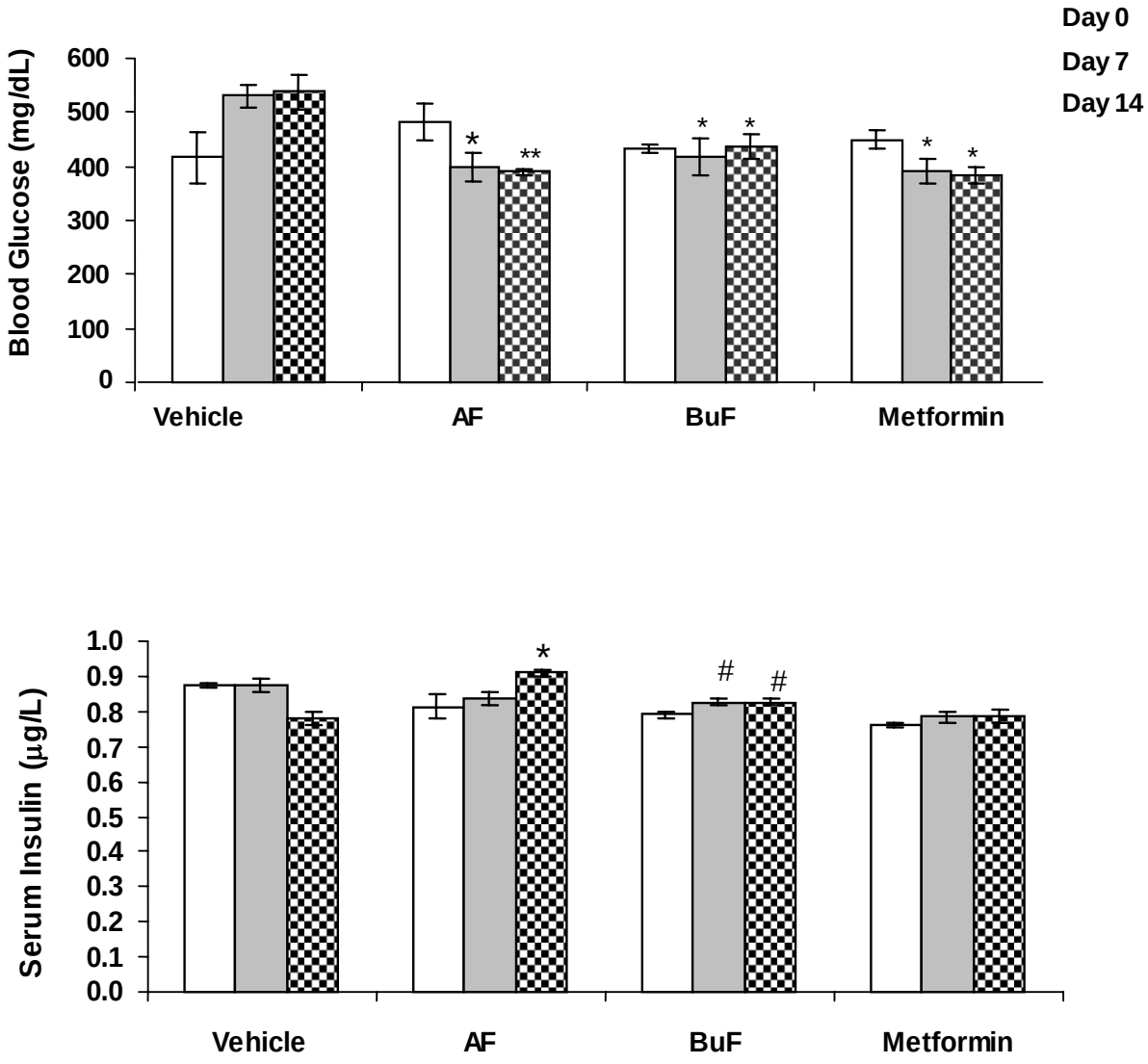

Figure 11. Effects of AF and BuF treatment on FBG and insulin levels in STZ-diabetic rats.
The blood glucose and insulin levels were measured on day 0, day 7, and day 14 at 9.00 a.m. after 16 hour fast in the vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg)-treated STZ-diabetic rats. Columns represent the mean ± SEM (n= 6).

*P<0.05 compared with the diabetic untreated rats (Student's t-test).
**P<0.01 compared with the diabetic untreated rats (Student's t-test).
$^{\#}$P<0.05 compared with the corresponding day 0 value (Student's t-test).

AF caused a significant ($P<0.01$) time-dependent hypoglycemic effect after twice-daily oral administration at a dose of 125 mg/kg for 7 and 14 days (Figure 11). BuF also showed a significant ($P<0.05$) hypoglycemic property on day 7 as well as on day 14 compared to the vehicle-treated control group. However, BuF did not cause a statistically significant reduction in blood glucose on day 7 and 14 when compared to day 0 value (of BuF-treated group). This indicates that the AF is better than BuF in controlling the blood glucose of diabetic animals. The serum insulin level in the AF-treated group was significantly higher on day 14 compared to both the control ($P<0.05$) and day zero levels ($P<0.05$) (Figure 11). On the other hand, the serum insulin level in the BuF-treated group was significantly higher on both day 7 ($P<0.05$) and day 14 ($P<0.05$) when compared to its day zero value.

The elevation in serum insulin in the AF- and BuF-treated STZ-diabetic rats could either be due to the presence of insulinotropic substances in the fractions, (which induce the intact functional β-cells to produce insulin), or the protection of the functional β-cells from further deterioration so that they remain active and produce insulin. However, except for the level in AF-treated group on day 14, the insulin levels were well below the normal insulin level in control rats, suggesting that they may not be sufficient to lower the blood glucose to its normal level in the STZ-diabetic rats. Our results indicate a possible insulin-releasing action of ABe in STZ-diabetic rats. Similarly the extracts of *Medicago sativa* (Gray and Flatt, 1997), *Eucalyptus globulus* (Gray and Flatt, 1998) and *Sambucus nigra* (Gray et al., 2000) have been shown to possess insulin-releasing action both *in vitro* and *in vivo*.

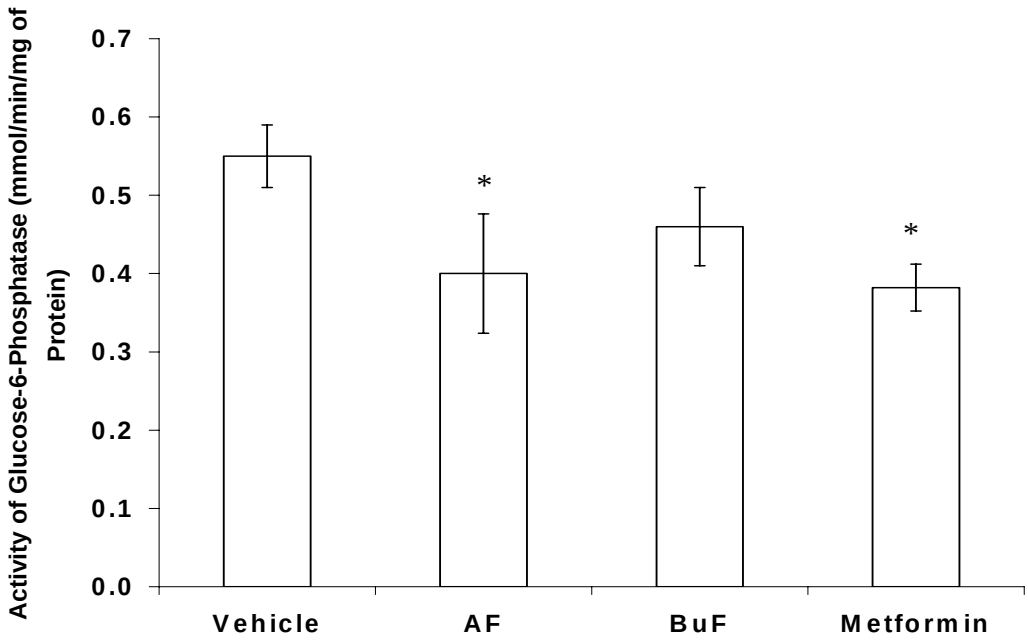

Figure 12. Effects of AF and BuF treatment on hepatic Glc-6-Pase activity in STZ-diabetic rats.
Hepatic Glc-6-Pase activity after 2- week oral treatment with vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg). Columns represent the mean ± SEM (n= 6).

*P<0.05 compared with control (Student's t-test).

Glc-6-Pase activity (Figure 12) in the liver was significantly reduced ($P<0.05$) in both AF-and metformin-treated groups when compared to the vehicle-treated diabetic control group. However, there was no significant change in the activity of BuF–treated group. Glc-6-Pase catalyzes the final step in glucose production by the liver and kidney. STZ has been reported to increase the expression of Glc-6-Pase mRNA, which contributes to the increased Glc-6-Pase activity in diabetes mellitus (Liu et al., 1994). Overproduction of glucose by the liver is the major cause of fasting hyperglycemia in both insulin-dependent and non-insulin-dependent diabetes mellitus. 90% of partially pancreatectomized diabetic rats have a >5-fold increase in the mRNA and a 3-4-fold increase in the protein level of the catalytic subunit of hepatic Glc-6-Pase. Prolonged hyperglycemia may thus result in overproduction of glucose via increased expression of this protein (Massillon et al., 1996). Normalization of the plasma glucose concentration in diabetic rats with either insulin or the glycosuric agent, phlorizin, normalized the hepatic Glc-6-Pase mRNA and protein within approximately 8 h. However, phlorizin failed to decrease Glc-6-Pase gene expression in diabetic rats when the fall in the plasma glucose concentration was prevented by glucose infusion. These data indicate that *in vivo* gene expression of Glc-6-Pase in the diabetic liver is regulated by glucose independently of insulin. AF-fraction, like the biguanide drug, metformin, controls the increase in blood glucose in STZ-diabetic rats by

decreasing the activity of Glc-6-Pase in the liver. This could be one of the mechanisms for the suppression of blood glucose concentration in the diabetic rats. Other workers have also reported that extracts of some plants such as *Zizyphus spina-christi* significantly reduced serum glucose level, liver phosphorylase and Glc-6-Pase activities, and significantly increased serum pyruvate level and liver glycogen content after 4 weeks of treatment (Glombitza et al., 1994). Similarly, 60 % ethanolic extract of *Coccinia indica* and 95 % ethanolic extract of *Momordica charantia* extracts were found to lower blood glucose by depressing its synthesis, on the one-hand, through depression of the key gluconeogenic enzymes, Glc-6-Pase and fructose-1, 6-bisphosphatase (Fru-1, 6-P2ase) and on the other by enhancing glucose oxidation by the shunt pathway through activation of its principal enzyme, G6PDH (Shibib et al., 1993).

The liver is an attractive target organ for insulin gene expression in type 1 diabetes as it contains appropriate cellular mechanisms of regulated gene expression in response to blood glucose and insulin. The expression of the promoter of the Glc-6-Pase gene in the liver is induced by glucose and suppressed by insulin (Chen et al., 2000). Insulin suppresses hepatic glucose production (HGP) in euglycemia by solely decreasing the Glc-6-P concentration; when combining hyperinsulinemia with hyperglycemia, the suppression of HGP involves the inhibition of the Glc-6-Pase activity; and a sustained glucose-phosphorylation flux might be a crucial determinant in the inhibition of Glc-6-Pase and of HGP (Guignot and Mithieux, 1999). The operation of Glc-6-Pase stems from the interaction of at least two

highly hydrophobic proteins embedded in the ER membrane, a heavily glycosylated catalytic subunit of molecular weight 36 kDa (P36) and a 46-kDa putative Glc-6-P translocase (P46). P36 gene expression is increased by glucose, fructose 2, 6-bisphosphate (Fru-2, 6-P2) and free fatty acids, as well as by glucocorticoids and cyclic AMP; the latter are counteracted by insulin. P46 gene, like P36 gene, expression is affected by glucose, insulin and cyclic AMP (Van de Werve et al., 2000). The insulinotropic effect of AF might play a crucial role in the control of hyperglycemia in STZ-diabetic rats. Insulin thus inhibits the activity of glucose-6-phosphatase in the liver of STZ-diabetic rats and thereby controls HGP. Hence the suppression of Glc-6-P hydrolysis could also be one of the reasons for the hypoglycemic effect of AF in STZ-diabetic rats. Similar effects were reported for other hypoglycemic agents such as vanadate (Mosseri et al., 2000).

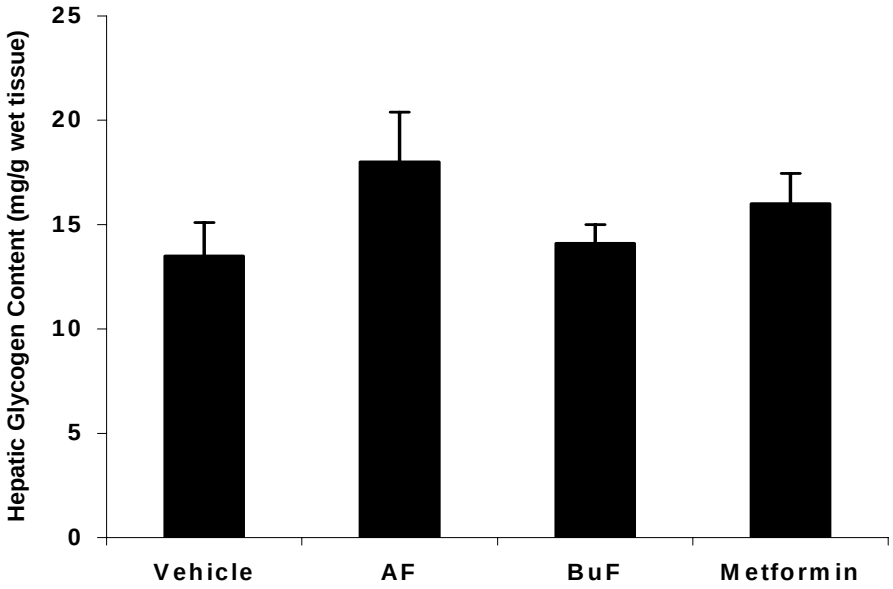

Figure 13. Effects of AF and BuF treatment on hepatic glycogen content in STZ-diabetic rats. Hepatic glycogen content after 2- week oral treatment with vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg). Columns represent the mean ± SEM (n= 6).

Table 6. Liver cytochrome P_{450} content and TBARS levels in the kidney and liver of STZ-diabetic rats after twice-a-day oral treatment for 2 weeks with vehicle, AF, BuF, and metformin

Treatment group (n = 6)	TBARS level (nmol of malonaldehyde per 25 mg of tissue) (mean ± SEM)		Hepatic cytochrome P_{450} content (nmol/mg protein) (mean ± SEM)
	Liver	Kidney	
Vehicle	4.0 ± 0.2	4.7 ± 0.3	1.25 ± 0.04
AF	3.8 ± 0.1	3.9 ± 0.2*	1.15 ± 0.04
BuF	3.9 ± 0.2	4.0 ± 0.3	1.20 ± 0.06
Metformin	3.7 ± 0.2	3.7 ± 0.2*	1.07 ± 0.03*

*P < 0.05 compared with vehicle-treated rats (Student's t-test).

However, there was no significant difference in the level of hepatic glycogen content in AF, BuF and also metformin-treated rats compared to vehicle-treated rats although the hepatic glycogen content tended to be higher in the AF-treated group when compared to the control group (Figure 13). Similarly, Vanadate compounds have been shown to inhibit hepatic Glc-6-Pase activity, thereby reducing blood glucose levels in non-obese diabetic (NOD) mice. However no significant difference was found in the hepatic glycogen stores of the treated groups compared to control (Schulz, 1988). The kidney TBARS in AF- and metformin-treated diabetic rats were significantly lower (Table 6, $P < 0.05$) than in the vehicle-treated rats. On the other hand, the kidney TBARS value in BuF-treated rats was not significantly different from that in the vehicle-treated rats. There was also no difference in liver TBARS values between AF-, BuF- and metformin-treated rats and vehicle-treated control rats. The liver microsomal cytochrome P_{450} content was significantly lower in the metformin-treated rats when compared to that in the corresponding vehicle-treated rats. However, there was no significant difference in the liver cytochrome P_{450} content in AF- and BuF-treated rats when compared with that in the corresponding vehicle-treated rats.

The level of increase in the hepatic cytochrome P_{450} content depends on the duration of diabetes (Barnett et al., 1994).Reduced insulin level in the diabetic state also causes an increase in cytochrome P_{450} content (Woodcraft et al., 1997). In this study, AF and BuF did not cause any change in the cytochrome P_{450} enzymes in the liver. However a reduction was found in the metformin-treated group.

Hypoinsulinemia in diabetes increases the activity of fatty acyl coenzyme A oxidase, which initiates β-oxidation of fatty acids, resulting in lipid peroxidation (Bruch and Thayer, 1983). The enormous increase in lipid peroxidation leads to the alteration of the transbilayer fluidity gradient (Oberley, 1988), which could hamper the activities of membrane-bound enzymes and receptors. The products of lipid peroxidation, such as lipid radicals and lipid peroxides, are extremely harmful to most of the cells in the body and are associated with a variety of diseases, such as atherosclerosis and brain damage (Elangovan et al., 2000). It has been suggested that oxidative stress plays an important role in the development of chronic complications of diabetes (Tatsuki et al., 1997). In our present study, there was no significant change in the level of TBARS in the liver of AF-, BuF- and metformin-treated diabetic rats when compared to the vehicle-treated rats. The assessment of lipid peroxidation by conjugate diene levels showed a significant increase in all diabetic tissues except the liver. This suggests a unique response of the liver in STZ-induced diabetic rats to oxidative stress and supports the observation of Oberley (1988) that it has higher capacity to cope with the stress compared to other organs. Tatsuki et al. (1997) also reported that there was no change in the liver

lipid peroxides of STZ-diabetic rats after 2-weeks of STZ-administration at an i.v. dose of 32-mg/kg per day. Hence the lack of change in the TBARS levels in the liver of AF-and BuF-treated and metformin-treated diabetic rats could again reflect the resistance of the liver to the oxidative stress in the diabetic state. It is significant to note that neither AF nor BuF affects this capacity adversely. AF has the ability to reduce the formation of TBARS like ABe; it could also potentially prevent platelet aggregation and thrombosis.

Experiment 3: Studies on the pancreatic β-cell protective effects of ABe, AF and BuF against STZ in SD rats

3.1 Aims

To evaluate the pancreatic β-cell protective action of ABe, AF, and BuF against STZ in SD rats.

3.2 Experimental procedure

3.2.1 Pancreatic β-cell protective study with ABe

Twenty-eight SD rats were randomly divided into four groups of seven rats each. On day zero, the body weights were recorded. The two treatment groups were given ABe at a dose of 125 mg/kg twice a day for 7 and 14 days respectively, whereas the other two groups were untreated and served as controls. On day 8 or 15, the body weights were recorded in the respective treatment and control groups and blood samples were taken from the tail vein for glucose estimation, followed by STZ injection (i.p. 60 mg/kg). On day 11 or 18, the rats were fasted till the next morning, and decapitated to collect blood for the estimation of glucose. The liver, pancreas, kidneys and heart were removed and weighed.

3.2.2 Studies on the pancreatic β-cell protective effect of AF and BuF

Twenty-eight SD rats were randomly divided into four groups of seven rats each. The treatment groups were given AF or BuF at a dose of 125 mg/kg twice a day for 14 days; the other two groups were untreated. On day 15, after overnight fasting, the AF and BuF-treated groups and their corresponding diabetic control

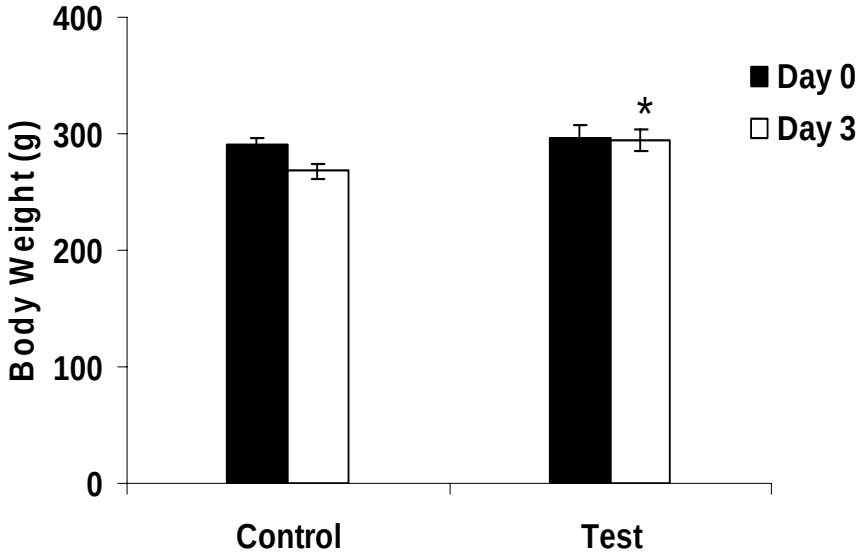

Figure 14. Effects of STZ on body weights in control and 1-week ABe-treated SD rats. Columns represent the mean ± SEM (n=7).

*P< 0.05 compared with control (Student's t-test).

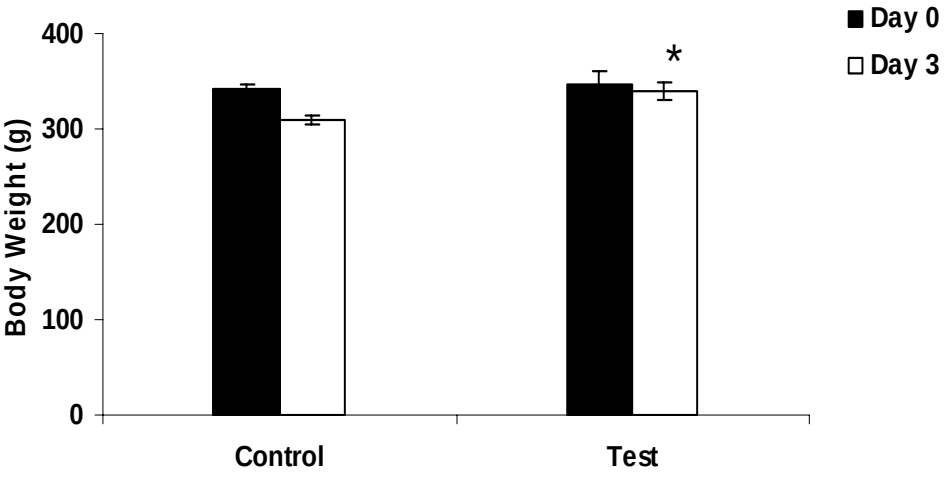

Figure 15. Effects of STZ on body weights in control and 2-week ABe-treated SD rats. Columns represent the mean ± SEM (n=7).

*P< 0.05 compared with control (Student's t-test).

group were injected intraperitoneally with a single dose of 60 mg/kg STZ. Another untreated group served as normal control. Fasting blood samples were collected on day -14 (before giving the AF and BuF), day 0 (before injecting the STZ) and on day 3 after injecting STZ, for the determination of serum glucose and insulin levels. 14 days after injecting STZ, all rats were fasted overnight and killed by decapitation. Blood samples were collected for determination of glucose and insulin levels. Pancreases were weighed and immediately frozen in liquid nitrogen and stored at –70°C for assays.

3.3 Results and discussion

The body weights of the control groups, on day 3 after STZ injection, were significantly lower than day 0 values ($P<0.05$), whereas there was no significant change in the ABe-pre-treated groups (Figure 14 & 15).

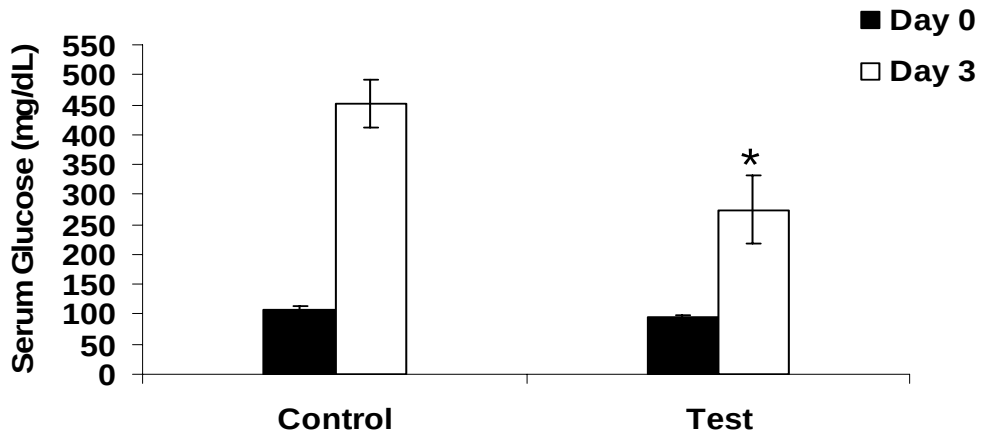

Figure 16. Effects of STZ on FBG levels in control and 1-week ABe-treated SD rats. Columns represent the mean ± SEM (n=7).

*P < 0.05 compared with control (Student's t-test).

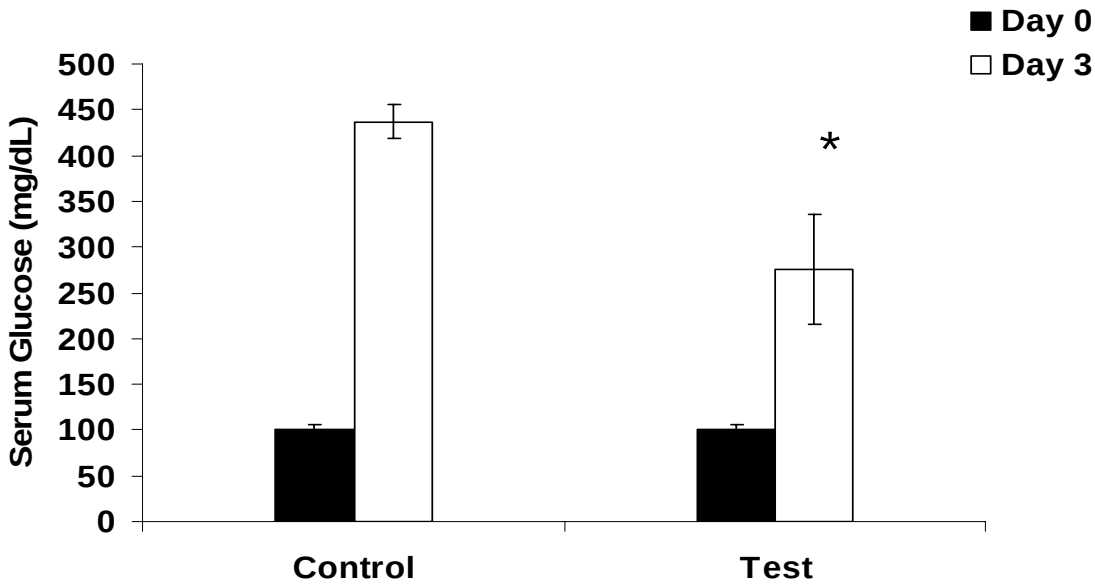

Figure 17. Effects of STZ on FBG levels in control and 2-week ABe-treated SD rats. Columns represent the mean ± SEM (n=7).

*P < 0.05 compared with control (Student's t-test).

As shown in Figure 16 & 17, the fasting serum glucose levels were significantly increased in both control as well as ABe-pre-treated groups on day 3 after STZ injection (both $P< 0.05$).

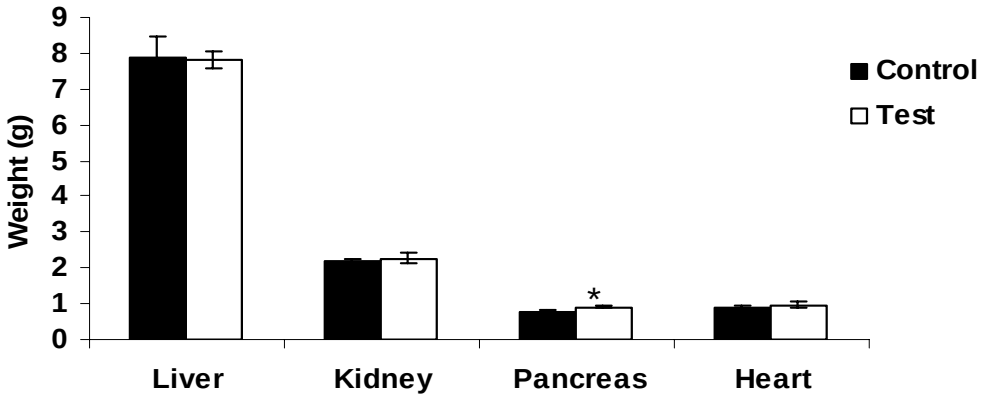

Figure 18. Effects of STZ on weights of vital organs in control and 1-week ABe-treated SD rats. Columns represent the mean ± SEM (n=7).

*P < 0.05 compared with control (Student's t-test).

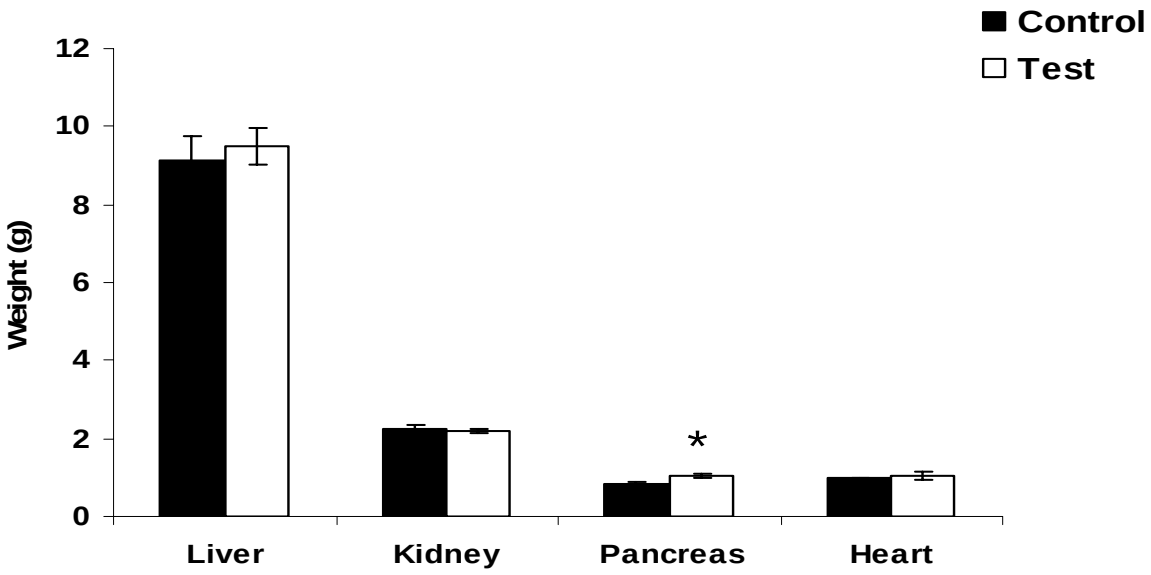

Figure 19. Effects of STZ on weights of vital organs in control and 2-week ABe-treated SD rats. Columns represent the mean ± SEM (n=7).

* P < 0.05 compared with control (Student's t-test).

The weights of the pancreases of both 1 and 2 week ABe pre-treated rats were significantly ($P < 0.05$) higher than in respective control groups (Figure 18 & 19).

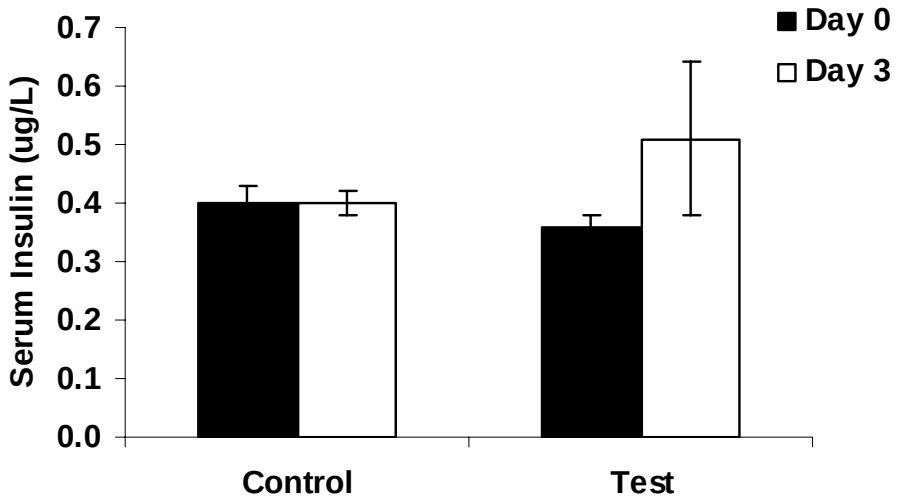

Figure 20. Effects of STZ on fasting serum insulin levels in control and 1-week ABe-treated SD rats. Columns represent the mean ± SEM (n=7).

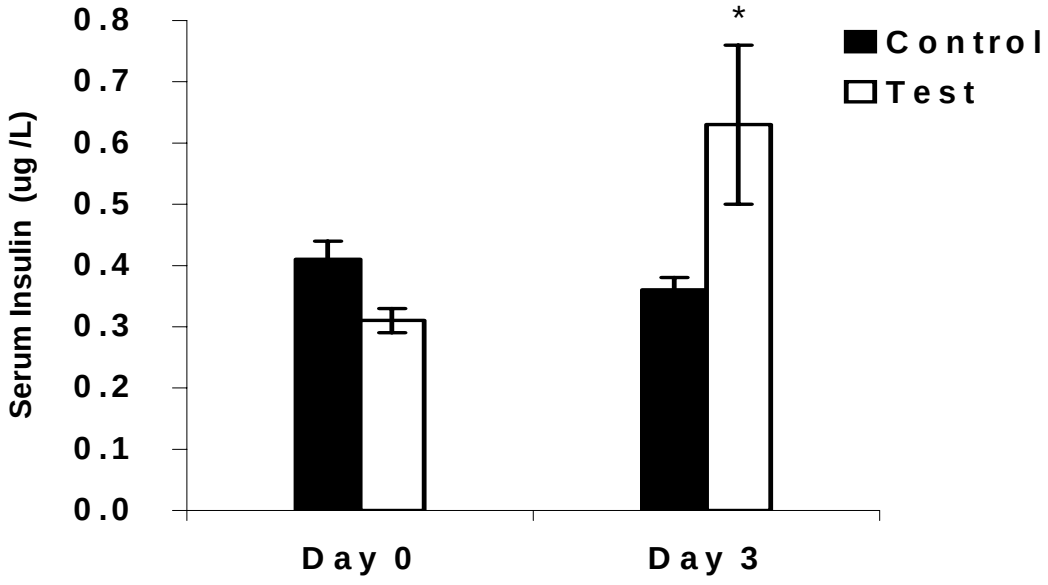

Figure 21. Effects of STZ on fasting serum insulin levels in control and 2-week ABe-treated SD rats. Columns represent the mean ± SEM (n=7).

The serum insulin content on day 3 after STZ injection was significantly higher ($P < 0.05$) in 2-week ABe-pre-treated group when compared to the control group. However there was about 20% increase in the serum insulin in the 1 week ABe pre-treated group even though no statistical significance was found (Figure 20 & 21). This indicates that ABe could protect the pancreatic β-cell destruction against STZ-toxicity. Hence the insulin level tends to be higher (~20%) than the control group.

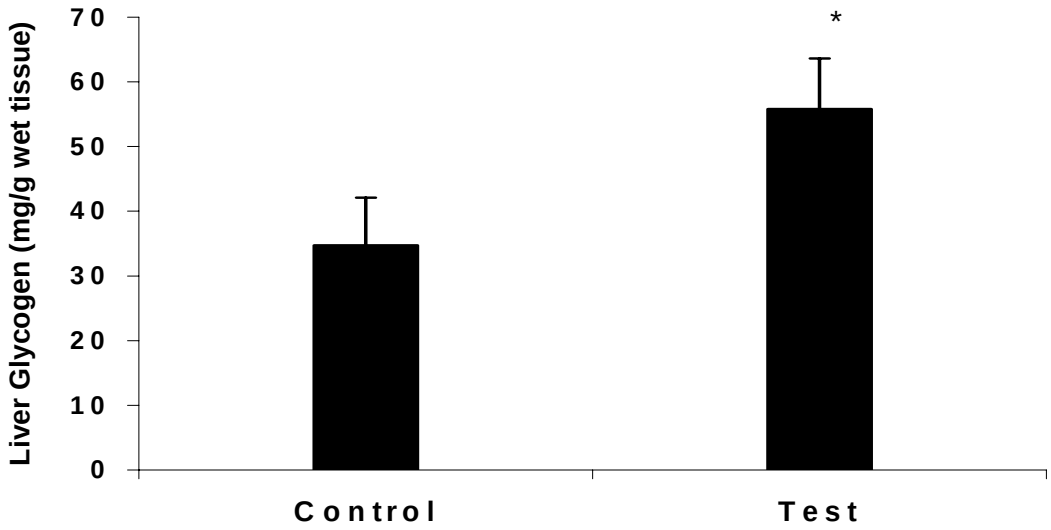

Figure 22. Effects of STZ on liver glycogen content in control and 1-week ABe-treated SD rats. Columns represent the mean ± SEM (n=7).

*P<0.05 compared with control (Student's t-test).

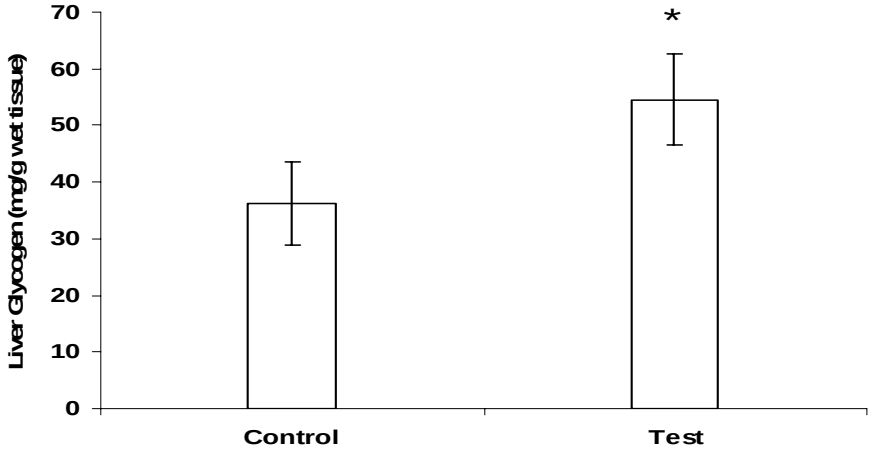

Figure 23. Effects of STZ on liver glycogen content in control and 2-week ABe-treated SD rats. Columns represent the mean ± SEM (n=7).

*P<0.05 compared with control (Student's t-test).

The hepatic glycogen content (Figure 22 & 23) was significantly higher ($P < 0.05$) than the control rats in both 1 and 2 week ABe pre-treated rats.

The fasting serum glucose levels were significantly increased in diabetic control group on 7 and 14 days after STZ injection (both $P < 0.001$), and also in the STZ-injected AF and BuF pre-treated groups as compared to normal control group (both $P < 0.05$) [Figure 24].

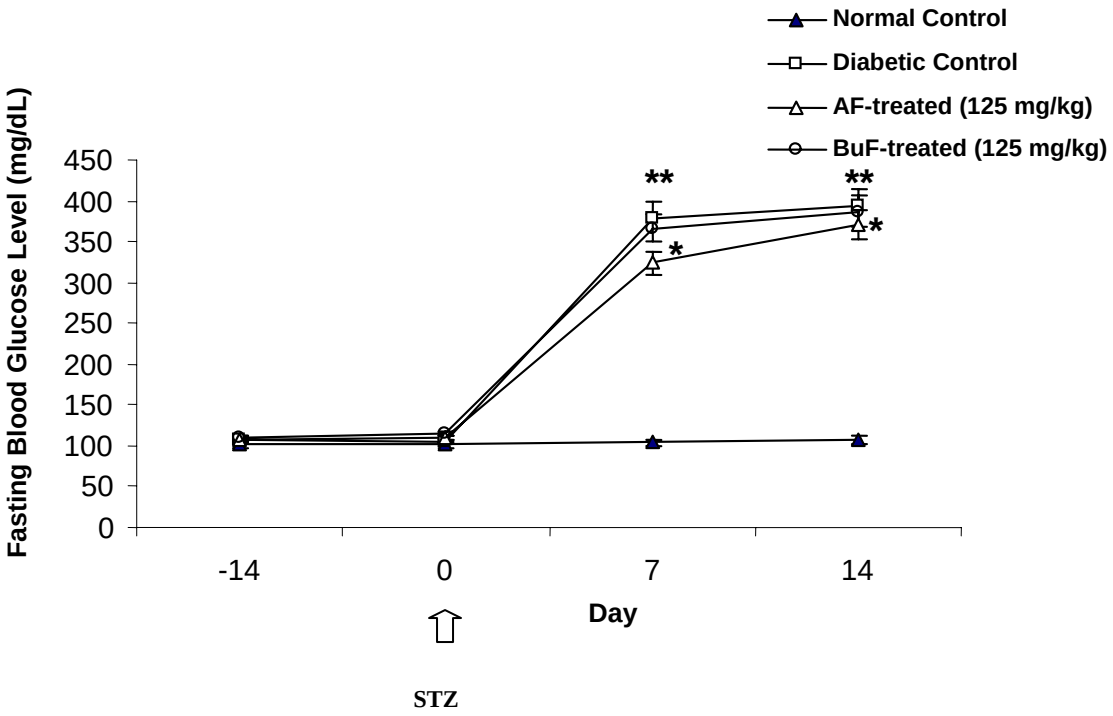

Figure 24. Effects of 14-day pre-treatment with AF and BuF at a dose of 125 mg/kg on fasting blood glucose levels on STZ-induced diabetic rats. Columns represent the mean ± SEM (n=7).

*$P < 0.05$ compared to the normal control (Student's t-test).
**$P < 0.001$ compared to the normal control (Student's t-test).

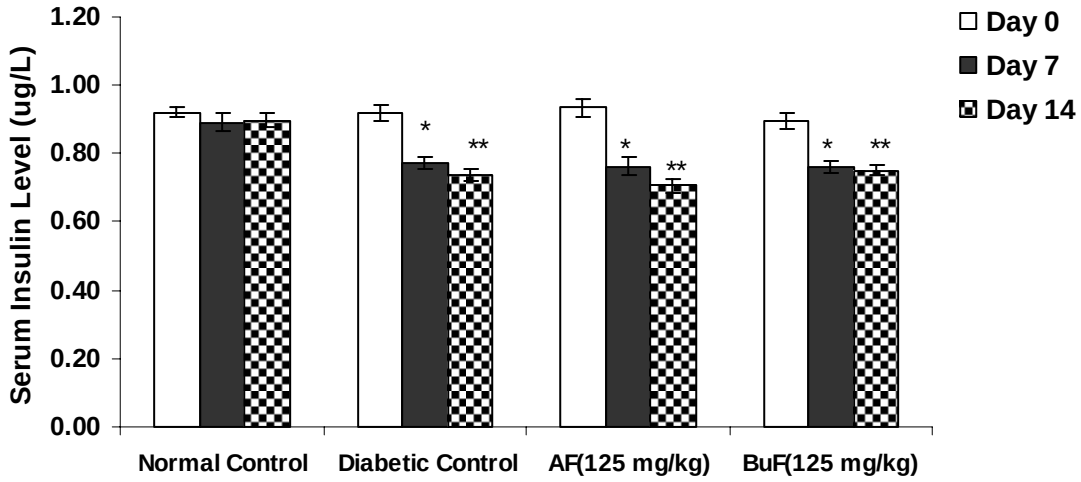

Figure 25. Effects of 14-day pre-treatment with AF and BuF at a dose of 125 mg/kg on serum insulin content in STZ-induced diabetic rats. Columns represent the mean ± SEM (n=7).

* P< 0.05 compared to the normal control (Student's t-test).
** P<0.001 compared to the normal control (Student's t-test).

The levels in the STZ-injected AF pre- treated group were much lower ($P<0.05$) than in the STZ-injected control group. As shown in Figure 25, the serum insulin level was decreased markedly in the STZ-injected control group on day 7 after STZ injection ($P < 0.001$), and this level was further decreased on day 14 ($P < 0.001$). However, there was a fall in serum insulin in all the three groups on day 7 and 14 compared to day 0. Hence, there was no difference in terms of percentage change between diabetic control, AF and BuF-pre-treated group. These results indicate that there is no much change in serum insulin observed in the AF and BuF pre-treated groups when compared to diabetic control.

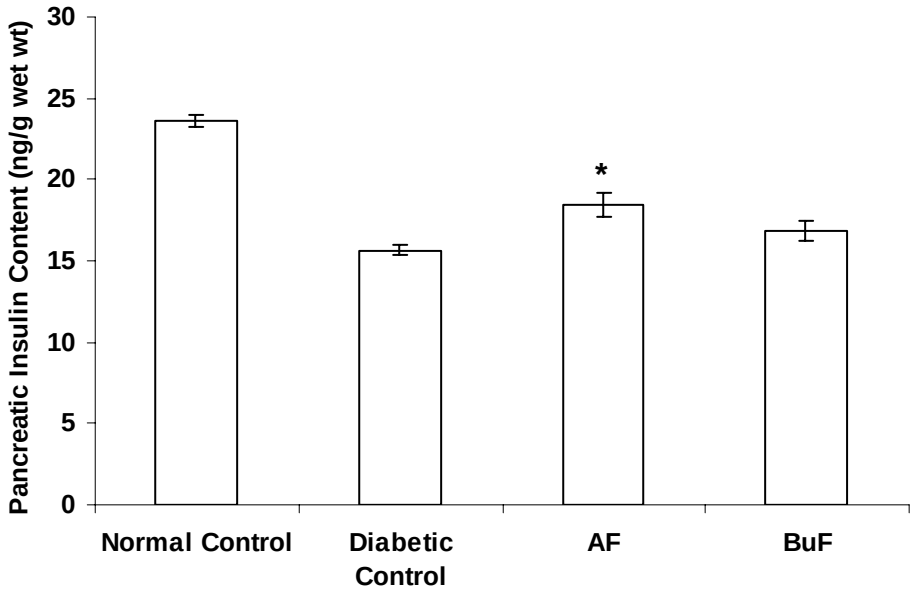

Figure 26. Effects of 14-day pre-treatment with AF and BuF at a dose of 125 mg/kg on pancreatic insulin content in STZ-induced diabetic rats. Columns represent the mean ± SEM (n=7).

***** P< 0.05 compared to the diabetic control (Student's t-test).

Pancreatic insulin content was decreased by 33.6% in control animals on day 7 after STZ injection (Figure 26). The levels in the STZ-injected AF and BuF pre-treated rats were decreased by 21.6% and 28.5% respectively compared to the normal control rats, but were markedly higher compared to levels in the STZ-injected control rats ($P < 0.05$).

As shown in Figure 27, pancreatic TBARS values were higher in the STZ-injected control, AF and BuF-treated groups than in the normal group on day 14 after STZ injection ($P < 0.05$). However there was about 7% reduction in TBARS in both AF and BuF-treated groups when compared to diabetic control group.

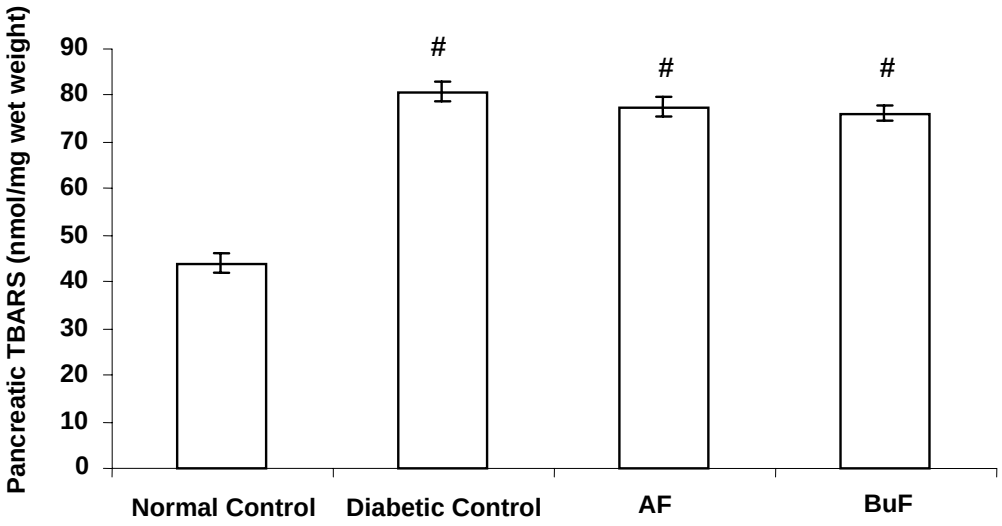

Figure 27. Effects of 14-day pre-treatment with AF and BuF at a dose of 125 mg/kg on pancreatic TBARS in STZ-induced diabetic rats. Columns represent the mean ± SEM (n=7).

# $P< 0.05$ compared with normal control (Student's t-test).

The diabetogenic action of STZ in a single high dose (60 mg/kg) injection destroys most of the β-islet cells of pancreas. Thus high dose STZ-induced diabetes is an animal model of IDDM. The 2 weeks pre-treatment with ABe caused a significant attenuation of the severity of high dose STZ-induced diabetes when compared to the 1 week pre-treatment. Similarly, pretreatment with the semi-purified fraction for 2 weeks AF could attenuate the severity of high dose STZ-induced diabetes. Both acute and chronic elevations in glucose concentration stimulate the rate of insulin biosynthesis. Acute stimulation results in increased insulin at the translational level (Itoh and Okamoto, 1980), whereas chronic stimulation enhances the formation and/or stability of insulin mRNA (Giddings, 1985). So a steady-state insulin mRNA level is important for regulation of insulin production (Permutt, 1984). Two non-allelic insulin genes (insulin I and insulin II) have been identified in rats (Clark, 1969; Lomedico, 1979). The variation was taken as evidence for an independent regulation of the two genes, whereby the insulin I gene is preferentially expressed under stimulatory conditions (Kakita, 1982). Ling et al., (1998) noted that an elevated ratio of insulin I over insulin II in pancreatic tissue is a marker for a prolonged exposure to elevated glucose levels, since there is a transcriptional and/or a posttranscriptional failure in elevating insulin II formation while insulin I production is stimulated in the glucose-activated β-cells. The β-cells in the diabetic control group could not synthesize enough insulin products under stimulated conditions. This was confirmed by the finding that the pancreatic insulin content was significantly lower in the diabetic control group as compared to the normal group. However the pancreatic insulin level was

significantly higher than the diabetic control group in the AF-pretreated group. Moreover, AF-pretreatment causes better preservation of pancreatic insulin content than BuF. These findings showed that the pre-treatment of ABe and AF at a dose of 125 mg/kg can protect β-cells against STZ.

A glucose molecule with a highly reactive nitrosourea side-chain in STZ is considered to initiate its cytotoxic action as this glucose moiety binds to a membrane receptor, which most likely is a glucose transporter, to cause structural damage (Rodrigues, 1999). Some works have previously reported that various carbohydrate compounds, such as 5-thio-D-glucose (Wang et al, 1993), 3-*O*-methyl-D-glucose (Ganda et al., 1976), 2-deoxy-D-glucose (Dulin and Wyse, 1969) and 4, 6-O-ethylidene glucose (Kawada et al., 1987), protected against the diabetogenic effect of a single high dose of STZ.

The methyl-nitrosourea moiety of STZ can cause DNA breaks by alkylating DNA bases at various positions and lead to profound NAD depletion linked to stimulation of the activity of the nuclear enzyme, poly (ADP-ribose) synthetase, for the excision and repair of the broken DNA strands (Yamamoto et al., 1981). These are responsible for the deterioration in insulin synthesis and secretion (Okamoto et al., 1996). Therefore, poly (ADP-ribose) synthetase inhibitors such as nicotinamide and 3-aminobenzamide could prevent the onset of diabetes (Uchigata et al., 1983). Whether ABe and AF can inhibit poly (ADP-ribose) synthetase activity to protect β-cells from NAD^+ depletion is not clear .and further

experiments are necessary to confirm it. It has been proposed that oxygen free radicals are involved secondarily in the mechanism of action of STZ (Baynes, 1991). Gandy et al (1982) found that hydrogen peroxide was produced in pancreatic islets upon STZ exposure *in vivo*.

Some free radical scavengers, such as nickel chloride ($NiCl_2$) (Novelli, 1988) and metallothionein (Yang and Cherian, 1994), have been demonstrated to provide some protection against the diabetogenic properties of STZ. In this study, the finding of a markedly increased pancreatic lipid peroxidation level in the diabetic control group is in agreement with the previous report of Yang and Cherian (1994). The percentage of reduction in TBARS in the AF and BuF-pre-treated groups were about 7%. However the pancreatic insulin content in the AF and BuF-pre-treated groups were 18% and 10% respectively. Based on the increase in pancreatic insulin content in the AF-pre-treated group as well as partial reduction in the TBARS when compared to diabetic control group, we can conclude that AF-treatment could protect pancreatic beta cells against streptozotocin toxicity better than BuF.

Experiment 4: Evaluation of the anti-diabetic effects of AF and BuF in a rat model of type 2 diabetes

4.1 Aims

In the present study, the antidiabetic effects of AF (125 mg/kg) and BuF (125 mg/kg) were evaluated in a rat model of type 2 diabetes.

4.2 Experimental procedure

4.2.1 Induction of type 2 diabetes mellitus in SD Rats

Diabetes was induced according to Method 2.3 (refer to page 31)

4.2.2 The OGTT in HFD-STZ - diabetic SD rats treated with semi-purified fractions of ABe

Prior to OGTT, HFD-STZ-diabetic rats were fasted for 16 h. Distilled water (Control), Four different fractions of ABe viz., AF, BuF, EF, and HF each at a dose of 125 mg/kg…….(as done previously in Experiment 2.2.1, page 58).

4.2.3 Twice daily administration of AF and BuF in HFD-STZ-diabetic SD rats

The HFD fed-STZ-diabetic rats (blood glucose >350 mg/dl) were divided on day zero into four groups (each with 9 rats). The fasting blood glucose level was measured on day zero at 9.00 am. Distilled water, AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg) were then administered orally twice a day at 9.00 am and 9.00 pm to diabetic control, treatment and positive control groups respectively for 2 weeks. Body weight, food and water intakes were monitored

every day between 9.00 and 10.00 am for 2 weeks. On the morning of the 15th day, after overnight fasting, the rats were decapitated and the blood was collected for estimation of the FBG, TC, TG, and HDL-C. The organs such as liver and kidney were isolated, weighed and stored at -70°C for the assay of hepatic Glc-6-Pase, glycogen, cytochrome P_{450} and TBARS in both liver and kidney.

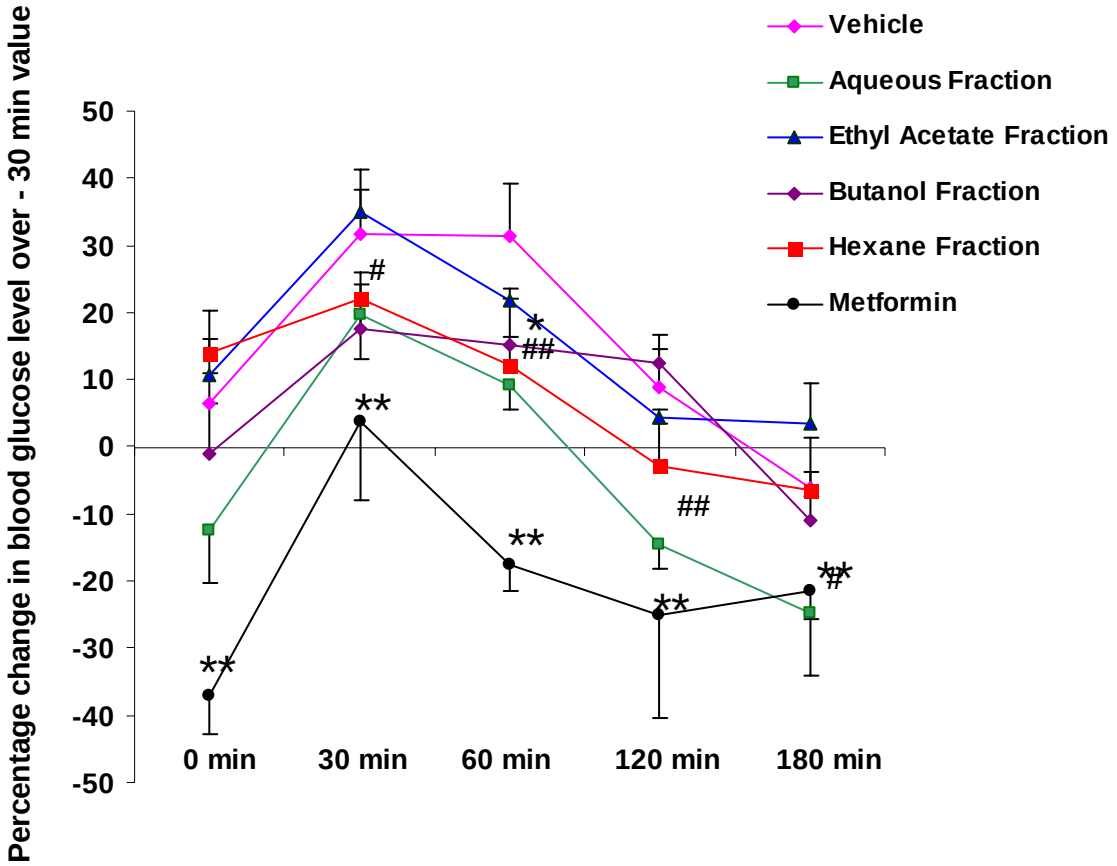

Figure 28. Effects of the semi-purified fractions of ABe on OGTT in HFD-STZ-diabetic rats. The graph represents the mean percentage changes in blood glucose concentration over – 30 min level. The bars represent SEM (n = 6). The oral glucose tolerance test (OGTT) in HFD-STZ-diabetic rats using the semi-purified fractions of *A.bilimbi* leaf (ABe). The graph represents the mean percentage changes in blood glucose concentration over – 30 min level in vehicle (465 ± 25), Aqueous Fraction [AF] (446± 34), Butanol Fraction [BuF] (510 ± 42), Ethyl acetate Fraction [EF] (467 ± 24), and Hexane Fraction [HF] (526 ± 21) of ABe, each at a dose of 125 mg/kg and metformin (522 ± 37)–treated (500 mg/kg) diabetic rats, while bars represent SEM (n = 5-6). The blood glucose concentration (mg/dL) of each group at –30 min is given in brackets

*P<0.05 BuF-treated group vs vehicle-treated group (Student's t-test).
**P<0.01 Metformin-treated group vs vehicle-treated group (Student's t-test).
#P<0.05 AF-treated group vs vehicle-treated group (Student's t-test).
P<0.01 AF-treated group vs vehicle-treated group (Student's t-test).

4.3 Results and discussion

The HFD-STZ-induced diabetic rat is one of the animal models of human NIDDM or type 2 diabetes mellitus (Reed et al., 1999). The present study revealed that AF and BuF have potent hypoglycemic and hypotriglyceridemic properties when given for 2 weeks to HFD-STZ-diabetic rats. In the OGTT (Figure 28), AF (125 mg/kg) caused a significant hypoglycemic effect at 30 min, 60 min, 120-min and 180 min when compared with vehicle control. BuF (125 mg/kg) had no significant effect on blood glucose except at 60 min after oral administration ($P<0.05$). The other two-fractions, EF and HF did not cause any reduction in blood glucose level at any time point.

Table 7. Body weight, water and food intakes in HFD-STZ-diabetic rats before and after oral treatment with vehicle, AF, BuF, and metformin twice a day for 2 weeks

Treatment group (n = 9)	Body weight (g) (mean ± SEM)		Water (mL/rat/day) (mean ± SEM)		Food (g/rat/day) (mean ± SEM)	
	Before	After ¶	Before	After ¶	Before	After ¶
Control	224 ± 5	239 ± 11 (7)	176 ± 21	186 ± 16 (6)	42 ± 4	60 ± 5 (43)
AF	225 ± 6	246 ± 19 (9)	148 ± 29	157 ± 24 (6)	35 ± 5	43 ± 4 (23)
BuF	231 ± 11	253 ± 25 (9)	155 ± 12	178 ± 15 (14)	45 ± 3	46 ± 4 (2)
Metformin	226 ± 9	247 ± 14 (9)	163 ± 14	167 ± 16 (2)	42 ± 2	43 ± 3* (2)

¶The percentage change from day 0 is indicated in the brackets.
*P<0.001 compared with vehicle-treated rats (two-way ANOVA).

The body weight, food and water intakes of the rats did not differ significantly in AF- and BuF-treated diabetic rats (Table 7). However the change in mean body weight, food and water intakes in AF-treated group were 9%, 23% and 6% and in BuF-treated group were 9%, 2% and 15% respectively when compared to the day 0 values. On the other hand, the change in mean body weight, food and water intakes in diabetic control group were 6%, 5% and 43% when compared to the day 0 values. The water intake in the AF and BuF-treated groups were much lower when compared to the diabetic control group. However the water intake in the BuF-treated group was much higher than the AF-treated group. The control of blood glucose level in the AF-treated group could have been the reason for the better control of polydipsia than the BuF-treated group.

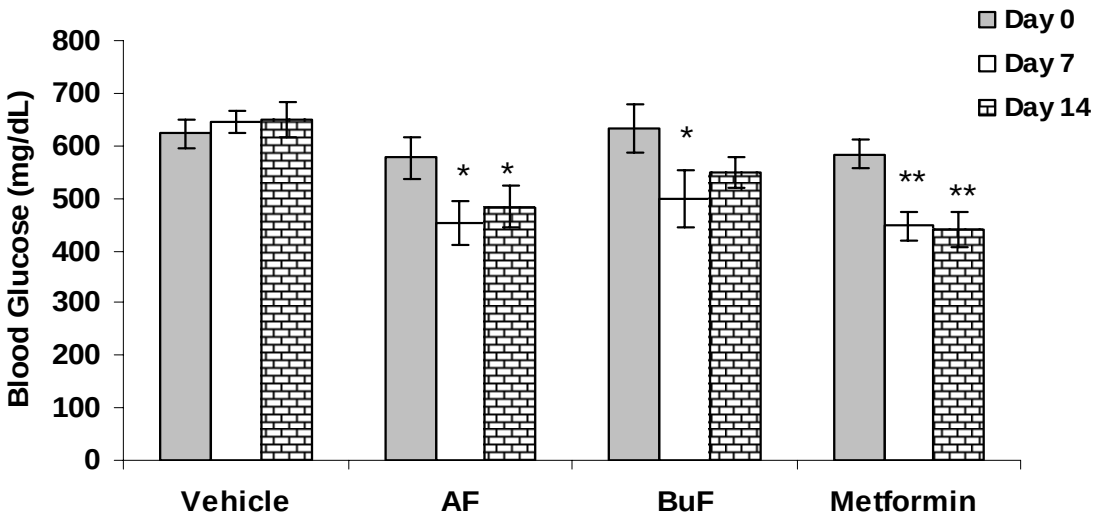

Figure 29. Effects of the semi-purified fractions of ABe, AF and BuF, on FBG levels in HFD-STZ-diabetic rats.
The FBG levels were measured on day 0, day 7, and day 14 at 9.00 a.m. after a 16-hour fast in the vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg)-treated HFD-fed STZ-diabetic rats. Columns represent the mean ± SEM (n = 9).

*P<0.05 compared with the diabetic untreated rats (Student's t-test).
**P<0.01 compared with the diabetic untreated rats (Student's t-test).

AF caused a significant ($P<0.01$) time-dependent hypoglycemic effect (Figure 29) after twice-daily oral administration of 125 mg/kg BW for 7 and 14 days. BuF also showed a significant ($P<0.05$) hypoglycemic property on day 7 as well as on day 14 compared to the vehicle-treated control group (Figure 29).

Both AF and BuF administration significantly (P<0.01) reduced the serum triglycerides (Figure 30) on day 7 and 14. The reduction in the serum TG after daily administration of AF (125 mg/kg) and BuF (125 mg/kg) to HFD-STZ-diabetic rats twice a day for two weeks could be due to the reduction of serum non-esterified fatty acids (NEFA) in the HFD-STZ-diabetic rats similar to masoprocol (nordihydroguaiaretic acid), a pure compound isolated from *Larrea tridentata* (Reed et al., 1999).

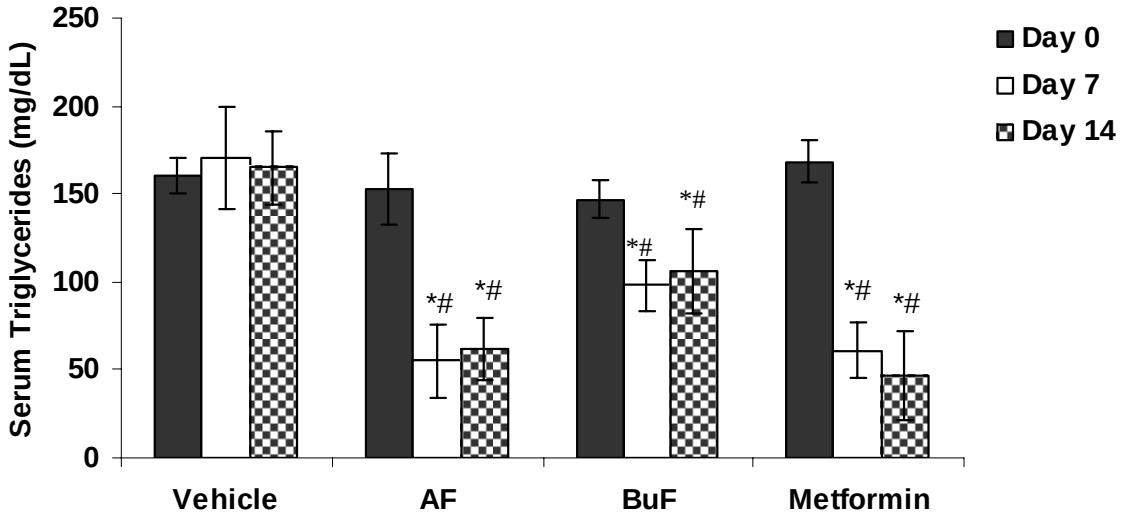

Figure 30. Effects of the semi-purified fractions of ABe, AF and BuF, on the serum TG levels in HFD-STZ- diabetic rats.
The serum TG levels were measured on day 0, day 7, and day 14 at 9.00 a.m. after a 16-hour fast in the vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg)-treated HFD-fed STZ-diabetic rats.Columns represent the mean ±SEM(n =9).

*P<0.01 compared with control rats (Student's t-test).
#P<0.01 compared with day 0 value (Student's t-test).

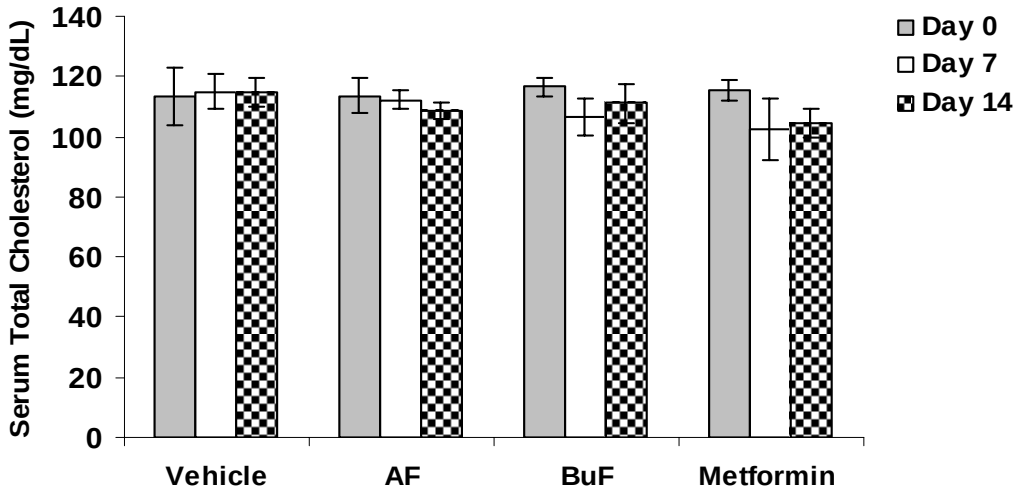

Figure 31. Effects of the semi-purified fractions of ABe, AF and BuF, on serum TC levels in HFD-STZ- diabetic rats.
The serum TC levels were measured on day 0, day 7, and day 14 at 9.00 a.m. after a 16-hour fast in the vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and Metformin (500 mg/kg)-treated HFD-fed STZ-diabetic rats. Columns represent the mean ± SEM (n = 9).

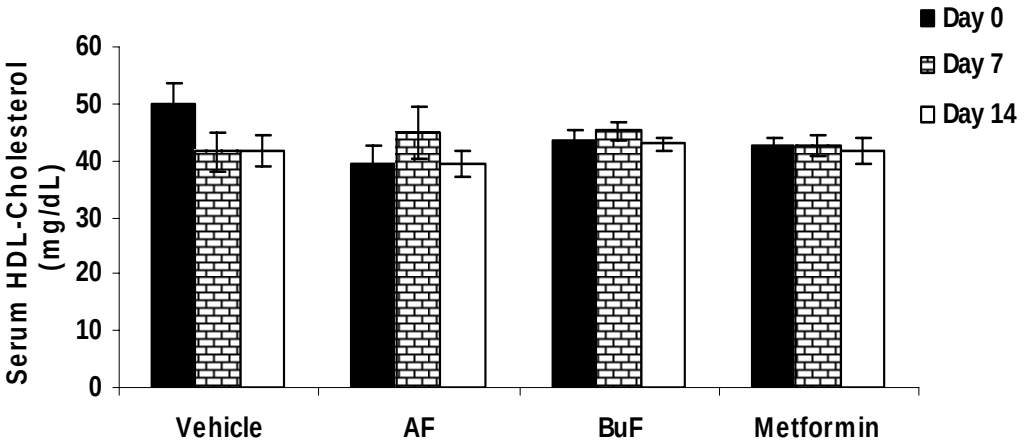

Figure 32. Effects of the semi-purified fractions of ABe, AF and BuF, on the serum HDL-C levels in HFD-STZ- diabetic rats.
The serum HDL-C levels were measured on day 0, day 7, and day 14 at 9.00 a.m. after a 16-hour fast in the vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg)-treated HFD-fed STZ-diabetic rats. Columns represent the mean ± SEM (n = 9).

However there was no significant change in serum TC (Figure 31) and HDL-C content (Figure 32).

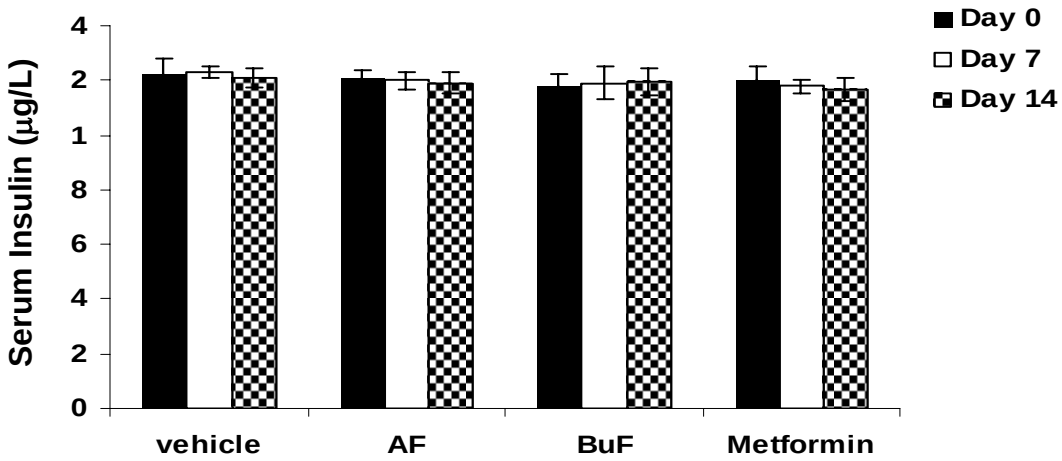

Figure 33. Effects of the semi-purified fractions of ABe, AF and BuF on the serum insulin levels in HFD-STZ- diabetic rats.
The serum insulin levels were measured on day 0, day 7, and day 14 at 9.00 a.m. after a 16-hour fast in the vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg)-treated HFD-fed STZ-diabetic rats. Columns represent the mean ± SEM (n = 9).

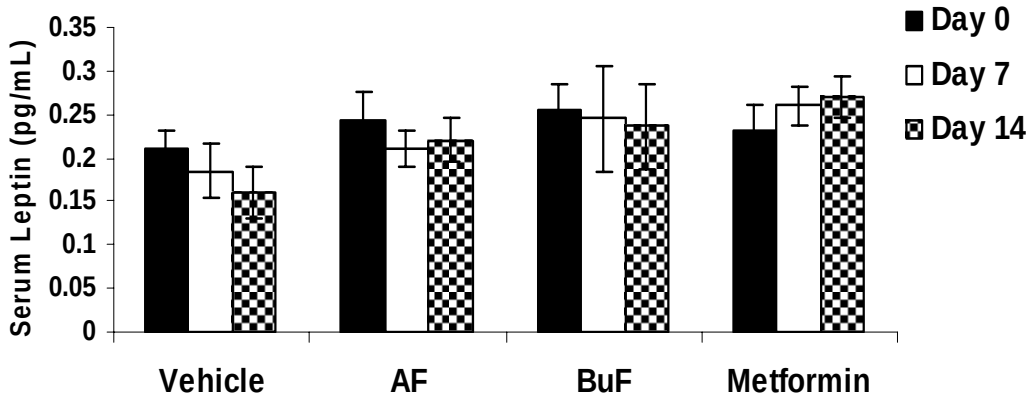

Figure 34. Effects of the semi-purified fractions of ABe, AF and BuF on the serum leptin levels in HFD-STZ- diabetic rats.
The serum leptin levels were measured on day 0, day 7, and day 14 at 9.00 a.m. after a 16-hour fast in the vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg)-treated HFD-fed STZ-diabetic rats. Columns represent the mean ± SEM (n = 9).

Moreover, the serum insulin (Figure 33) and leptin levels (Figure 34) were not significantly influenced either by the fractions or metformin.

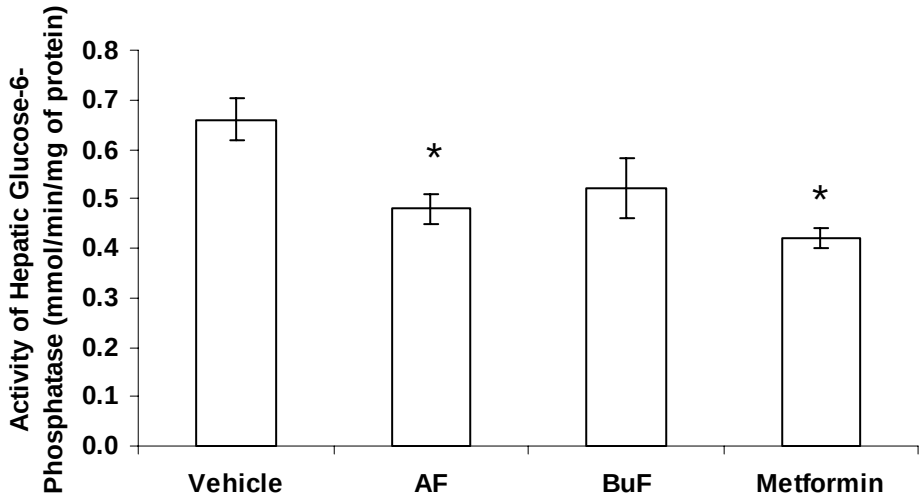

Figure 35. Effects of the semi-purified fractions of ABe, AF and BuF, on the Glc-6-Pase activity in HFD-STZ- diabetic rats.
The Glc-6-Pase activity in the vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg)-treated HFD-fed STZ-diabetic rats. Columns represent the mean ± SEM (n = 9).

*P<0.05 compared with the diabetic untreated rats (Student's t-test).

In this study, Glc-6-Pase activity (Figure 35) in the liver was significantly reduced ($P<0.05$) in both AF- and metformin-treated groups when compared to the vehicle-treated diabetic control group. However, there was no significant change in the Glc-6-Pase activity of BuF –treated group. Excessive production of glucose is the major cause of fasting hyperglycemia in human (Bell et al., 1986; Defronzo, 1988) and experimental diabetes mellitus (Chen et al., 1975; Rossetti et al., 1993). The hydrolysis of hepatic Glc-6-Pase is the “final common pathway” for the release of glucose into the circulation. Recent experimental evidence supports the notion that this final step is rate-determining for the increased rate of hepatic glucose output in diabetic states.

In fact, marked changes in the rate of formation of hepatic Glc-6-P through gluconeogenesis fail to alter hepatic glucose production (Puhakainen et al., 1991), and in experimental diabetes hepatic glucose production is markedly elevated in the presence of a significant *decrease* in the hepatic Glc-6-P pool (Rossetti et al., 1993; Barzilai and Rossetti, 1993). Haber et al. (1995) have recently reported a marked increase in hepatic Glc-6-Pase mRNA and protein in diabetic BB rats. In the same study, *in vivo* treatment with 0.5 unit of insulin normalized the plasma glucose concentration and the hepatic Glc-6-Pase mRNA levels in diabetic rats within 4 h. Similarly, Liu *et al.* (15) have shown increased hepatic Glc-6-Pase mRNA and activity in STZ-induced diabetic rats. Massillon et al. (1996) confirmed that prolonged insulin deficiency and hyperglycemia (experimental diabetes) cause a marked increase in the hepatic Glc-6-Pase mRNA and protein and indicate that short-term (8 h) correction of hyperglycemia in diabetic rats leads

to normalization of the hepatic gene expression of this enzyme, regardless of the circulating insulin concentrations. These studies confirmed that *in vivo* gene expression of Glc-6-Pase in the diabetic liver is regulated by glucose independent of insulin.

It has been documented that consumption of high fat or high simple sugar diets can lead to insulin resistance in rats (Grundlege and Thenen, 1982; Storlien et al., 1991; Storlien et al., 1993). Kragen et al. (1991) reported that high fat feeding induced insulin resistance initially in rat liver and adipose tissues, followed by impaired glucose metabolism in skeletal tissues. The rats fed high simple carbohydrate or high fat diets had significantly higher fasting plasma insulin and glucose levels compared to control animals, characteristic symptoms of insulin resistance. The nutritional state is a factor known to affect hepatic glycogen levels and the glycogenolytic state, as seen in livers from fasted, fed and fasted-refed rats (Nur et al., 1995; Tosh et al., 1994). In comparison to control animals, hepatocytes from rats fed high simple sugar or high fat diets had significantly decreased activated glycogen synthase activity. This was true for both basal GS activity and activity stimulated by insulin. A significant inhibition of glucose incorporation into glycogen was observed in rats fed a high fat diet (Oakes et al., 1997). These results support the findings that high fat and high simple sugar diets lead to insulin resistance as observed in lower rates of both GS activity and expression than in control animals. Hepatic insulin resistance is a major defect of non-insulin dependent diabetes mellitus. It has been previously demonstrated that glycogen

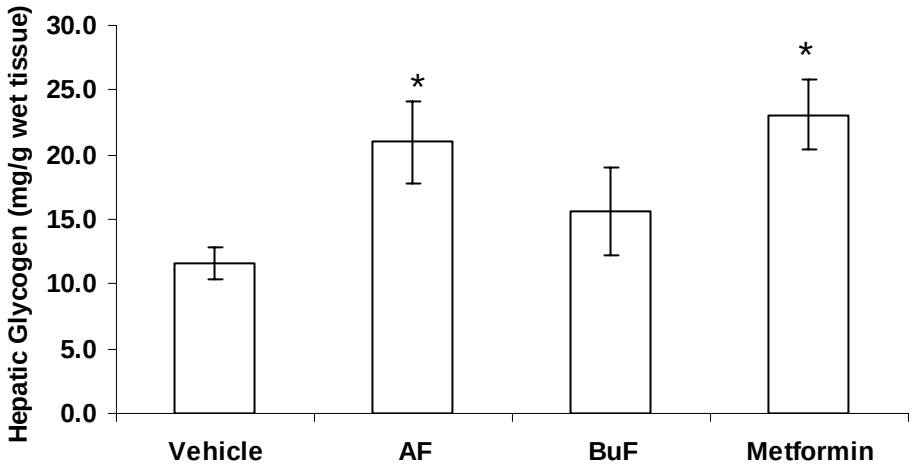

Figure 36. Effects of the semi-purified fractions of ABe, AF and BuF, on hepatic glycogen content in HFD-STZ- diabetic rats.
The hepatic glycogen content in the vehicle (distilled water), AF (125 mg/kg), BuF (125 mg/kg) and metformin (500 mg/kg)-treated HFD-fed STZ-diabetic rats. Columns represent the mean ± SEM (n = 9).

*P<0.05 compared with the diabetic untreated rats (Student's t-test).

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deposition from glucose is impaired in diabetic animals (Ciudad et al., 1988; Bollen et al., 1998).

Glycogen deposition was also impaired in diabetic rats where the activation of glycogen synthase is either impaired or absent, in proportion to the severity of insulin deficiency (Langdon and Curnow, 1980). In this study, the liver glycogen content was markedly lower in diabetic control rats whereas the glycogen content was significantly higher in AF-treated rats (Figure 36)

Insulin regulates blood glucose homeostasis by stimulating the utilization of glucose by the liver, muscle and adipose tissue. In the liver, insulin stimulates glycogen synthesis, glycolysis and fatty acid synthesis, but unlike muscle and adipose tissue, it does not stimulate glucose transport. The conversion of glucose to glycogen in liver cells is dependent on the extracellular glucose concentration and on the presence of insulin, which stimulates glycogen synthesis over a wide range of glucose concentrations (Stalmans et al., 1997). Hepatic glycogen synthase, the rate-limiting enzyme of the glycogen synthetic pathway, is controlled by intricate mechanisms involving multisite phosphorylation as well as allosteric regulation (Wititsuwannakul and Kim, 1979; Nuttall et al., 1988). Its activity is increased in liver from diabetic rats (Bahnak and Gold, 1982; Niewoehner et al., 1986). This increase in activity could be due to an increased mass of the enzyme or to the presence of a more catalytically efficient form(s) of the enzyme. Although total activity may be enhanced in diabetic animals, the percent of activated GSI was significantly lower compared to hepatocytes from healthy control animals

(Gannon and Nuttall., 1997). Conflicting data has been reported by other investigators. Rao et al. (1995) reported no change in total glycogen synthase activity in livers of diabetic rats, but a significantly lower GSI activity. Recently, Wang et al. (1998) reported that both total GS and GSI activity were significantly lower in livers of STZ-diabetic rats. In primary hepatocyte culture isolated from normal and diabetic adult rats, it was shown that both glucose and insulin activate glycogen synthesis in primary culture hepatocytes from normal but not diabetic rats (Miller et al., 1986). Van Auken et al. (1996) reported that total GS activity in the diabetic cells at physiologic glucose concentrations was significantly higher than that for normal cells; however, the amount of active synthase was twofold lower than that of normal cells. Additionally, in normal hepatocytes, chronic (48 h) exposure to increasing concentrations of glucose was found to up-regulate total synthase activity, synthase protein and synthase mRNA levels. All three of these responses were lost in hepatocytes from diabetic animals.

Table 8. Liver cytochrome P_{450} content and TBARS levels in the kidney and liver of HFD-STZ-diabetic rats after twice-a-day oral treatment for 2 weeks with vehicle, AF, BuF, and metformin

Treatment group (n = 9)	TBARS level (nmol of malonaldehyde per 25 mg of tissue)		Hepatic cytochrome P_{450} content (nmol/mg protein) (mean ± SEM)
	Liver (mean ± SEM)	Kidney (mean ± SEM)	
Vehicle	4.5 ± 0.4	4.8± 0.3	1.34 ± 0.02
AF	4.3± 0.3	4.5± 0.6	1.28 ± 0.06
BuF	4.2 ± 0.3	4.3 ± 0.3	1.35 ± 0.09
Metformin	4.6 ± 0.2	4.2 ± 0.3	1.22 ± 0.07*

*P<0.05 compared with vehicle-treated rats (Student's t-test).

The kidney TBARS in metformin-treated diabetic rats were significantly lower than ($P < 0.05$) in the vehicle-treated rats (Table 8). On the other hand, the kidney TBARS value in AF and BuF-treated rats were not significantly different from that in the vehicle-treated rats. However the percentage of reduction in kidney TBARS was about 6% and 11% in AF and BuF-treated groups respectively when compared to the vehicle-treated control group. Similarly, there was no difference in liver TBARS values between AF, BuF and metformin-treated rats and vehicle-treated control rats. However the percentage of reduction in liver TBARS was about 6% and 7% in AF and BuF-treated groups when compared to the vehicle-treated rats. However there was about 2% increase in TBARS in metformin-treated group when compared to vehicle-treated rats. Based on the observation, the BuF reduced the TBARS better than AF. The liver microsomal cytochrome P450 content was significantly lower in the metformin-treated rats when compared to that in the corresponding vehicle-treated rats. However, there was no significant difference in the liver cytochrome P450 content in AF- and BuF-treated rats when compared with that in the corresponding vehicle-treated rats. Similar effects on kidney and liver TBARS and liver microsomal P450 content were produced by AF and BuF in the type 1 rat model of diabetes (refer to Experiment 2).

Experiment 5: Identification of bioactive principle (s) in ABe, AF and BuF

5.1 Aims

In this study, ABe, AF and BuF were analyzed by RP-HPLC and AAS for the presence of anti-diabetic principle (s). Its effects on glucose tolerance in animals with experimental diabetes mellitus were evaluated.

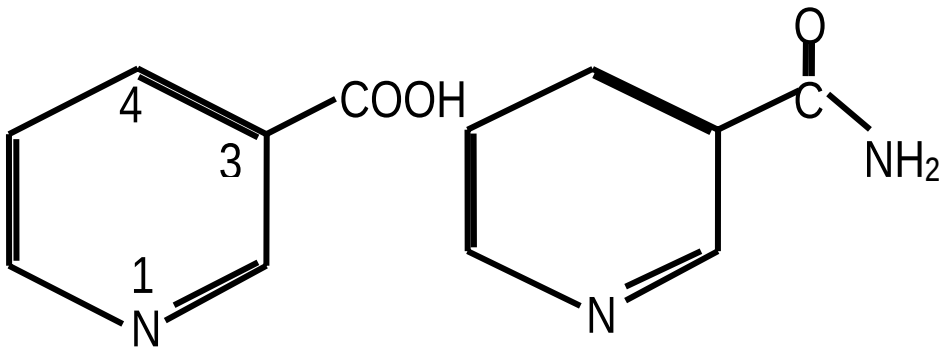
5.2 Experimental procedure

5.2.1 RP-HPLC of ABe, AF and BuF

The RP-HPLC was done according to the procedure described in Method 2.20 (page 40).

5.2.2. AAS analysis of ABe, AF and BuF

The AAS analysis of ABe, AF, and BuF was done according to the procedure described in Method 2.21 (page 41).



Nicotinic Acid Nicotinamide

Figure 37. Molecular structure of nicotinic acid (NA) and niacinamide (NAM).

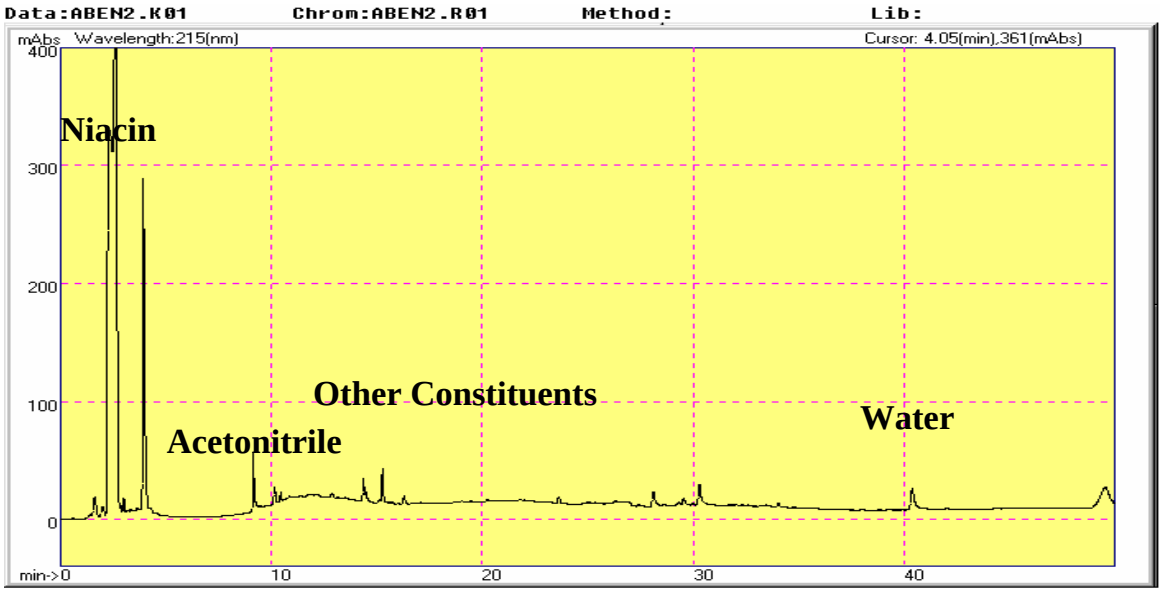


Figure 38. RP-HPLC finger print of ABe.

5.3. Results and discussion

Nicotinic acid (NA) and nicotinamide (NAM) are commonly called niacin (Figure 37). NA is the predominant form of niacin found in plant products. In our studies, the RP-HPLC analysis of the 80% ethanolic leaf extract of *A. bilimbi* (ABe), as well as AF and BuF showed the presence of nicotinic acid or niacin (Figure 38, 39 & 40) at the amount of 174.4 ± 1.8, 73.9 ± 2.7, and 55.8 ± 2.1mg/g respectively (Table 9). NA and NAM are the dietary precursors for NAD (nicotinamide adenine dinucleotide), which is required for DNA synthesis, as well as for the activity of the enzyme poly (ADP-ribose) polymerase-1 (PARP-1) for which NAD is the sole substrate. The enzyme PARP-1 is highly activated by DNA strand breaks during the cellular genotoxic stress response, is involved in base excision repair, plays a role in p53 expression and activation, and hence, is thought to be important for genomic stability. *In vitro* as well as animal studies indicate that niacin deficiency increases genomic instability especially in combination with genotoxic and oxidative stress. NA and NAM are rapidly absorbed from the stomach and the intestine. Within normal dietary intakes, most of the dietary NA is converted to NAD in the intestine or liver, and is cleaved by NAD glycohydrolase to release NAM into the portal or systemic circulation. NAM is the major form in the bloodstream and is obtained from NAD and NADP in animal products in the diet (Hageman and Stierum, 2001).

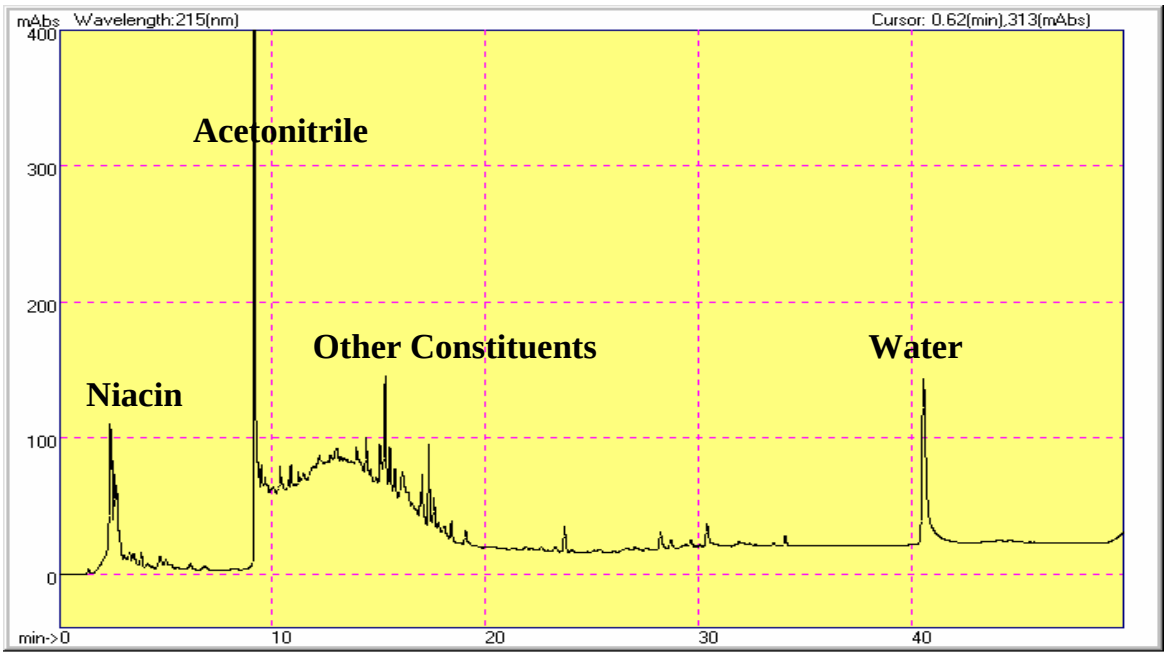


Figure 39. RP-HPLC finger print of AF of ABe.

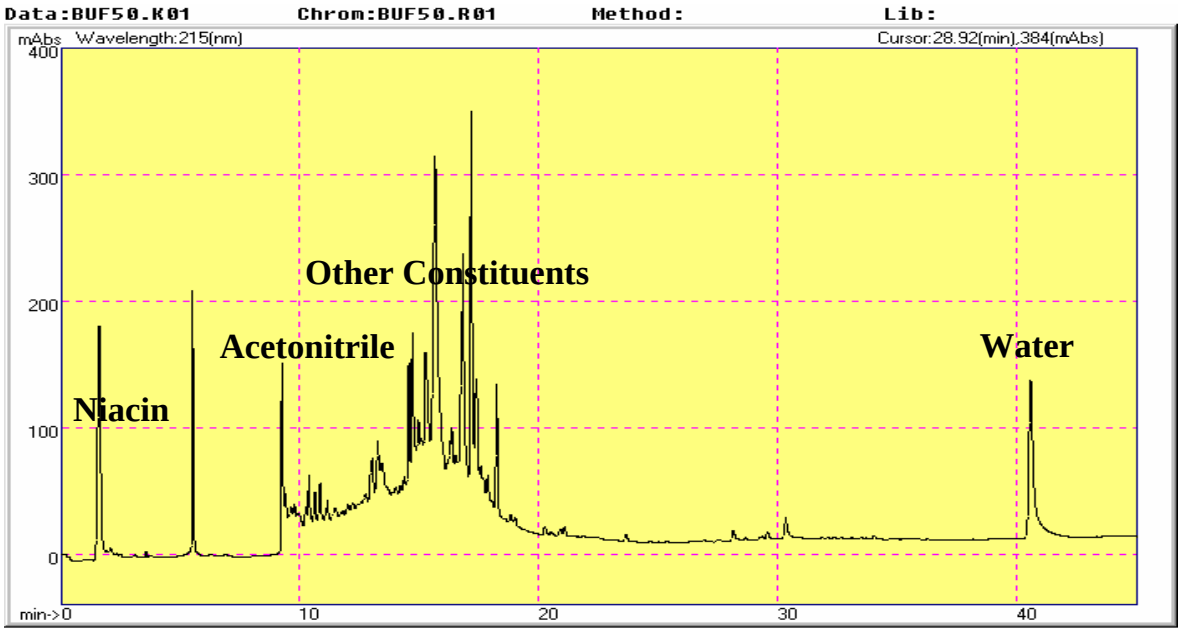


Figure 40. RP-HPLC finger print of BuF of ABe.

Table 9. Niacin, magnesium, zinc, vanadium, and manganese content in ABe, AF, and BuF

Name of the sample	Niacin* (mg/g)	Magnesium (ppm)	Zinc (ppm)	Vanadium (ppm)	Manganese (ppm)
ABe	174.4 ± 1.8	12.8	<0.1	1.9	7
AF	73.9 ± 2.7	11.0	Undetected	1.0	5.6
BuF	55.8 ± 2.1	1.6	Undetected	<0.1	<0.1

*The values are expressed as the mean ± SEM of HPLC quantitations (n = 6) of each sample.

AAS analysis showed the presence of magnesium (Mg) and other metals such as vanadium and manganese. Mg level was low in BuF (Table 9). Intracelluar deficiency of some trace elements has been shown in diabetes, obesity, and hypertension (Failla and Kiser, 1981; Paolisso et al., 1987; Paolisso et al., 1988; Raz., et al., 1988). Over the last several years, numerous reports have confirmed the *in vitro* and *in vivo* insulin-like activity of several elements. Vanadate and lithium have been shown to stimulate glucose uptake and glycogen synthase activity in heptatocytes, adipocytes, diaphragm, and skeletal muscle (Bosch et al., 1986; Bhattacharya, 1964; Dubyak and Kleinzellar, 1980; Green, 1986; Duckworth et al., 1988). Recently, Ho et al., (2000) reported that high zinc intake significantly reduced the severity of type 1 diabetes in alloxan and STZ animal models.

Since NA and Mg were identified in ABe and its bio-active fractions, their effects on glucose tolerance had been tested in animals with both type 1 and type 2 diabetes mellitus.

Experiment 6: Evaluation of the synergistic interaction of magnesium and nicotinic acid on glucose tolerance in animals with experimental diabetes mellitus

6.1 Aims

In this study the synergistic interaction of Mg and NA on glucose tolerance was tested in animals with both type 1 and type 2 diabetes mellitus.

6.2 Experimental procedure

6.2.1 The OGTT in STZ - diabetic SD rats using $MgCl_2$

Prior to OGTT rats were fasted for 16 h. Distilled water (normal and diabetic control), a reference drug metformin (500 mg/kg), or each of four different doses of $MgCl_2$ (10 mg, 50 mg, 100 and 600 mg/kg) was then orally administered to groups of 6 rats each….. (as done previously in Experiment 2.2.1, page 58).

6.2.2 The OGTT in STZ - diabetic SD rats using NA

Prior to OGTT rats were fasted for 16 h. Distilled water (control), a reference drug metformin (500 mg/kg), or each of four different doses of nicotinic acid (10 mg, 50 mg, 100 and 2 g/kg) was then orally administered to groups of 6 rats each…..(as done previously in Experiment 2.2.1, page 58).

6.2.3 The OGTT in STZ - diabetic SD rats using $MgCl_2$ and NA

Prior to OGTT rats were fasted for 16 h. Distilled water (normal and diabetic control), a reference drug metformin (500 mg/kg), or each of four different doses of $MgCl_2$ and NA ($MgCl_2$ 10 mg + NA 10 mg/kg, $MgCl_2$ 50 mg + NA 50 mg/kg,

$MgCl_2$ 100 mg + NA 100 mg/kg, and $MgCl_2$ 600 mg + NA 2 g /kg) was then orally administered to groups of 6 rats each….(as done previously in Experiment 2.2.1, page 58).

6.2.4 The OGTT in HFD-STZ - diabetic SD rats using $MgCl_2$

The OGTTs were performed in HFD-STZ-diabetic rats using $MgCl_2$ by the same procedure as described for the STZ-diabetic rats (refer to Experiment 6.2.1).

6.2.5 The OGTT in HFD-STZ - diabetic SD rats using NA

The OGTTs were performed in HFD-STZ-diabetic rats using NA by the same procedure as described for the STZ-diabetic rats (refer to Experiment 6.2.2).

6.2.6 The OGTT in HFD-STZ - diabetic SD rats using $MgCl_2$ and NA

The OGTTs were performed in HFD-STZ-diabetic rats using MgCl2 and NA by the same procedure as described for the STZ-diabetic rats (refer to Experiment 6.2.3).

6.2.7 The IPGTT in STZ – C57BL/6J mice using $MgCl_2$

Before the IPGTT, animals were fasted for 4 h (Starting from 9.00 a.m.) Distilled water (normal and diabetic controls), a reference drug metformin (500 mg/kg), or each of four different doses of $MgCl_2$ (10 mg, 50 mg, 100 mg and 600 mg/kg) was then orally administered to groups of 6 mice each and followed by an i.p.

administration of glucose (2 g/kg). Blood glucose levels were determined in tail blood samples at -30 (just before the $MgCl_2$ and metformin administration), 0 (just before the i.p. administration of glucose), 120 and 180 min after glucose load for the estimation of glucose by i-STAT blood glucose analyzer.

6.2.8 The IPGTT in STZ – C57BL/6J using NA

Before the IPGTT, animals were fasted for 4 h (Starting from 9.00 a.m.) Distilled water (normal and diabetic controls), a reference drug metformin (500 mg/kg), or each of four different doses of NA (10 mg, 50 mg, 100 mg and 2 g/kg) was then orally administered to groups of 6 mice each and ….(as done previously in Experiment 6.2.7).

6.2.9 The IPGTT in STZ – C57BL/6J using $MgCl_2$ and NA

Before the IPGTT, animals were fasted for 4 h (Starting from 9.00 a.m.) Distilled water (normal and diabetic controls), a reference drug metformin (500 mg/kg), or each of four different doses of $MgCl_2$ and NA ($MgCl_2$ 10 mg + NA 10 mg/kg, $MgCl_2$ 50 mg + NA 50 mg/kg, $MgCl_2$ 100 mg + NA 100 mg/kg, and $MgCl_2$ 600 mg + NA 2 g /kg) was then orally administered to groups of 6 mice each and …… (as done previously in Experiment 6.2.7).

6.2.10 The IPGTT in HFD – C57BL/6J mice using $MgCl_2$

The IPGTTs were performed in HFD-C57BL/6J-mice using MgCl2 by the same procedure as described for the STZ- C57BL/6J-mice (refer to Experiment 6.2.7).

6.2.11 The IPGTT in HFD – C57BL/6J using NA

The IPGTTs were performed in HFD-C57BL/6J-mice using NA by the same procedure as described for the STZ- C57BL/6J-mice (refer to Experiment 6.2.8).

6.2.12. The IPGTT in HFD – C57BL/6J using $MgCl_2$ and NA

The IPGTTs were performed in HFD-C57BL/6J-mice using $MgCl_2$ and NA by the same procedure as described for the STZ- C57BL/6J-mice (refer to Experiment 6.2.9).

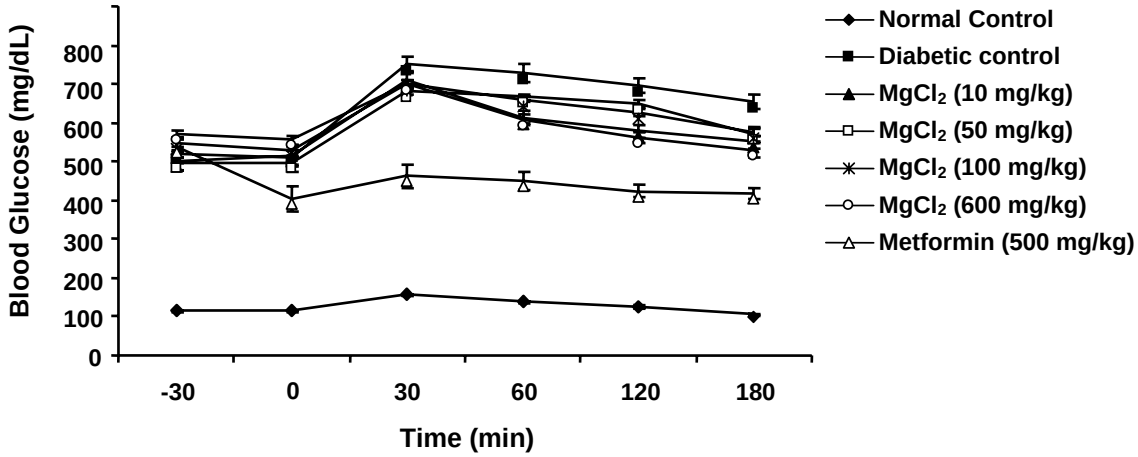


Figure 41. Effects of $MgCl_2$ on glucose tolerance in STZ-diabetic rats.
The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the OGTT.

$P < 0.001$ Metforim-treated group vs diabetic control at 0, 30, 60, 120 and 180 min (Student's t-test).

$P < 0.05$ $MgCl_2$ (600 mg/kg)-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

6.3 Results and discussion

In the OGTT (Figure 41 & 42), administration of $MgCl_2$ at a dose of 600 mg/kg per orally caused a significant attenuation ($P<0.05$) of blood glucose at 60, 120 and 180 min after the glucose load was given orally at 0-h in both STZ and HFD-STZ-diabetic rats. On the other hand, the administration of $MgCl_2$ at doses of 10 mg, 50 mg and 100 mg/kg did not cause any improvement in the glucose tolerance in both STZ as well as HFD-STZ-diabetic rats.

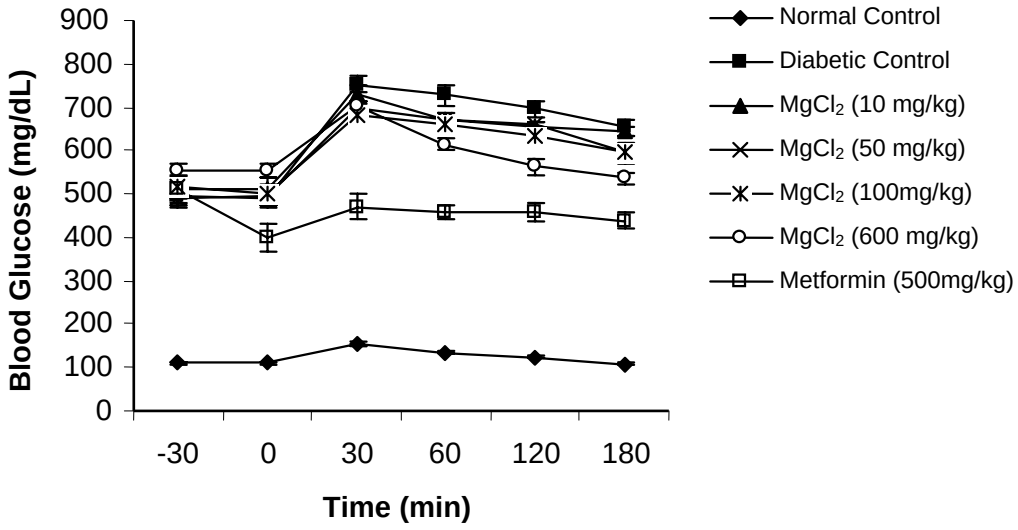


Figure 42. Effects of $MgCl_2$ on glucose tolerance in HFD-STZ diabetic rats.

The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the OGTT.

$P < 0.001$ Metforim-treated group vs diabetic control at 0, 30, 60, 120 and 180 min (Student's t-test).

$P < 0.05$ $MgCl_2$ (600 mg/kg)-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

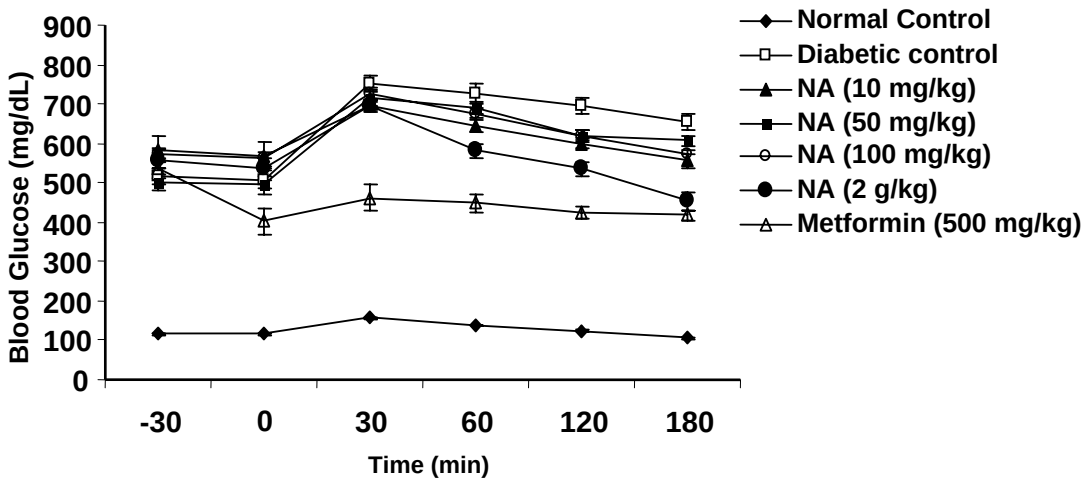


Figure 43. Effects of NA on glucose tolerance in STZ-diabetic rats.

The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the OGTT.

$P < 0.001$ Metforim-treated group vs diabetic control 0, 30, 60, 120 and 180 min (Student's t-test).

$P < 0.05$ NA (2 g/kg)-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

Similarly, in the OGTT (Figure 43 & 44), administration of NA at doses of 10 mg, 50 mg and 100 mg/kg per orally did not cause attenuation of blood glucose, where as the administration of NA at a dose of 2 g/kg caused significant improvement ($P<0.05$) in the glucose tolerance in both STZ as well as HFD-STZ-diabetic rats at 60, 120 and 180 min after the oral glucose load at 0-h.

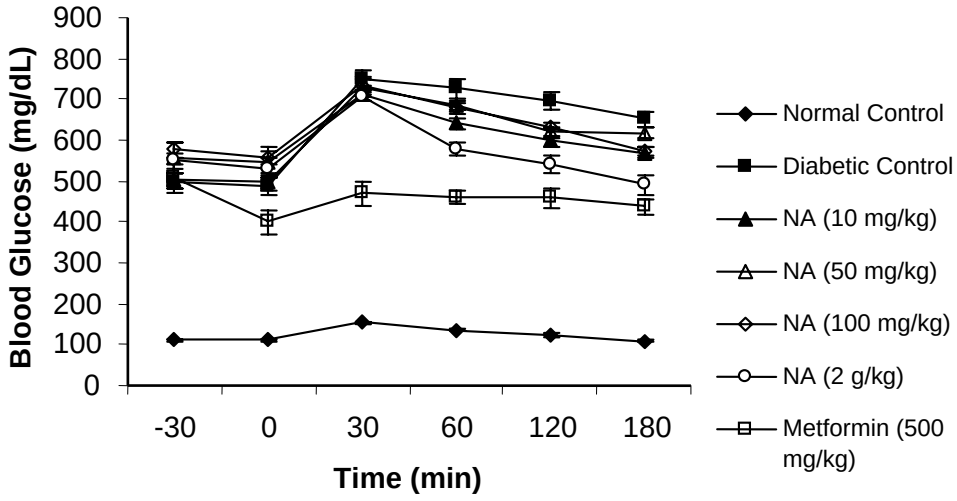


Figure 44. Effect of NA on glucose tolerance in HFD-STZ-diabetic rats.

The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the OGTT.

$P < 0.001$ Metforim-treated group vs diabetic control at 0, 30, 60, 120 and 180 min (Student's t-test).

$P < 0.05$ NA (2 g/kg)-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

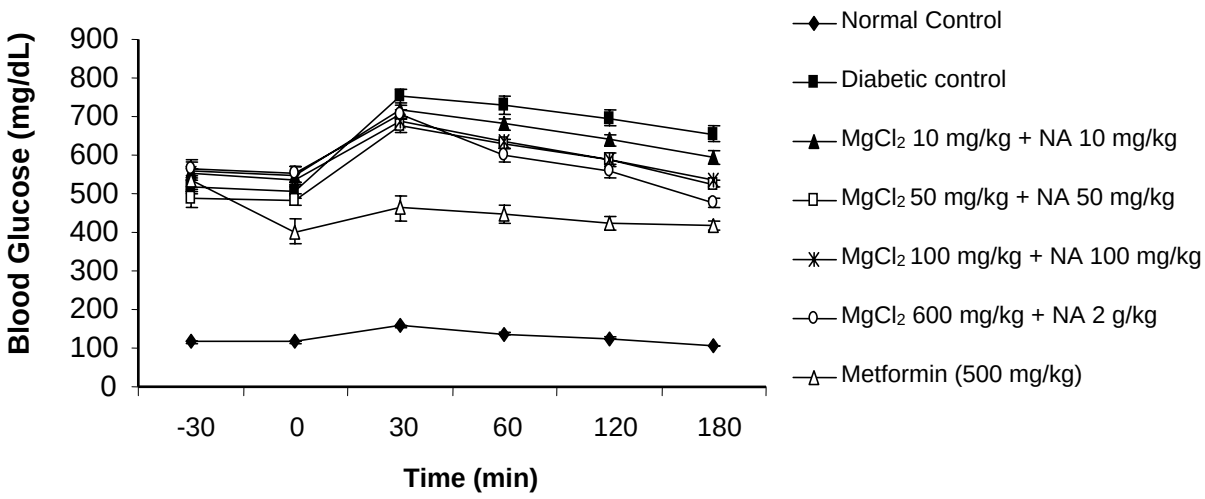


Figure 45. Effects of $MgCl_2$ and NA on glucose tolerance in STZ-diabetic rats. The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the OGTT.

$P < 0.05$ $MgCl_2$ + NA (each 10 mg/kg) -treated group vs diabetic control at 120 and 180 min (Student's t-test).

$P < 0.05$ $MgCl_2$ + NA (each 50 mg/kg)-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

$P < 0.05$ $MgCl_2$ + NA (each 100 mg/kg)-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

$P < 0.01$ $MgCl_2$ (600 mg/kg) + NA (2 g/kg)-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

$P < 0.001$ Metforim-treated group vs diabetic control at 0, 30, 60, 120 and 180 min (Student's t-test).

However, the administration of each of four different doses of $MgCl_2$ and NA (MgCl2 10 mg + NA 10 mg/kg, MgCl2 50 mg + NA 50 mg/kg, $MgCl_2$ 100 mg + NA 100 mg/kg, and MgCl2 600 mg + NA 2 g /kg) increases glucose tolerance significantly ($P < 0.05$ and $P<0.01$ respectively) in both STZ and HFD-STZ-diabetic rats (Figure 45 & 46).

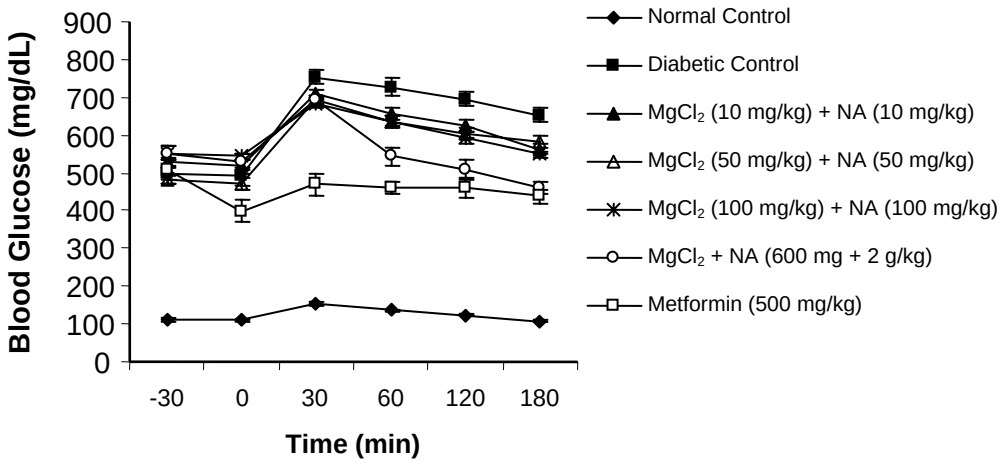


Figure 46. Effects of $MgCl_2$ and NA on glucose tolerance in HFD-STZ-diabetic rats. The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the OGTT.

$P < 0.05$ $MgCl_2$ + NA (each 10 mg/kg) -treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

$P < 0.05$ $MgCl_2$ + NA (each 50 mg/kg)-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

$P < 0.05$ $MgCl_2$ + NA (each 100 mg/kg)-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

$P < 0.01$ $MgCl_2$ (600 mg/kg) + NA (2 g/kg)-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

$P < 0.001$ Metforim-treated group vs diabetic control at 60, 120 and 180 min (Student's t-test).

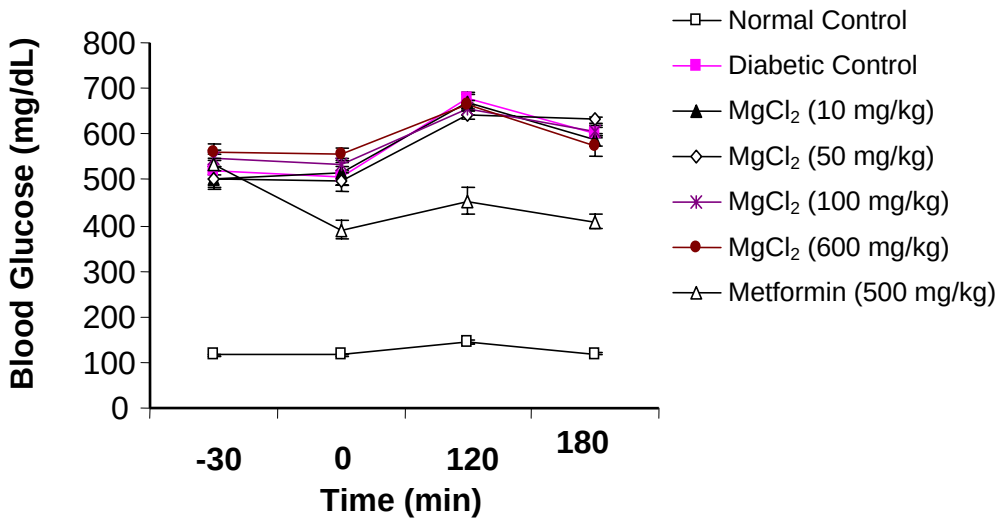


Figure 47. Effects of $MgCl_2$ on glucose tolerance in STZ-diabetic C57BL/6J mice. The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the IPGTT.

$P < 0.01$ Metformin-treated group vs diabetic control at 0, 120 and 180 min (Student's t-test).

On the other hand, in the IPGTT (Figure 47 & 48), the administration of $MgCl_2$ at doses of 10 mg, 50 mg, 100 mg and 600 mg/kg did not cause any improvement in the glucose tolerance in both STZ as well as HFD-diabetic C57BL/6J mice after the intraperitoneal glucose load at 0-h.

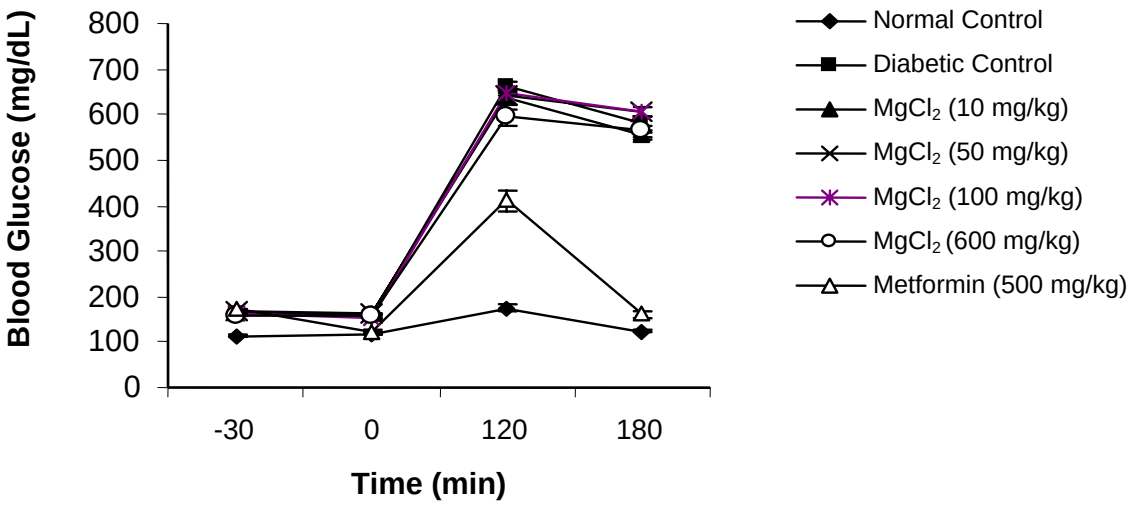


Figure 48. Effects of $MgCl_2$ on glucose tolerance in HFD-induced diabetic C57BL/6J mice.
The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the IPGTT.

P < 0.01 Metformin-treated group vs diabetic control at 120 and 180 min (Student's t-test).

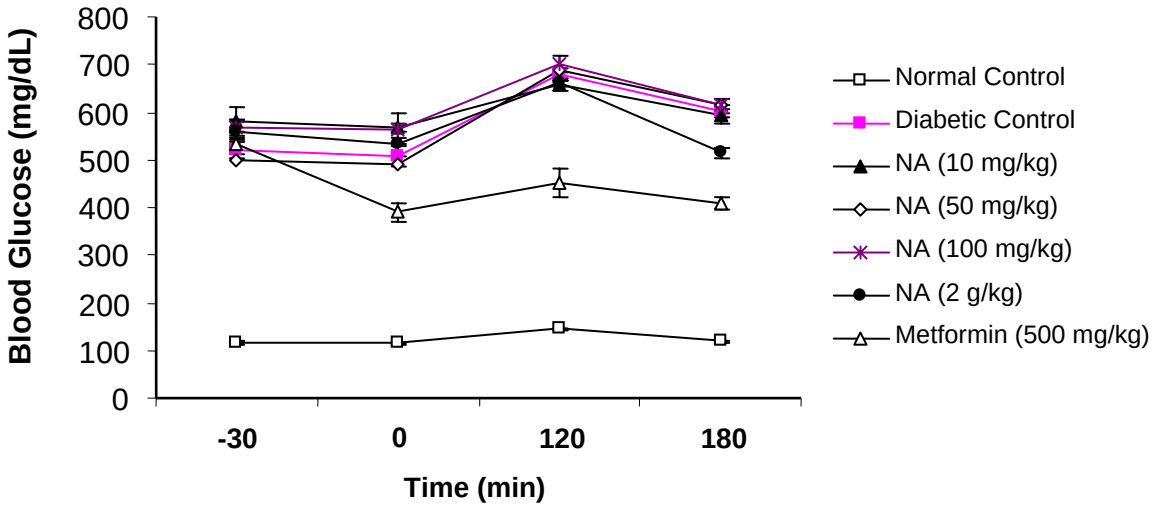


Figure 49. Effects of NA on glucose tolerance in STZ-diabetic C57BL/6J mice. The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the IPGTT.

P < 0.01 Metformin-treated group vs diabetic control at 0, 120 and 180 min (Student's t-test).

Similarly, (Figure 49), administration of NA at doses of 10 mg, 50 mg, 100 mg and 2 g/kg per orally did not cause attenuation of blood glucose, in STZ-diabetic C57BL/6J mice in the IPGTT. However, administration of 2 g/kg of NA per orally to HFD-diabetic C57BL/6J mice (Figure 50) caused a significant attenuation ($P<0.05$) of blood glucose at 180 min after the intraperitoneal glucose load at 0-h.

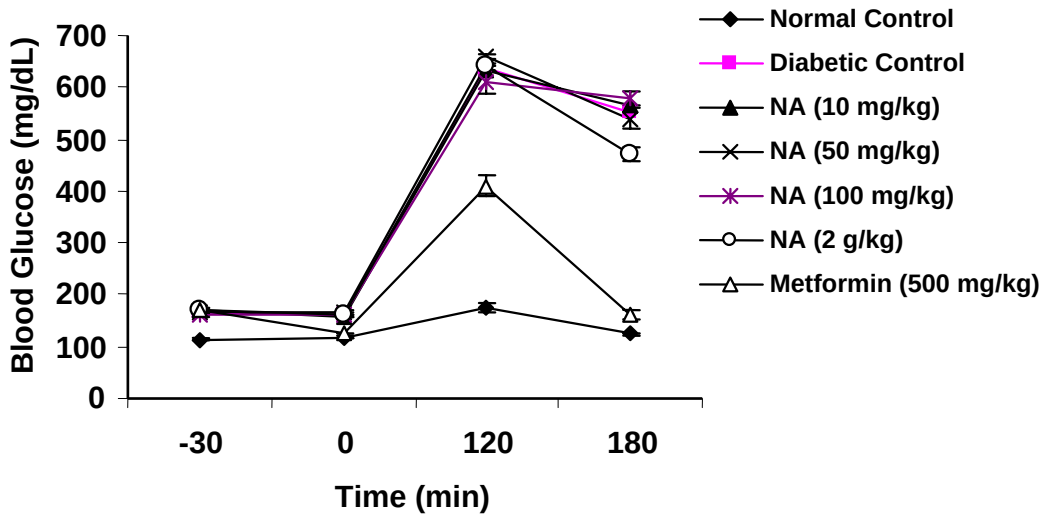


Figure 50. Effects of NA on glucose tolerance in HFD-induced diabetic C57BL/6J mice.
The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the IPGTT.

$P < 0.05$ NA (2 g/kg) vs diabetic control at 180 min (Student's t-test).

$P < 0.05$ Metformin-treated group vs diabetic control at 120 and 180 min (Student's t-test).

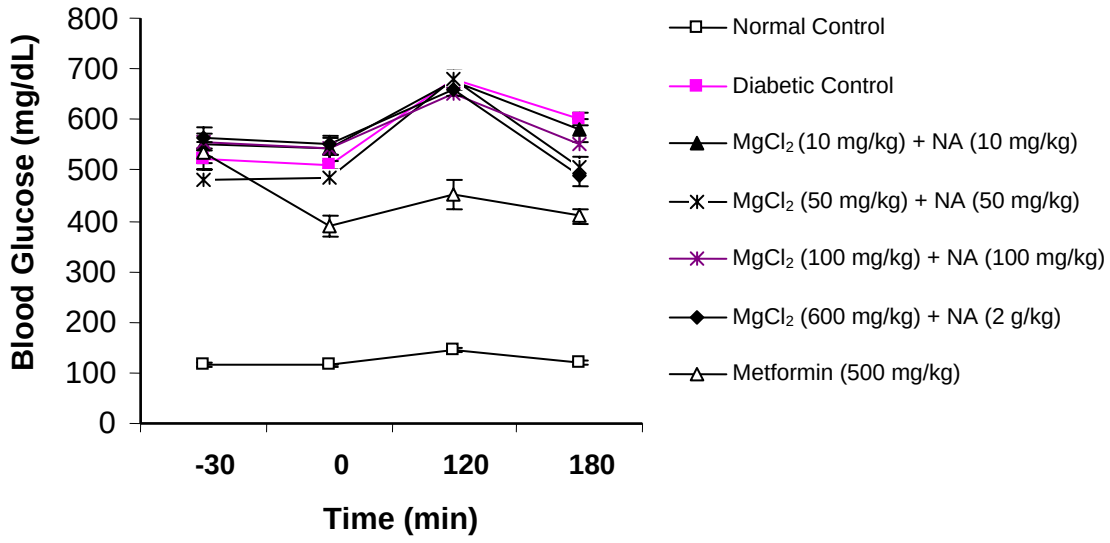


Figure 51. Effects of $MgCl_2$ and NA on glucose tolerance in STZ-diabetic C57BL/6J mice.

The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the IPGTT.

$P < 0.05$ $MgCl_2$ + NA (600 mg + 2 g/kg) vs diabetic control at 120 and 180 min (Student's t-test).

$P < 0.01$ Metformin-treated group vs diabetic control at 0, 120 and 180 min (Student's t-test).

The administration of each of four different doses of $MgCl_2$ and NA ($MgCl_2$ 10 mg + NA 10 mg/kg, $MgCl_2$ 50 mg + NA 50 mg/kg, $MgCl_2$ 100 mg + NA 100 mg/kg) did not increase glucose tolerance in both STZ and HFD-diabetic C57BL/6J mice (Figure 51 & 52). However, the administration of high doses of $MgCl_2$ and NA (600 mg/kg of $MgCl_2$ and 2 g/kg of NA in combination) caused a significant attenuation of blood glucose at 180 min in STZ-diabetic C57BL/6J mice and at both 120 and 180 min in HFD-diabetic C57BL/6J mice.

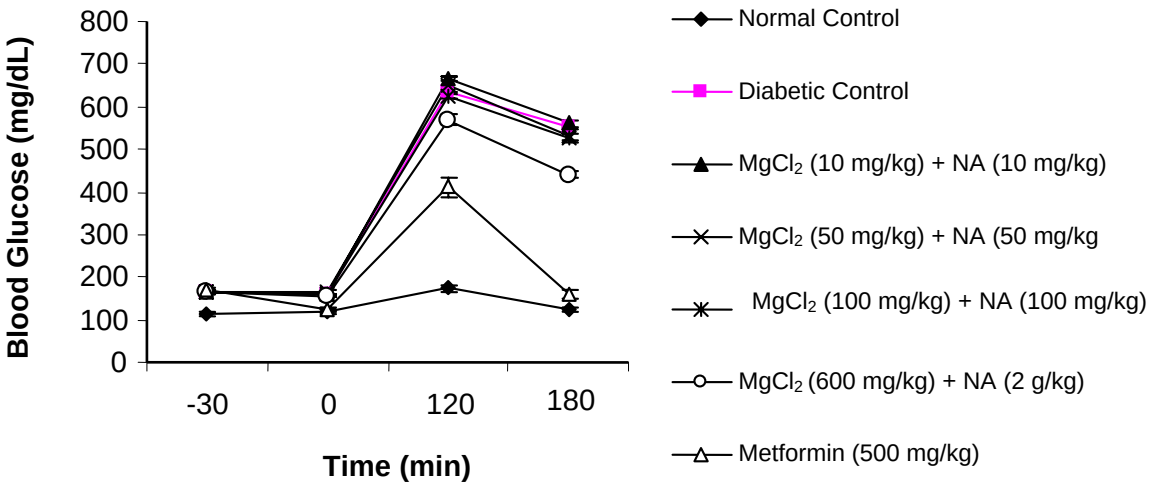


Figure 52. Effects of $MgCl_2$ and NA on glucose tolerance in HFD-induced diabetic C57BL/6J mice.
The graph represents the mean changes in blood glucose concentration, while bars represent ± SEM (n = 6) in the IPGTT.

$P < 0.05$ $MgCl_2$ + NA (600 mg + 2 g/kg) vs diabetic control at 120 and 180 min (Student's t-test).

$P < 0.05$ Metformin-treated group vs diabetic control at 120 and 180 min (Student's t-test).

These results indicate a synergistic interaction of $MgCl_2$ and NA in diabetic animals in improving the glucose tolerance in diabetic animals. Similarly, a decrease in fasting blood glucose level in rats following the intraperitoneal administration of NA was observed (Ammon et al., 1971). The measurement of insulin-stimulated glucose disposal and hepatic glucose production in STZ-diabetic rats during an infusion of nicotinic acid has shown a decrease in plama NEFA accompanied by an increase in peripheral glucose disposal and a fall in hepatic glucose production and fasting plasma glucose was lowered (Reaven et al., 1988). NA does exhibit hypolipidaemic properties, and effectively lowers both TC and TG (Boberg et al., 1971). It inhibits fatty acid mobilization from adipose tissue (Carlson et al., 1968), thereby lowering plasma NEFA and inhibiting the synthesis of VLDL-C synthesis.

Chronic magnesium supplementation (4.5 g) to NIDDM patients produced a significant reduction of the lipid profile and an increase of HDL-C. These results suggest that oral supplementation of magnesium may be useful in the treatment of hyperlipidemia in patients with non-insulin-dependent diabetes mellitus (Corica et al., 1994). This was recently confirmed by Lal et al. (2003). Moreover, magnesium supplementation in male obese Zucker diabetic fatty rats prevents deterioration of glucose tolerance and delayed the development of diabetes (Balon et al., 1995).

Vanadate and lithium have been shown to stimulate glucose uptake and glycogen synthase activity in heptatocytes, adipocytes, diaphragm, and skeletal muscle (Bosch et al., 1986; Bhattacharya, 1964; Dubyak and Kleinzellar, 1980; Green, 1986; Duckworth et al., 1988). Zinc has been shown to stimulate insulin action and

insulin receptor tyrosine kinase (IRTK) activity (Ezaki, 1989; Coulston and Dandona, 1980). The oral administration of lithium (Rossetti, 1989) and vanadate (Rossetti and Laughlin, 1989) improves glucose tolerance and normalizes insulin-mediated glucose uptake, primarily through the stimulation of skeletal muscle glycogen synthesis.

Mg is known to have a synergistic effect with V to increase muscle glycogen synthesis in diabetic rats. It was reported that neither magnesium sulfate (MgSO4) nor sodium vanadate (NaV) had any effect on glucose utilization. However, MgV was superior to either V alone or Mg alone in improving insulin sensitivity and glycogen synthesis in diabetic rats (Matsuda et al., 1999). So the trace elements potentiate insulin action at the cellular level.

CHAPTER 4

SUMMARY OF RESULTS AND OVERALL DISCUSSION

Section 1: Summary of results

This research project was designed to examine the anti-diabetic properties of ABe *in vivo.* In our initial experiment, ABe was evaluated for its antidiabetic activity in STZ-induced diabetic SD rats. At a dose of 125 mg/kg body weight, ABe increased the glucose tolerance in OGTT. Moreover, it showed potent hypoglycemic, hypotriglyceridemic, anti-lipid peroxidative and anti-atherogenic activities when administered twice a day for 2 weeks.

In the next experiment, anti-diabetic effects of semi-purified fractions of ABe were evaluated in STZ-diabetic SD rats. To obtain the semi-purified fractions, ABe was partitioned with organic solvents such as butanol, ethyl acetate and hexane to obtain AF, BuF, EF and HF soluble fractions. The hypoglycemic property of each fraction was then assessed by OGTT at a dose of 125-mg/kg-body weight in STZ-diabetic rats. Fractions AF and BuF produced significant blood glucose-lowering effect. In the long-term study, twice a day administration of AF and BuF at a dose of 125 mg/kg-body weight for 14 days in both STZ-diabetic rats showed a significant blood glucose lowering action. Among AF and BuF, AF was more potent in lowering blood glucose and increased the serum insulin level and lowered hepatic Glc-6-Pase activity significantly in STZ-diabetic rats.

The third experiment was designed to investigate the β-cell protective effects of ABe and its bio-active fractions, AF and BuF against STZ. The pre-treatment of ABe at a dose of 125 mg/kg for 14 days caused a protection against STZ induction of diabetes in male SD rats. The FBG levels in the AF pre- treated STZ-diabetic

rats were much lower than in the vehicle-treated control group. The serum insulin level on day 7 after STZ was decreased markedly in both control as well as AF and BuF pre-treated groups and this level was further decreased on day 14 after STZ injection, while pancreatic insulin content was markedly higher compared to levels in the diabetic control rats. On the other hand, the pancreatic TBARS values were higher in the diabetic control, AF and BuF-treated groups than in the normal control on day 14 after STZ injection. However the values were significantly lower when compared with diabetic control.

In the fourth experiment, hypoglycemic property of semi-purified fractions of ABe was examined in HFD-STZ-diabetic rats. Each fraction was assessed by OGTT at a dose of 125-mg/kg-body weight in HFD-STZ diabetic rats. Of which, fractions AF and BuF produced significant blood glucose-lowering effect. In the long-term study, twice a day administration of AF and BuF at a dose of 125 mg/kg-body weight for 14 days in HFD-STZ-diabetic rats showed a significant blood glucose lowering action. Moreover, among the AF and BuF, AF was more potent in lowering blood glucose and Glc-6-Pase activity and increased the hepatic glycogen content in HFD-STZ-diabetic rats compared to BuF. These results indicated that AF is more potent than BuF in the amelioration of diabetes in both STZ and HFD-STZ-diabetic rats.

In our fifth experiment, the RP-HPLC analysis of ABe, AF, and BuF showed the presence of NA, whereas the AAS analysis showed the presence of magnesium in ABe, AF and BuF in higher amount than other elements such as V, Mn^{++}, and

Zn^{++}. Hence in our final experiment, the effects of both Mg and NA on glucose tolerance were assessed in type 1 and type 2 animal models of diabetes. When given, $MgCl_2$ and NA, together in the OGTT at doses of 10 mg, 50mg, 100 mg/kg each and 600 mg of $MgCl_2$ + 2 g/kg of NA, blood glucose was attenuated significantly in STZ as well as HFD-STZ-diabetic rats.

In the IPGTT of STZ-induced C57BL/6J and HFD-induced C57BL/6J mice, the administration of $MgCl_2$ at the doses of 10 mg, 50 mg, 100 mg, and 600 mg/kg and NA at the doses of 10 mg, 50 mg, 100 mg and 2 g/kg, did not influence the glucose tolerance in both STZ-induced C57BL/6J and HFD-induced C57BL/6J mice. On the other hand, the administration of $MgCl_2$ with NA at a dose of 600 mg/kg and 2 g/kg respectively caused a significant blood glucose attenuating effect in both STZ-induced C57BL/6J and HFD-induced C57BL/6J mice.

Section 2: Overall discussion

The present work is an attempt to evaluate the anti-diabetic properties of ABe in experimental animal models of diabetes mellitus. This study suggested the following findings.

ABe possesses a definite hypoglycemic, hypotriglyceridemic, anti-atherogenic, and anti-lipid peroxidative properties in STZ-diabetic rats after 2 weeks of treatment. The hypoglycemic activity of ABe was observed at the lowest dose (125 mg/kg) in normal as well as STZ-diabetic rats. Moreover, the daily administration of ABe (125 mg/kg) to STZ-diabetic rats twice a day for two weeks caused a significant reduction in food and water intakes, and an increase in the body weight. Since ABe has the ability to reduce the formation of TBARS in the kidneys of STZ-diabetic rats, it could prevent platelet aggregation and thrombosis (Sushil Jain et al., 1998). However, the lack of change in the TBARS levels in the liver of AF-and BuF-treated and metformin-treated STZ/HFD-STZ diabetic rats could again reflect the resistance of the liver to the oxidative stress in the diabetic state as observed by Oberley (1988) and Tatsuki et al (1997). It is significant to note that neither AF nor BuF affects this capacity adversely. Similarly, AF and BuF did not cause any change in the cytochrome P_{450} enzymes in the liver of both STZ and HFD-STZ-diabetic rats, since the level of increase in hepatic cytochrome P_{450} depends on the duration of diabetes (Barnett et al., 1994) and insulin levels (Woodcraft et al., 1997).

Further studies showed that semi-purified fractions of ABe such as AF and BuF have potent hypoglycemic property in both STZ and HFD-STZ-diabetic rats when administered twice a day per orally for 2 weeks at a dose of 125 mg/kg. Although the body weight of the rats did not differ significantly in both STZ and HFD-STZ-diabetic rats, food and water intakes of AF-and BuF-treated STZ-diabetic rats were reduced significantly when compared to the vehicle-treated diabetic rats. Similar effects were reported for hypoglycemic agents such as tungstate and vanadate (Gil et al., 1988; Barbera et al., 1994).

The elevation of serum insulin in AF and BuF-treated STZ-diabetic rats could either be due to the insulinotropic substances present in the fractions, which induce the intact functional β-cells to produce insulin, or the protection of the functional β-cells from further deterioration so that they remain active and produce insulin. Similarly the extracts of *Medicago sativa* (Gray and Flatt, 1997), *Eucalyptus globulus* (Gray and Flatt, 1998), and *Sambucus nigra* (Gray et al., 2000) have been shown to possess insulin-releasing action both *in vitro* and *in vivo*. Since insulin inhibits the activity of Glc-6-Pase in the liver of STZ-diabetic rats and controls HGP, the insulinotropic effect of AF might play a crucial role in the control of hyperglycemia in STZ-diabetic rats. The suppression of Glc-6-P hydrolysis could also be one of the reasons for the hypoglycemic effect of AF in both STZ and HFD-STZ-diabetic rats. Similar effects were reported for other hypoglycemic agents such as vanadate compounds (Mosseri et al., 2000) which have been shown to inhibit hepatic Glc-6-Pase activity, thereby reducing blood glucose levels in NOD mice. However no significant difference was found in the hepatic glycogen

stores of the treated groups compared to control (Schulz, 1988). However the liver glycogen content was significantly higher in AF-treated HFD-STZ-diabetic rats. It could be due to the HFD-feeding of rats, which causes peripheral and hepatic insulin resistance expressed by a decrease in peripheral glucose disposal and an increase in hepatic glucose uptake (Kraegen et al., 1991).

Further investigations on the β-cell protective effects of ABe and its bio-active fractions, AF and BuF, at a dose of 125 mg/kg for 14 days caused protection against STZ induction of diabetes in male SD rats. The ability of ABe and its semi-purified fraction, AF, to protect β-cells suggest that ABe and AF may prevent β-cell DNA break by STZ. The methyl-nitrosourea moiety of STZ can cause DNA breaks by alkylating DNA bases at various positions and lead to profound NAD depletion linked to stimulation of the activity of the nuclear enzyme, poly (ADP-ribose) synthetase, for the excision and repair of the broken DNA strands (Yamamoto et al., 1981). These are responsible for the deterioration in insulin synthesis and secretion (Okamoto et al., 1996). Therefore, poly (ADP-ribose) synthetase inhibitors such as nicotinamide and 3-aminobenzamide could prevent the onset of diabetes (Uchigata et al., 1983). Whether ABe and AF can inhibit poly (ADP-ribose) synthetase activity to protect β-cells from NAD^+ depletion is not clear and further experiments are necessary to confirm it. Gandy et al., (1982) found that hydrogen peroxide was produced in pancreatic islets upon STZ exposure *in vivo* and the oxygen free radicals are involved secondarily in the mechanism of action of STZ (Baynes, 1995). The markedly increased pancreatic lipid peroxidation level found in the diabetic control groups in our experiments is

in agreement with the previous report of Yang and Cherian (1994). Hence, the partial inhibition of lipid peroxidation in the pancreas of AF pre-treated rats could contribute to the reduction of cytotoxicity of STZ against the β-cells of the pancreas.

Since NA and Mg were identified in both ABe and AF, the influence of these two components were tested on glucose tolerance in both type 1 and type 2 animal models of diabetes. The administration of NA and Mg together increased glucose tolerance significantly in both STZ and HFD-STZ-diabetic rats in lower doses as well as in STZ and HFD-C57BL/6J mice at high doses. This indicates the synergistic interaction of NA and Mg in controlling the glucose tolerance in animals with experimental diabetes mellitus. Similar interaction of NA with other trace metal chromium was reported by Thomas and Gropper (1996). A decrease in FBG level in rats following the intraperitoneal administration of NA was observed by Ammon et al (1971).

Even though the effects of NA on the experimental animal models of diabetes are relatively consistent, the acute findings in humans, on the other hand, are more complex and subject of controversy. The glucose tolerance in diabetic patients has been both worsened (Gaut and Taylor-Russell, 1968) and improved (Carlson and Ostman, 1965) by NA. Though species differences may partly explain the discrepant findings in animals and man, the considerable variation in experimental conditions and in particular the lack of homogeneity in the study population also makes different studies difficult to compare (Reaven et al., 1988).

However, Mg supplementation in male obese Zucker diabetic fatty rats prevents deterioration of glucose tolerance and delayed the development of diabetes (Balon et al., 1995). Moreover, Mg is known to have a synergistic effect with V to increase muscle glycogen synthesis in diabetic rats (Matsuda et al., 1999). Thus the Mg in ABe and AF could interact with NA as well as other hypoglycemic metal elements such as vanadium and manganese (Rubenstein et al., 1962; Rubenstein et al., 1962; Fore, 1963; Swanston-Flatt et al., 1990) and improve glucose tolerance in experimental animal models of diabetes.

CHAPTER 5

CONCLUSION AND FUTURE STUDIES

Section 1: Conclusion

The following results have been demonstrated

1. The ABe at a dose of 125 mg/kg body weight caused hypoglycemic, hypotriglyceridemic, anti-lipid peroxidative, anti-atherogenic and β-cell protective activities in STZ-diabetic rats.
2. The semi-purified fractions of ABe such as AF and BuF at a dose of 125 mg/kg body weight caused hypoglycemic and hypotriglyceridemic properties in both STZ and HFD-STZ-diabetic rats.
3. Phytochemical analysis revealed the presence of both NA and Mg in ABe and AF.
4. Further studies in STZ/HFD-STZ-diabetic rats and STZ/HFD-diabetic C57BL6j mice showed that the administration of both NA and Mg together improved the glucose tolerance significantly than Mg or NA alone.

The results indicate the following:

1. ABe, AF and BuF have potent anti-diabetic activity in animals with experimental diabetes.
2. AF was more potent than BuF in reducing blood glucose and lipids as well as β-cell protection against streptozotocin toxicity in diabetic animals.
3. Significant amounts of NA and Mg were present in the *A bilimbi* leaves.
4. NA and Mg together had a synergistic effect on glucose tolerance in animal models of type 1 and type 2 diabetes.

Section 2: Future studies

1. The anti-diabetic activity of ABe, AF and BuF can be tested in genetically-diabetic animal models such as the BB rats, C57BL/KsJ-db/db mice and obese non-diabetic C57BL/6j-ob/ob mice.
2. The synergistic interaction of NA and Mg can also be further tested in genetically diabetic animal models.
3. The effects of NA and Mg on glucose uptake can be investigated *in vitro* in cell lines such as 3T3 adipocytes.
4. ABe, AF and BuF can further be analyzed using modern liquid chromatographic techniques (LC) coupled with mass spectrometry (LC-MS), nuclear magnetic resonance spectroscopy (LC-NMR), ultra-violet spectroscopy (LC-UV) and infrared spectroscopy (LC-IR) for the identification and isolation of novel anti-diabetic component(s).

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