EFFECT OF CURCUMIN ON BLOOD GLUCOSE LEVEL, NEUROBEHAVIORAL RESPONSES AND OXIDATIVE STRESS BIOMARKERS IN ALLOXAN-INDUCED DIABETIC SWISS ALBINO MICE

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ABSTRACT

Cognitive deficit is an emerging health concern in diabetic patients. Hyperglycemia and reactive oxygen species are believed to be among the prime candidates mediating the behavioral impairments and memory deficits. The aim of this study was to evaluate the effect of curcumin on blood glucose level, neurobehavioral responses and some oxidative stress biomarkers in Alloxan-induced diabetic Swiss Albino mice. The animals were divided into five (5) groups of four mice each (n=4). Diabetes was induced using a single dose of Alloxan (150 mg/kg) intra peritoneally. Group I served as normoglycemic control and received distilled water, group II, III, IV and V were diabetic and received olive oil 1 ml/kg, glibenclamide 1 mg/kg, curcumin 50 mg/kg and curcumin 100mg/kg respectively. All administrations were done for a duration of 21 days. Blood glucose level was determined using glucose oxidase method and cognitive impairment was determined using spontaneous alternation in the Y-maze and novel object recognition task (NORT). The result obtained from this study showed that curcumin at both doses (50 mg/kg and 100 mg/kg) decrease significantly ($p < 0.05$) the fasting blood glucose level (108.25 ± 16.01 mg/dl and 114.75 ± 5.56 mg/dl respectively) when compared with the diabetic control group (221.50 ± 14.03 mg/dl). Also the result demonstrated that curcumin at 100 mg/kg significantly ($p < 0.05$) increase the percentage alternation (74.39 ± 8.06%) when compared with the diabetic control group (47.50 ± 13.65%) in the Y- maze test and significantly ($p < 0.05$) improve the memory, recognition and discrimination indices (14.85 ± 6.46s, 0.39 ± 0.04s and 63.71 ± 2.95s) compared to the pretreatment (-10.90 ± 6.40s, -0.27 ± 0.11s and 36.04 ± 8.19s respectively) using NORT. Also, both doses of curcumin recorded increase in SOD (12.84 ± 0.84) and catalase (85.05 ± 3.23) compared to the diabetic control group (5.75 ± 0.96 and 62.27 ± 7.07) respectively. The findings of this study suggest that

curcumin has both antihyperglycemic and antioxidant activity and may ameliorate diabetes-induced cognitive impairment in Swiss albino mice.

CHAPTER ONE

1.0 INTRODUCTION

Diabetes mellitus has been considered as one of the major health concerns all around the world today (Stoler *et al.,* 2008). Experimental animal models are one of the best strategies for the understanding of pathophysiology of any disease in order to design and develop the drugs for its treatment (Chatzigeorgiou *et al.,* 2009). Numerous animal models have been developed for the past few decades for studying diabetes mellitus and testing anti-diabetic agents that include chemical, surgical and genetic manipulations (Etuk, 2012). One of the most potent methods to induce experimental diabetes mellitus is chemical induction by Alloxan (Tyberg *et al.,* 2001). It is a well- known diabetogenic agent that is used to induce Type I diabetes in experimental animals (Viana *et al.,* 2004).

Diabetes mellitus is recognized by chronic hyperglycaemia and is associated with long term damage, dysfunction and failure of various body organs by involvement of micro and macro-vasculature (Hink *et al.,* 2005). The micro-vascular involvement mostly effects retina, renal glomeruli and peripheral nerves, while macro-vascular involvement results in dyslipidemia, formation of reactive oxygen species (ROS), advance glycation end product (AGEs), platelet hyper-reactivity and endothelial dysfunction (Cosentino *et al*., 2003). Disturbance in endothelial function and coagulation pathway may lead to platelet activation, adhesion and aggregation (Ronald and Goldberg, 2012).

A large number of anti-diabetic medicines are available in the pharmaceutical market for diabetes and its related complications; however, currently no effective therapy is available to cure the disease (Sundaram and Mitra, 2007). WHO Expert Committee on Diabetes has recommended investigating traditional herbal medicines, and in this regard more than 400 medicinal plant species

have been compiled (WHO, 2010). These herbal products are gaining popularity in developing and developed countries due to their lesser side effects and low cost (Sundaram and Mitra, 2007). Cognitive function is defined as cerebral activities that lead to knowledge, including all means and mechanisms of acquiring information. It encompass reasoning, memory, attention, and language and lead directly to the attainment of information and thus, knowledge. Cognitive impairments in the diabetic population are emerging problems that warrant immediate research attention. Evidences from neurocognitive tests suggest that cognitive dysfunction should be listed along with retinopathy, neuropathy, nephropathy and cardiovascular complications as one of the complications of diabetes (Sima, 2010).

The major concerns regarding cognitive impairments in the diabetic population are the associated dramatic rise in morbidity and mortality rates and the substantial impact on the medical cost for the public health sectors as well as the patient (Katon *et al.,* 2004; Kodl and Seaquist, 2008; Luchsinger, 2012). Although, comorbid cognitive impairments in the diabetic population is evident in clinical practice (Gispen and Biessels, 2000; Anderson *et al.,* 2001; Laron, 2009), it has not been studied comprehensively in the preclinical setting where emphasis is significantly given to researches on the amelioration of the macro-vascular and micro-vascular complications of diabetes (Arora *et al.,* 2008; Senador *et al.,* 2009). However, the positive correlations that has been found to exist between diabetes and cognitive impairment, the data advocate the central nervous system (CNS) as a crucial target for the diabetic complications that includes micro-vascular diseases, insulin resistance, activation of polyol pathway and increased formation of advanced glycation end products (AGEs) and activation of protein kinase C and that these complications could serve as gateways to the cognitive impairments in diabetes (Kodl and Seaquist, 2008; Laron, 2009; Sima, 2010; Luchsinger, 2012).

Turmeric (*Curcuma longa*) is an intriguing ingredient with a rich history as a dietary spice and herbal supplement in ancient China and India. This distinctive yellow- colored spice, derived from the rhizome of the plant (*C. longa*), is a member of Zingiberaceae family and is widely cultivated in India and Southeast Asia. The use of turmeric in Asia dates back more than 2000 years where it was used in cooking, medicine, cosmetics and fabric dyes (Lee *et al.,* 2013). In traditional medicine turmeric is used to enhance the immune system and as a cure for different respiratory diseases such as asthma and for allergy (Araujo *et al.,* 2001). Tumeric has also been traditionaly used for the treatment of diabetes, cough, sinusitis, flu, rheumatism and liver disorders (Araujo *et al.,* 2001). Meanwhile, traditional Chinese medicine practitioners regularly use turmeric for treating abdominal pain associated diseases (Gupta *et al.,* 2012). It has widely been accepted since ancient times that this polyphenol compound possesses anti-inflammatory properties (Gupta *et al.,* 2012). Advancements in modern medicine have revealed many unkown medicinal properties of turmeric which include anti-oxidant, anti-mutagenic, anti-cancer, anti-microbial and anti-cardiovascular activities (Araujo *et al.,* 2001). Studies have also strongly indicated that curcumin, the active compound in turmeric, is the key ingredient responsible for the major therapeutic activities of turmeric (Gupta *et al.,* 2012)

1.1 Statement of the Research Problem

Diabetes mellitus affected more than 415 million people in 2015 and this is projected to double by the year 2040. Nigeria has a prevalence of 0.8% to 11% involving both rural and urban dwellers with about 2% reported in Zaria (Dahiru *et al.,* 2008). The management of diabetes place an enormous burden on individuals and government. In 2015, the total global expenditure on diabetes was estimated to be between USD 673 billion to USD 1,197 billion and this is projected to rise to

about USD 802 to USD 1,452 billion (IDF, 2015). In Nigeria, about USD 500 million to USD 5 billion is spent annually on diabetes (IDF, 2015).

Also, cognitive impairment has been associated with diabetes. Although much research has been done, the pathophysiology of the neurobehavioral deficit in diabetes is still not well understood and the most appropriate methods to diagnose, treat, or prevent cognitive deficits in diabetes has not yet been defined. However, it appears that inflammation, oxidative stress and apoptosis are central factors and major drivers in the etiology of the comorbid cognitive impairments in diabetes (Sima, 2010) and that reactive oxygen species (ROS), inflammatory processes and apoptosis operates in conjunction through multiple pathways to cause metabolic perturbations of chemical transmitters in the brain and the atrophies of both the white and the gray matters of the brain which usually precedes the cognitive deficits in diabetes (Sima, 2010).

1.2 Justification

Neurobehavioral deficit can impact negatively on one's behaviour. Whilst healthcare professionals offer education, treatment, and support, the patient is principally responsible for the day to day management of the condition. Self-management tasks are many and varied and can include dietary management, monitoring of blood glucose, and insulin injection. People with diabetes must employ a variety of skills in order to successfully self-manage their condition. Neurobehavioral deficit in diabetic patients could lead to apathy towards self-care regimens such as regular physical exercise, dietary habits and towards medication for diabetes (Sinclair *et al.*, 2000; Munshi *et al.*, 2006). However, given that oxidative stress and inflammation appear to be major drivers in the pathogenic mechanisms underlying the cognitive impairments in diabetes, antioxidants and anti-inflammatory drugs could be potential therapeutic tools for the prevention and treatment of cognitive impairments in diabetes.

Additionally, literatures on the ameliorative effect of curcumin on the neurobehavioral deficit, disturbed lipid distribution and formation of ROS associated with alloxan-induced diabetes in Wistar rats are sparse. It is believed that this study will add to the data base of related studies and also provide useful information that may serve as a lead to the development of novel clinical procedure for the prevention and treatment of cognitive impairments in diabetes.

Starting from 1815, when curcumin was first isolated from turmeric, there were only a few reports till the 1970s on its chemical structure, synthesis, biochemical and antioxidant activity (Vogel and Pelletier, 1815; Sharma, 1976).

1.3 Research Hypothesis

Curcumin has no effect on blood glucose level, neurobehavioral responses and some oxidative stress biomarkers in alloxan-induced diabetic Swiss albino mice

1.4 General Aim and Specific Objectives

1.4.1 General Aim

The aim of the study is to determine the effect of curcumin on blood glucose level, neurobehavioral responses and some oxidative stress biomarkers in alloxan-induced diabetic Swiss albino mice

1.4.2 Specific Objectives

The objectives of the study include the following:

1. To determine the effect of curcumin on blood glucose level in alloxan-induced diabetic Swiss Albino Mice
2. To determine the effect of curcumin on spatial working memory using Y-maze test in alloxan-induced diabetic Swiss Albino Mice

3. To determine the effect of curcumin on social memory using novel object recognition test (NORT) in alloxan-induced diabetic Swiss Albino Mice
4. To determine the effect of curcumin on oxidative stress biomarkers (Catalase, Superoxide dismutase, Glutathione peroxidase) in alloxan-induced diabetic Swiss Albino Mice

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes mellitus is a complex metabolic disorder characterized by persistent hyperglycaemia resulting from defects in insulin secretion, insulin action or both. The two main types of diabetes mellitus are type 1 (formerly known as insulin-dependent diabetes), and type 2 (formerly known as non-insulin-dependent diabetes). Type 1 diabetes is caused by the autoimmune destruction of

the β-cells of the pancreatic islets, whereas type 2 diabetes results from both impaired insulin secretion and resistance to the action of insulin (Jonsson, 2002).

2.2 Epidemiology of Diabetes Mellitus

The prevalence of type 2 diabetes is increased in patients with schizophrenia compared with the general population (Holt *et al.*, 2004). Although many factors, including genetic and lifestyle issues, contribute to this association, there has been a recent surge in interest in the subject because of the possible link between the use of atypical antipsychotic drugs and the development of diabetes. The increased prevalence of type 2 diabetes among people with schizophrenia has implications for the delivery of care by psychiatrists, as well as for diabetologists and general practitioners (Holt *et al.*, 2004; Zimmet *et al.*, 2005).

Globally, as of 2015, an estimated 415 million people had diabetes, with type 2 making up about 90% of the cases. Its incidence is increasing rapidly, and by 2040, this number is estimated to almost double (IDF, 2015). Diabetes mellitus occurs throughout the world, but is more common (especially type 2) in the more developed countries. The greatest increase in prevalence is, however, expected to occur in Asia and Africa, where most patients will probably be found by 2040 (IDF, 2015). Incidence in developing countries follows the trend of urbanization and lifestyle changes, perhaps most importantly a "Western-style" diet. This has suggested an environmental (i.e., dietary) effect, but there is little understanding of the mechanism(s) at present, though there is much speculation, some of it most compellingly presented (IDF, 2015).

2.3 Mechanisms of Blood Glucose Regulation

Blood glucose levels are regulated by negative feedback in order to keep the body in homeostasis. The levels of glucose in the blood are monitored by the cells in the pancreas Islets of Langerhans. If the blood glucose level falls to dangerous levels (as in very heavy exercise or lack of food for extended periods), the Alpha cells of the pancreas release glucagon, a hormone whose effects on liver cells act to increase blood glucose levels. They convert glycogen into glucose through the process of glycogenolysis. The glucose is released into the bloodstream, increasing blood glucose levels (Bowen, 2009)

When levels of blood glucose rise, whether as a result of glycogen conversion, or from digestion of a meal, a different hormone is released from beta cells found in the Islets of Langerhans in the pancreas. This hormone, insulin, causes the liver to convert more glucose into glycogen through the process of glycogenesis. And to force about 2/3 of body cells (primarily muscle and fat tissue cells) to take up glucose from the blood through the GLUT4 transporter, thus decreasing blood glucose (Bowen, 2009). When insulin binds to the receptors on the cell surface, vesicles containing the GLUT4 transporters come to the plasma membrane and fuse together by the process of exocytosis, thus enabling a facilitated diffusion of glucose into the cell. As soon as the glucose enters the cell, it is phosphorylated into Glucose-6-Phosphate in order to preserve the concentration gradient so glucose will continue to enter the cell. Insulin also provides signals to several other body systems, and is the chief regulatory metabolic control in humans (Bowen, 2009)

There are also several other causes for an increase in blood glucose levels. Among them are the 'stress' hormones such as epinephrine (also known as adrenaline), several of the steroids, infections, trauma, and of course, the ingestion of food.

2.4 Classification of Diabetes Mellitus

Diabetes mellitus is classified into four broad categories: type 1, type 2, gestational diabetes and "other specific types" (Plonsky, 2012). The "other specific types" are a collection of a few dozen individual causes. The term "diabetes", without qualification, usually refers to diabetes mellitus. The rare disease diabetes insipidus has similar symptoms as diabetes mellitus, but without disturbances in the glucose metabolism (insipidus means "without taste" in Latin) (Plonsky, 2012).

2.4.1 Diabetes mellitus type 1

Type 1 diabetes mellitus is characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas, leading to insulin deficiency (Gray and Heart, 2010). This type can be further classified as immune-mediated or idiopathic. The majority of type 1 diabetes is of the immune-mediated nature, in which beta cell loss is a T-cell-mediated autoimmune attack. There is no known preventive measure against type 1 diabetes, which causes approximately 10% of diabetes mellitus cases in North America and Europe (Polonsky, 2012). Most affected people are otherwise healthy and of a healthy weight when onset occurs. Sensitivity and responsiveness to insulin are usually normal, especially in the early stages (Rother, 2007).

2.4.2 Diabetes mellitus type 2

Type 2 diabetes mellitus is characterized by insulin resistance, which may be combined with relatively reduced insulin secretion. The defective responsiveness of body tissues to insulin is believed to involve the insulin receptor (Rother, 2007). However, the specific defects are not known.

In the early stage of type 2, the predominant abnormality is reduced insulin sensitivity. At this stage, hyperglycemia can be reversed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the liver (Rother, 2007).

2.4.3 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) resembles type 2 diabetes in several respects, involving a combination of relatively inadequate insulin secretion and responsiveness. It occurs in about 2% 5% of all pregnancies and may improve or disappear after delivery. Gestational diabetes is fully treatable, but requires careful medical supervision throughout the pregnancy. About (20% to 50%) of affected women develop type 2 diabetes later in life (Seo *et al*., 2008).

Though it may be transient, untreated gestational diabetes can damage the health of the fetus or mother. Risks to the baby include macrosomia (high birth weight), congenital cardiac and central nervous system anomalies, and skeletal muscle malformations (Rother, 2007). Increased fetal insulin may inhibit fetal surfactant production and cause respiratory distress syndrome. Hyperbilirubinemia may result from red blood cell destruction. In severe cases, perinatal death may occur, most commonly as a result of poor placental perfusion due to vascular impairment. Labor induction may be indicated with decreased placental function (Seo *et al*., 2008)

2.4.4 Other types

Pre-diabetes indicates a condition that occurs when a person's blood glucose levels are higher than normal but not high enough for a diagnosis of type 2 DM. Many people destined to develop type 2 DM spend many years in a state of prediabetes which has been termed "America's largest healthcare epidemic (Cooke and Plotnick, 2008; Boussageon, *et al*., 2011).

Latent autoimmune diabetes of adults (LADA) is a condition in which type 1 DM develops in adults. Adults with LADA are frequently initially misdiagnosed as having type 2 DM, based on age rather than etiology (Boussagion *et al*., 2011).

2.5 Symptoms of Diabetes

People can often have diabetes and be completely unaware (MNT, 2016). The main reason for this is that the symptoms, when seen on their own, seem harmless. However, the earlier diabetes is diagnosed the greater the chances are that serious complications, which can result from having diabetes, can be avoided (MNT, 2016). It is characterized by frequent urination (polyuria), disproportionate thirst, intense hunger, weight gain (this might be the result of the above symptom (intense hunger)), unusual weight loss (this is more common among people with Diabetes Type 1. As the body is not making insulin it will seek out another energy source. Muscle tissue and fat will be broken down for energy. As Type 1 is of a more sudden onset and Type 2 is much more gradual, weight loss is more noticeable with Type 1), increased fatigue, irritability, blurred vision (this can be caused by tissue being pulled from eye lenses. This affects the eyes' ability to focus), cuts and bruises don't heal properly or quickly, more skin and/or yeast infections, itchy skin, gums are red and/or swollen (gums pull away from teeth), frequent gum disease/infection, sexual dysfunction among men, numbness or tingling, especially in the feet and hands (MNT, 2016)

2.6 Complications of Diabetes Mellitus

2.6.1 Diabetic neuropathies

Diabetic neuropathies are a family of nerve disorders caused by diabetes. People with diabetes can, over time, develop nerve damage throughout the body. Some people with nerve damage have no

symptoms. Others may have symptoms such as pain, tingling, or numbness (loss of feeling) in the hands, arms, feet, and legs. Nerve problems can occur in every organ system, including the digestive tract, heart, and sex organs (Solomon *et al*., 2010)

2.6.2 Diabetes mellitus and cardiovascular diseases

Diabetes or prediabetes increases the risk for heart disease and stroke. Maintaining the blood glucose, blood pressure, and blood cholesterol close to the recommended target numbers-the levels suggested by diabetes experts is essential for good health (Ronald and Goldberg, 2012).

2.6.1.1 Stroke

A stroke results when the blood supply to the brain is suddenly cut off, which can occur when a blood vessel in the brain or neck is blocked or bursts. Brain cells are then deprived of oxygen and die. Most strokes are caused by fatty deposits or blood clots (jelly-like clumps of blood cells) that narrow or block one of the blood vessels in the brain or neck. A blood clot may stay where it formed or can travel within the body. People with diabetes are at increased risk for strokes caused by blood clots (Ronald and Goldberg, 2012)

2.6.3 Diabetes mellitus and sexual problems

Both men and women with diabetes can develop sexual problems because of damage to nerves and small blood vessels. Damage to the autonomic nerves can hinder normal function. Reduced blood flow resulting from damage to blood vessels can also contribute to sexual dysfunction (Saigal *et al.,* 2006)

2.6.4 Diabetes mellitus and urologic problems

Diabetes is the most common cause of kidney failure, accounting for nearly 44 percent of new cases (Solomon *et al*., 2010). Urologic problems that affect men and women with diabetes include bladder problems and urinary tract infections. More than half of men and women with diabetes have bladder dysfunction because of damage to nerves that control bladder function. Bladder dysfunction can have a profound effect on a person's quality of life (Jeanette *et al*., 2008). Common bladder problems in men and women with diabetes include the following:

I. **Overactive bladder.** Damaged nerves may send signals to the bladder at the wrong time, causing its muscles to squeeze without warning. The symptoms of overactive bladder include; urinary frequency, urinary urgency, urge incontinence

II. **Poor control of sphincter muscles**

III. **Urine retention.** For some people, nerve damage keeps their bladder muscles from getting the message that it is time to urinate or makes the muscles too weak to completely empty the bladder. If urine remains in the bladder for too long, an infection can develop in the kidneys or bladder. Urine retention may also lead to overflow incontinence-leakage of urine when the bladder is full and does not empty properly (Jeanette *et al*., 2008).

2.6.5 Diabetic retinopathy

Diabetic retinopathy is a condition occurring in persons with diabetes, which causes progressive damage to the retina, the light sensitive lining at the back of the eye (Lynn Grieger, 2008). It is a serious sight-threatening complication of diabetes. Diabetic retinopathy is the result of damage caused by diabetes to the small blood vessels located in the retina. Blood vessels damaged from diabetic retinopathy can cause vision loss (Lynn Grieger, 2008).

2.6.6 Diabetes and Hypoglycemia

Hypoglycemia, also called low blood glucose, occurs when blood glucose drops below normal levels (Lynda and Lee, 2012).

In adults and children older than 10 years, hypoglycemia is uncommon except as a side effect of diabetes treatment. Hypoglycemia can also result, however, from other medications or diseases, hormone or enzyme deficiencies, or tumors (Lynda and Lee, 2012).

2.7 Cognitive Impairment in Diabetes Mellitus

Poor glycemic control has been associated with progression of cognitive dysfunction (Cukierman-Yaffe *et al.,* 2009). An increasing number of studies have reported an acceleration of cognitive decline in patients with DM, independent of common cardiovascular risk factors (Lu *et al.,* 2009). To date, DM is recognised as an independent risk factor for the development of cognitive dysfunction (Brands *et al.,* 2005). In a meta-analysis based on twenty-five studies, it was estimated that T2DM patients have 1.5-fold greater risk of cognitive dysfunction and 1.6-fold increased risk of dementia, when compared to people without diabetes (Cukierman, 2005). Similarly, a recent report has shown a 1.5-fold higher risk of Alzheimer's disease (AD) in people with diabetes than those without diabetes (Cheng *et al.,* 2012). Most reports so far have suggested an increased risk of global cognitive dysfunction in diabetes (Brands *et al.,* 2005; Cukierman, 2005; Cheng *et al.,* 2012), while some reports showed more selective cognitive impairment, mainly affecting learning, mental speed, and visuospatial process (Messier, 2005). It is important to point out that the discrepancy between these studies may simply be due to the variation of neurocognitive testing

variables, such as age, education, sex, history of other illnesses, and the duration/severity of diabetes (Schoenle *et al.,* 2002; Wessels, 2007).

Many complications of diabetes, such as retinopathy, lower limb ulcers, and atherosclerosis, usually take years to develop before becoming clinically apparent (Brands *et al.,* 2005). However, cognitive function decrement has been observed in the early stage of T2DM (Ruis *et al.,* 2009). In children with T1DM, deficits in cognitive development, including vocabulary, block design, general intelligence, speed of processing, and learning, have been observed as early as 2 years after the onset of T1DM (Northam *et al.,* 1999). These findings suggested that deficits in regulation of blood glucose level, even at an early stage, would have detrimental effects on cognitive function. Interestingly, recent studies have demonstrated that elevated blood glucose levels may be a risk factor for impaired cognitive function leading to dementia, even among people without diabetes (Crane *et al.,* 2013) highlighting the relationship between high blood glucose level and dementia outcome.

There is ample evidence from neuropsychological studies reporting that people who have DM also suffer from mild cognitive impairment (MCI) (Crane *et al.,* 2013). Longitudinal studies have shown that approximately by 55% of patients with MCI developed probable Alzheimer's dementia over 3 years (Modrego *et al.,* 2005) and the progression rate reached 100% after 9.5 years (Morris *et al.,* 2001). It has been suggested that DM patients have 50% higher chance of developing Alzheimer's disease than those without DM (Cheng *et al.,* 2012). A longitudinal study has also shown a relationship between diabetes and incidence of MCI (Artero *et al.,* 2008). Progression of MCI to dementia has been shown to be markedly accelerated by diabetes in elderly subjects who were either cognitively intact or diagnosed with MCI at baseline (Xu *et al.,* 2010). Brain imaging studies have provided direct evidence to support DM-mediated MCI and dementia (Chen *et al.,*

2014; Garcia-Casares *et al.,* 2014). Resting-state functional magnetic resonance imaging (rs-fMRI) studies have revealed abnormalities in amplitude of low-frequency fluctuations (ALFF) in T2DM patients in multiple brain regions. These present as decreased ALFF in the bilateral middle temporal gyrus and left fusiform gyrus and increased ALFF in bilateral cerebellum posterior lobe and right cerebellum culmen (Crane *et al.,* 2013). Moreover, recent studies have shown that alternation of ALFF and reduced connectivity of the hippocampus are associated with the presence of diabetic vascular disease and poor cognitive performance in T2DM patients (Zhou *et al.,* 2010; Wang *et al.,* 2010). Although the mechanism between MCI and the increased risk of dementia under DM is not fully understood, it has been suggested that the DM-mediated MCI and dementia are not likely to form a continuum, given the difference in etiologies and risk factors between MCI and dementia (Reijmer *et al.,* 2010; Exalto *et al.,* 2012).

People suffering from DM over a long period have been shown to express an elevated level of dementia (Peila *et al.,* 2002; Bruce *et al.,* 2008). There are an extensive number of studies examining the effect of DM on cognitive functions in elder population (Croxson and Jagger, 1995; Katzman *et al.,*1998; Hassing *et al.,* 2004). It has been shown that the prevalence of dementia in T2DM patients increased with age, from 2.4% in the age group of 65–76 and 5% in 76–85 to 8.3% for patients over 85 years of age (Parikh *et al.,* 2011). Several studies have also reported an increased incidence of dementia in individuals who were diagnosed with DM in midlife after an extended follow-up of 25–35 years (Biessels *et al.,* 2006). However, the exact effect of midlife against late-life DM onset on cognitive impairment and dementia remains to be clarified (Parikh *et al.,* 2011).

2.8 Pathophysiology of Cognitive Dysfunction In Diabetes

The mechanisms underlying the development of cognitive dysfunction in diabetes have not been fully elucidated (Parikh *et al.,* 2011). Many hypotheses have been suggested based on the pathophysiological mechanisms through which diabetes might affect the initiation and progression of the pathology of dementia (Gispen and Biessels, 2000). These proposed mechanisms include various diabetic-specific factors or signalling pathways that may influence cognitive functioning, such as hyperglycemia, insulin deficiency, microvascular complications, and inflammation.

2.8.1 Vascular dysfunction

Vascular complications, including atherosclerosis, hypertension, stroke, and vascular comorbidity, are closely associated with DM. Increased cerebral infarcts and reduction of amyloid-beta load were observed in older DM patients compared to non-diabetics (Ahtiluoto *et al.,* 2010). A meta-analysis of longitudinal studies suggested that there is a stronger association of vascular-related cognitive impairment than AD with DM patients (Cheng *et al.,* 2012). Interestingly, less Alzheimer's-like pathology has been observed, but more ischemic lesions in T2DM patients with a clinical diagnosis of dementia have been observed (Sonnen *et al.,* 2009). An increasing number of studies are suggesting that the reduction of cerebral perfusion plays a significant role in the development of AD (de la Torre, 2014), supporting the hypothesis that cerebrovascular pathology such as stroke predisposes cognitive decline and dementia development. The detrimental effects of DM on cognitive function in vascular dementia have been demonstrated in a recent preclinical study. Kwon *et al*., (2015) showed that exacerbated cognitive functions caused by diabetes were mediated via augmentation of neuronal cell death in the hippocampus through CREB/BDNF signalling pathway in an animal model of vascular dementia.

Hypertension has been shown to be a significant risk factor for poor cognitive performance in both T1DM and T2DM patients (Elias *et al.,* 1997; van den Berg *et al.,* 2010). In addition to

hypertension, atherogenic dyslipidemia is another common vascular risk factor in DM (Hamilton and Watts 2013). Dyslipidemia contributes to atherosclerosis development (Chan *et al.,* 2014) and has been found to increase risk of dementia in diabetes (Kloppenborg *et al.,* 2008). Moreover, reduced cerebral blood flow, upregulation of inflammatory cytokines, endothelial dysfunction, and abnormalities in cerebral capillaries have been demonstrated in patients with diabetes (Biessels *et al.,* 1996; Yngen *et al.,* 2004; Suzuki *et al.,* 2006). Changes in cerebral vasculature by these factors are closely associated with stroke and brain damage, including brain infarct and white matter lesions (Thal *et al.,* 2012), contributing to cognition deterioration in diabetes.

2.8.2 Metabolic abnormalities

Blood glucose levels are regulated by the endocrine system involving multiple organs and signalling molecules and pathways. Upset of this precisely regulated process could lead to imbalance of blood glucose level, resulting in organ damage. Although the exact mechanisms behind the association between DM and cognitive impairment or dementia are unclear, studies have shown that it is a multifactorial process where metabolic condition plays a significant role (Thal *et al.,* 2012).

2.8.3 Hyperglycemia

Chronic high blood glucose levels have been shown to have negative effects on cognitive functions and brain structure (Gold *et al.,* 2007). Hyperglycemia is a characteristic in both T1DM and T2DM. Numerous studies have demonstrated a close relationship between glucose intolerance and cognitive decrements and dementia (Yaffe *et al.,* 2006). It has been shown that people with poor glycemic control, with glycosylated hemoglobin (HbA1c) higher than 7.0%, have a 4-fold higher

risk of developing cognitive impairment (Reaven *et al.,* 1990). Similarly, an inverse association of HbA1c and cognitive function such as working memory, learning, and executive functioning has been observed in T2DM patients (Gold *et al.,* 2007). The results of these studies highlight the contribution of poor glycemic control in cognitive function deterioration process. Multiple toxic effects of hyperglycemia on the brain, such as formation of advanced glycated end products (AGEs), generation of reactive oxygen species (ROS), and activation of polyol, diacylglycerol, and hexosamine pathways, have been suggested (Comin *et al.,* 2010).

It has been shown that hyperglycemia leads to enhanced formation of AGEs (Toth *et al.,* 2006). AGEs have been shown to contribute to microvascular complications, accelerated amyloid-beta deposition and senile plaque formation. A preclinical study has demonstrated that increased cerebral AGEs expression is associated with cognitive dysfunction in diabetic mice (Girones *et al.,* 2004). Similarly, increased AGEs levels have been observed in AD patients with T2DM, when compared to non-diabetic AD patients (Ahmad *et al.,* 2005). Moreover, AGEs lead to ROS generation via activation of a receptors for AGEs (RAGE); cell surface receptor for AGEs, which in turns leads to neuronal injury (Comin *et al.,* 2010; Valente *et al.,* 2010).

It is well established that oxidative stress is implicated in both the onset and progression of diabetes and its complications. It has been shown that cognitive deficit caused by hyperglycemia in diabetic rat is associated with an increase in ROS levels and reduction of antioxidant levels (Fukui *et al.,* 2002; Brownlee, 2005). In addition, increased ROS generation has been shown to activate various cellular signalling pathways, such as the polypol pathway, protein kinase C activation, and increase of glucose shunting via the hexosamine pathway, all of which are related to neuronal injury and cerebral damage (Auer, 2004). Interestingly, it was shown that administration of antioxidants could

reverse the cognitive dysfunction in the diabetic rats (Fukui *et al.,* 2002; Brownlee, 2005), suggesting a potential therapeutic target for DM-mediated cognitive impairment.

2.8.4 Hypoglycemia

Sufficient glucose supply is vital for normal brain function and it is well established that hypoglycemia has detrimental effects on the brain (McNay and Cotero, 2010). Repeated hypoglycemic episodes are a common side-effect in patients who receive intensive insulin therapy for diabetes (Whitmer *et al.,* 2009). In animal studies, it has been shown that exposure to low blood glucose levels can cause cerebral energy failure, neuronal necrosis, and brain damage leading to a flat electroencephalograph and cognition dysfunction. In human autopsy studies, multifocal or diffuse necrosis of the cerebral cortex, basal ganglia, and hippocampus was observed in patients who died of hypoglycemia (McNay and Cotero, 2010). A dose-response relationship between the occurrence of severe hypoglycemic episodes and risk of dementia development has been reported in a retrospective study involving 16,667 T2DM patients (Bruce *et al.,* 2009). Although contradicting results have been reported by some studies (Warren and Frier, 2005), arguing that tolerance to a hypoglycemic state can be developed in patients exposed to hypoglycemia chronically, the effect of hypoglycemia on some high-risk groups cannot be ignored. It has been shown that impairment of memory functioning is strongly correlated with severe hypoglycemia in T1DM patients (Banks *et al.,* 2004).

2.8.5 Changes in Insulin and Amyloid Metabolism

The blood glucose level is regulated by insulin, a hormone generated by the beta cells in the pancreas. Traditionally, it was believed that the brain is an insulin independent organ; however, recent studies have suggested otherwise (Woods *et al.,* 2003). It has been shown that insulin is actively transported across the blood brain barrier (Steen *et al.,* 2005) and is also produced locally

in the brain (Bondy and Cheng, 2004). Furthermore, insulin receptors are expressed in the hippocampus and the cortex, indicating its functional role in the brain (Gerozissis, 2004). In addition, being a regulator of food intake and energy homeostasis (Woods *et al.*, 2003), insulin also plays a role in memory and learning (Zhao and Alkon, 2001). Changes in insulin levels and receptor sensitivity could lead to deficits in cognitive function (Kalmijn *et al.*, 2000). In AD, impairments of cerebral insulin receptors activation and elevated insulin level in the CSF have been reported (Kuusisto *et al.*, 1997), indicating the contribution of insulin in cognitive decline and dementia development.

Hyperinsulinemia is a common characteristic of T2DM and has been identified as a risk factor for cognitive dysfunction and dementia progression (Comin, 2010). It has been suggested that hyperinsulinemia is associated with reduction of amyloid metabolism, due to down regulation of insulin-degrading enzyme (IDE) levels in the brain. IDE is responsible for the degradation of insulin and amyloid-β peptide (Aβ) (Bhat *et al.*, 2000). Therefore reduced IDE levels would lead to Aβ accumulation in the brain, contributing to AD and cognitive impairment (Bhat *et al.*, 2000). Hyperphosphorylation of tau protein is another pathological hallmark of AD. It has been suggested that inhibition of insulin-mediated pathways can lead to hyperphosphorylation of tau and Aβ production, via activation of the glycogen synthase kinase 3 (GSK3) signalling (Phiel *et al.*, 2003).

2.8.6 Inflammation

Inflammation has been implicated in the onset of DM and progression of its complications (Ferreira *et al.*, 2014). It has been suggested that people suffering from DM are under a state of subclinical chronic inflammation. Numerous proinflammatory markers and cytokines, such as C-reactive protein (CRP), tumour necrosis factor- (TNF-) α, interleukin- (IL-) 1β, and IL-6, have been shown to be upregulated in both T1DM and T2DM (King, 2008). Many of the

proinflammatory markers have been associated with cognitive decline and dementia development. Given the fact that many of these inflammatory markers found in DM patients are closely associated with the pathogenesis of AD (Ferreira *et al.,* 2014), there is an increasing interest in the link between DM and dementia.

Inflammation has been suggested to induce cerebral changes via multiple mechanisms. Firstly, it has been shown that chronic inflammation in DM can induce changes in blood brain barrier (BBB) permeability (Ownby, 2010). Increase in BBB permeability has been observed in brain biopsies from AD patients. Moreover, increased BBB permeability can also allow access of toxic substances and metabolites into the brain, leading to cerebral damage (Bell and Zlokovic, 2009). Secondly, neuroinflammation is a well-established factor in the development of cognitive decline, dementia, and other neurodegenerative diseases (Ownby, 2010; Pimplikar, 2014). It has been demonstrated that inflammatory cytokines can cause activation of glia cells leading to neuronal damage. For example, TNF-α has been shown to induce hippocampal dysfunction, via activation of the janus kinase (JNK) and the IκBα kinase/NFκB signalling pathway (Milanski *et al.*, 2009). Finally, inflammation plays a central role in the development of complications in vasculature, including stroke (Seto *et al*, 2006), contributing to cognitive impairment and dementia development.

2.9 Oxidative Stress and Free Radicals

Reactive species or free radicals which include reactive oxygen species (ROS) and reactive nitrogen species (RNS), collectively called RONS (reactive oxygen nitrogen species), are released from activated immune cells (macrophages, neutrophils and dendritic cells) in response to an inflammatory stimulus. During this, phagocytic cells release RONS and non-phagocytic

cells are stimulated to produce RONS by pro-inflammatory cytokines (Hussain *et al.*, 2003). These RONS are highly reactive radicals that contain unpaired valence shell electrons. Their proper regulation is vital for an efficient immune response and for limiting tissue damage.

Oxidative stress is a condition when the balance between the production of oxidants/RONS and their removal by antioxidants gets disturbed leading to increased production and accumulation of oxidants in the body. A large number of conditions and diseases including ageing, smoking, chronic inflammatory diseases (e.g. Rheumatoid arthritis, Crohn's disease, colitis ulcerosa), diabetes, cancer, neurodegenerative diseases etc lead to generation of oxidative stress (Hussain *et al.*, 2003). Most of the reactive oxygen species are generated in cells by the mitochondrial respiratory chain (Poyton *et al.*, 2009). It has been estimated that about 4-5% of molecular oxygen is converted to reactive oxygen species during aerobic respiration (Klaunig and Kamendulis, 2004). These RONS have an important role in the microbicidal activity of the innate immune response. Some internally generated sources of free radicals are mitochondria, peroxisomes, xanthine oxidase action, inflammation, phagocytosis, arachidonate pathways, exercise and during ischemia/ reperfusion injury. Various externally generated sources of free radicals are radiation, cigarette smoke, environmental pollutants, certain drugs, pesticides, industrial solvents and ozone (Hussain *et al.*, 2003)

To neutralise these free radicals and protect the body against oxidative damage, various antioxidants are present which under normal physiological conditions are capable of counterbalancing the production of reactive oxygen species.

2.10 Sources of Cellular ROS

There are many systems inside a cell that can generate ROS. Mitochondria are recognized as the major site for ROS production and both complexes I and III have been established to be the specific

sites for mitochondrial ROS generation (Friedberg and Meira, 2006). Besides mitochondria, many enzymes are also capable of producing ROS. These include, but not limited to, NADPH oxidase (Bylund *et al.,* 2010), xanthine oxidase (Agarwal *et al*., 2011), α-ketoglutarate dehydrogenase complex, d-amino acid oxidases and dihydrolipoamide dehydrogenase (Gupta *et al.,* 2012).

2.11 The Impact of ROS in Pancreatic beta Cells

Beta cells (b-cells) adequately deal with the physiological challenges of substrate availability imposed both acutely and chronically depending on the nutritional and metabolic states (Schmitt *et al*., 2011). The availability and the type of antioxidant defences in these cells are dictated by demand. There is now a consensus that the chronically high circulating levels of glucose (glucotoxic concentrations) and/or lipid (glucolipotoxic or lipotoxic concentrations) associated with type 2 diabetes induce oxidative stress in different cell types (Newsholme *et al.,* 2007, Gehrmann *et al*., 2010). In type 1 diabetes, induced by autoimmune b-cell destruction, death is associated with cytokine-mediated oxidative stress (Morgan *et al*., 2007, Lenzen, 2008). Oxidative stress is currently viewed as an imbalance between pro- and antioxidants in favor of the former, which implicates a loss of redox signalling. It can be triggered by excessive ROS production as well as by low antioxidant enzyme activities. A well-known source of electrons for reduction of molecular oxygen is the mitochondrion (Morgan *et al*., 2007).

The increase in superoxide formation in the electron transport chain is associated with a high (inner) mitochondrial membrane potential. This causes a decrease in the electron flow through the respiratory chain, increasing the probability of superoxide formation by the retained electrons at various sites in the mitochondrial respiratory chain. However, an additional specialized enzyme-based system can also generate superoxide in a regulated fashion. This enzyme complex, the NADPH oxidase (NOX), is a member of a family of enzyme isoforms that are able to transfer

electrons from NADPH to molecular oxygen to generate O_2. NOX activation is more widely associated with efficient killing of pathogens by phagocytes, such as macrophages, monocytes, dendritic cells, and neutrophils that form ROS from NOX2 within the phagosomal membrane (Bylund *et al.*, 2010). NOX-derived ROS have been shown to stimulate mitogenic signaling and proliferation (Arnold *et al.*, 2001).

It is now evident that NOX isoforms (NOX1, NOX2, and NOX4) are also expressed in pancreatic b-cells (Uchizono *et al.*, 2006), where their function is related to regulation of insulin secretion and cell integrity

Chronic glucose exposure also induces the expression and activity of inducible NO synthase (iNOS) in pancreatic b-cells (Meidute-Abaraviciene *et al.*, 2009). Moreover, other toxic stimuli as pro-inflammatory cytokines, saturated non esterified fatty acids, or direct addition of ROS induce stress resulting in upregulation of iNOS in the pancreatic b-cells, thus causing generation of high levels of NO that are associated with reduced insulin secretion and apoptotic cell death (Michalska *et al.*, 2010).

However, another example of a deleterious pathway that mediates substrate or cytokine toxicity is the formation of hydroxyl radicals (Michalska *et al.*, 2010). In this situation, an abnormal increase in mitochondrial electron leakage or in NOX2 activity could overcome the antioxidant defenses allowing the reaction of H_2O_2 with transition metals (i.e. Fe^{2+}) producing OH^- . In addition, another toxic pathway was recently described, which was associated with cytokine-dependent b-cell death, which was derived from an interaction between RNS and ROS. This pathway for cytokine-induced b-cell apoptosis involves an interaction of mitochondrion-derived hydrogen peroxide with NO, in which the presence of trace metals leads to hydroxyl radical formation resulting in b-cell death (Gurgul-Convey *et al.*, 2011). Likewise, the cytokine death pathway involving OH^- formation may

increase ROS production via increased NOX2 activity, as the treatment of b-cells with a cytokine mixture (IL1b, TNFa, and INFg) increased the expression of p47, the key cytosolic regulator protein of NOX2 (Michalska *et al*., 2010).

2.12 Physiological Function of RNS Generated in the Pancreatic b-cell

Numerous researchers have described the negative effects of NO generation in the b-cell, including attenuation of glucose stimulated insulin secretion and stimulation of apoptosis, originating from the observation that pro-inflammatory cytokines induced gene and protein expression of iNOS and subsequent NO generation, which was associated with caspase activation and cell death (Michalska *et al*., 2010). However, it is now clear that enhanced glucose metabolism in the b-cell, in response to an increase in glucose availability, apart from involving activation of glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation to produce ATP, also triggers additional signaling mechanisms involving the modulation of nitrogen species at intracellular levels (Gray and Heart, 2010). A family of NOS enzymes, for example, nNOS, eNOS, and iNOS, can synthesize NO endogenously from L-arginine, oxygen, and NADPH. iNOS is increased in concentration in response to fatty acids, pro-inflammatory cytokines, and L-arginine (Michalska *et al*., 2010).

2.12.1 Antioxidants in pancreatic b-cells and the role of glucose

Although b-cells express low levels of certain antioxidant defenses (i.e. SOD, catalase, and GSH peroxidase (Lenzen, 2008)), it has been previously shown that they are particularly rich in other peroxidase-based antioxidant defenses, such as glutaredoxin and thioredoxin. Interestingly, modulation of b-cell function may depend on the antioxidant system involved in the scavenging process, as specific manipulation in antioxidant defenses promoted differential results, e.g. modulation of either glutaredoxin or thioredoxin levels altered exocytosis in the presence of

NADPH in a reciprocal fashion (Ivarsson *et al.,* 2005) while mitochondrial SOD overexpression increased cytokine-induced apoptosis in b-cells (Lortz *et al.,* 2005). While long-term exposure to high glucose induces oxidative stress in b cells, conflicting results have been published regarding the levels of ROS on acute glucose exposure and their role in glucose stimulated insulin secretion (GSIS) (Rebelato *et al.,* 2011). Despite evidence from mouse cells that ROS enhanced insulin secretion at low glucose (Leloup *et al*., 2009), both previous and recent results in rat islets indicate that glucose acutely decreases ROS content in islets, an effect that may involve reduction in mitochondrial ROS production (Martens *et al.,* 2005) and/or increased antioxidant output from the pentose– phosphate pathway (Rebelato *et al.,* 2011). Indeed, the evidence from rat islets indicates that antioxidant production acutely surpasses ROS production on exposure to glucose load, (Martens *et al.,* 2005) and that the control of ROS levels is important for insulin secretion (Zhang *et al.,* 2010). Another class of metabolic substrate, fatty acids, acutely enhanced ROS levels in islets, at least in part through activation of NOX (Morgan *et al*., 2007). However, the mechanisms underlying this activation and their functional role seem to differ depending on the type of fatty acid analyzed. The palmitic acid activates NOX at high glucose level in a manner independent on fatty acid oxidation (Graciano *et al.,* 2011), whereas oleic acid-induced NOX activation is metabolism-dependent (Santos *et al*., 2011).

2.13 Antioxidants

Oxygen is an element indispensable for life (Bucci, 2009). When cells use oxygen to generate energy, free radicals are created as a consequence of ATP (adenosine triphosphate) production by the mitochondria. These byproducts are generally reactive oxygen species (ROS) that result from the cellular redox process. These species play a dual role as both toxic and beneficial compounds. Thus, the delicate balance between these two antagonistic effects is clearly an important aspect of

life. At low or moderate levels, ROS exert beneficial effects on cellular responses and immune function; whereas at high concentrations, they generate oxidative stress, a deleterious process, that can damage all cell structures (Genestra, 2007). Oxidant/antioxidant balance has been suggested as an important factor for initiation and progression of cancer (Gupta *et al*., 2012). The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ (endogenous) or externally supplied through foods and/or supplements (exogenous). Endogenous and exogenous antioxidants act as "free radical scavengers" by preventing and repairing damages caused by ROS; and therefore can enhance the immune defence and lower the risk of disease and cancer (Gupta *et al*., 2012).

2.14 Antioxidant Classification

Endogenous antioxidant compounds in cells can be classified as enzymatic antioxidants and non-enzymatic (metabolic and nutrient) antioxidants.

2.14.1 Enzymatic antioxidants

The major enzymatic antioxidants directly involved in the neutralization of ROS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx) (Halliwel, 2007). SOD, the first line of defence against free radicals, catalyzes the dismutation of superoxide anion radical (O_2^-) into hydrogen peroxide (H_2O_2) by reduction. The oxidant formed (H_2O_2) is transformed into water and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GPx).

The selenoprotein GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. Besides hydrogen peroxide, GPx also reduces lipid or nonlipid hydroperoxides while oxidizing glutathione (GSH) (Halliwel, 2007).

2.14.2 Metabolic antioxidants

Metabolic antioxidants, belonging to endogenous antioxidants, are produced by metabolism in the body, such as lipoid acid, glutathione, L-ariginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc (Willcox *et al.,* 2004); while, nutrient antioxidants, belonging to exogenous antioxidants, are compounds which cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc. Nutrient antioxidants have been shown to be involved in detoxification of the reactive oxygen species (ROS) (Gupta and Singh, 2013) and play an important role in helping endogenous antioxidants for the neutralization of oxidative stress (Donaldson, 2004). The nutrient antioxidant deficiency is one of the causes of numerous chronic and degenerative pathologies and cancer. Each nutrient is unique in terms of its structure and antioxidant function (Willcox *et al.,* 2004).

2.15 Antioxidant Process

When an antioxidant destroys a free radical, this antioxidant itself becomes oxidized (Bahorun *et al.,* 2006). Therefore, the antioxidant resources must be constantly restored in the body. Thus, while in one particular system, an antioxidant is effective against free radicals; in other systems, the same antioxidant could become ineffective. Also, in certain circumstances, an antioxidant may even act as a pro-oxidant, for example, it can generate toxic ROS (Young and Woodside, 2001). The antioxidant process can function in one of two ways: chain-breaking or prevention. For the chain-breaking process, when a radical releases or steals an electron, a second radical is formed. The last one exerts the same action on another molecule and continues until either the free radical formed is stabilized by a chain-breaking antioxidant (vitamin C, E, carotenoids, etc) or it simply disintegrates into an in-offensive product (Bahorun *et al.,* 2006). The classic example of such a

chain breaking reaction is lipid peroxidation. For the preventive way, an antioxidant enzyme like superoxide dismutase, catalase, and glutathione peroxidase can prevent oxidation by reducing the rate of chain initiation, for example, either by scavenging initiating free radicals or by stabilizing transition metal radicals such as copper and iron (Young and Woodside, 2001).

Signaling In order to prevent oxidative stress, the cell must respond to ROS by mounting an antioxidant defence system. Antioxidant enzymes play a major role in reducing ROS levels; therefore, redox regulation of transcription factors is significant in determining gene expression profile and cellular response to oxidative stress. Ref-1 is a multifunctional protein that not only regulates transcription factor activity but also mediates base excision repair. The transcriptional regulatory function of Ref-1 is mediated through its redox activity on several transcription factors such as activator protein 1 (AP-1), p53, nuclear factor kappa B (NFkB), and hypoxia inducible factor 1 (HIF-1α). The N-terminus region of Ref-1 is responsible for redox activity; while the AP-endonuclease activity domain is located at the C-terminal region (Xanthoudakis *et al*., 1994).

Cellular antioxidant defence machinery has been unequivocally established as an oxidative stress counteracting entity. Antioxidant supplementation/treatment has been adopted for either prevention of or protection against several disorders and pathophysiological states; wherein, oxidative stress has been established as a causative mechanism. Naturally occurring phytochemical antioxidants have occupied a prominent position as effective antioxidants for the prevention and/or treatment of several disorders and diseases among humans (Bahorun *et al.*, 2006). The premise for this has been the antioxidant actions of the phytochemicals as free-radical scavengers, oxidative stress relievers, and lipoperoxidation inhibitors (Scalbert *et al.*, 2005). Phytochemical antioxidants include simple molecule antioxidants such as vitamins C, E, and K; plant pigments such as carotenoids (β-carotene), xanthophylls, lycopene, anthocyanins, and phaeophytins; and secondary

plant metabolites, including simple phenolics to more complex polyphenols (Bors and Michel, 2002). Some of these phytochemical polyphenols, in addition to acting as antioxidants, will also function as pro-oxidants that cause oxidative stress (Lambert and Elias, 2010). The pro-oxidant action of tea polyphenols has been linked to their anticancer actions (Forester and Lambert, 2011). Also, Morinda citrifolia (Noni) has been found to alter oxidative stress marker, Malondialdehyde (MDA) and Antioxidant Activity (Superoxide Dismutase and Catalase activity) in Cervical Cancer Cell Lines (Gupta and Singh, 2013). Polyphenols are known for their complexing abilities (chelation) with trace metals (Scalbert *et al.,* 2005).

2.16 Mitochondrial Dysfunction and Neurodegenerative Diseases

The mitochondria are the most important and essential organelles for energy production and cellular metabolism. The mitochondria are critical energy conservation centers in human cells. Through oxidative phosphorylation they create ATP with the food that humans eat and the oxygen that humans breathe (Rego and Oliveira, 2003). ATP is the energy currency that every cell needs in order to carry out processes that are needed to allow an organism to survive. Some cells, like muscle cells, can survive without a lot of mitochondria, because they can survive on the "glycolytic oxidation of stored glucose molecules" (Fiskum *et al.,* 1998), however, this is not the case with neurons. Neurons need the mitochondria to be fully functional because they are highly metabolically active and do not have a backup system as some other cells do for energy production. (Fiskum *et al.,* 1998).

When mitochondria in neurons do become dysfunctional, many problems can occur. One way the mitochondria become damaged and dysfunctional is through ROS (Nicholson *et al.,* 2010). Which cause a decrease in the functionality of many needed mitochondrial enzymes. These enzymes include pyruvate dehydrogenase and cytochrome oxidase. The cell counts on these enzymes for

both the Krebs cycle and glucose metabolism and once deficient in these enzymes, the mitochondria become dysfunctional (Colca and Feinstein, 2012). The stress associated with enzyme malfunctions in metabolic pathways may also account for some mutagenesis in the mitochondrial DNA. Another way mitochondria are proposed to become dysfunctional is through mutations accumulated over a person's lifetime. The mtDNA is known for "large scale deletions or point mutations" that cause adverse effects to the mitochondria function by negatively affecting energy production processes (Lin and Beal, 2006).

Once some of the mitochondria are not working, less ATP are produced because oxidative phosphorylation becomes less prevalent in a given cell. With less ATP, fewer processes in the cell take place. These processes include autophagy and lysosomal degradation. Unfortunately, these processes are necessary for getting rid of proteins that are folded improperly and for getting rid of many other waste products in the cell. When the mitochondria do not make enough ATP to perform these processes, the cell cannot dispose or recycle these deformed proteins and they accumulate and aggregate (Duthie, 2011).

In addition, if the cell no longer has enough ATP, it cannot perform normal cell-to-cell communication via the synapses (Colca and Feinstein, 2012). This is an important function for the nervous system because it keeps the brain in communication with the rest of the body and the external environment. This loss of critical information exchange by the central nervous system leads to the visible signs and progressive pathology of neurodegenerative disease (Ferguson and Schlothauer, 2012). The mitochondria in a cell are also known for keeping calcium levels stable, as an excess in calcium can lead to cell death. (Castellani *et al.,* 2002) This means that dysfunction in the mitochondria is able to cause spikes in calcium levels, for it no longer has sufficient ATP to maintain membrane potential to keep the cells in a state of homeostasis. (Rego and Oliveira, 2003).

When calcium levels fluctuate in the cell, apoptosis is usually initiated because more calcium creates increased sensitivity to "apoptotic stimuli" (Rizzuto *et al.,* 2003). These apoptotic stimuli include the Bax and Bcl proteins, which activate the opening of the mitochondrial permeability transition pore and eventually this leads to cell death.

Studies have shown that with dysfunctional mitochondria comes an increase in reactive oxygen species that are produced in the mitochondria. This may be due to an alteration in the electron transport chain which causes defects in the enzymatic complexes, resulting in the release of an excess of ROS (Benzi and Moretti, 1995). Reactive oxygen species endanger the neuron by causing damage to membranes, proteins, and nucleic acids. Not only do reactive oxygen species cause more damage to the mitochondria, but they also initiate apoptosis, programmed death of the cell. Apoptosis is a very orderly process in the cell, and it happens only in extreme cases when the cell knows it cannot be repaired (Castellani *et al.,* 2002).

When mitochondria go through dysfunctional changes, there is a very good chance the cell will go through apoptosis. One trigger of apoptosis is through the opening of the mitochondrial permeability transition pore (MPT) on the inner membrane of the mitochondria (Castellani *et al.,* 2002). The MPT is activated during a decrease in mitochondrial function characterized by an increase in reactive oxygen species, a decrease in ATP production, and an associated rise in calcium. The opening of this pore induces the release of the cytochrome c protein which activates caspases (Castellani *et al.,* 2002) causing apoptosis (Colca and Feinstein, 2012). The sensitivity of the mitochondrial permeability transition pore to increased calcium has been shown to be increased by oxidative stress. Therefore dysfunctional mitochondria with increased ROS are creating an opportune environment for apoptosis through the opening of the MPT pore (Cho *et al.,* 2010; Colca and Feinstein, 2012).

2.17 Oxidative Stress and Neurodegenerative Diseases

Reactive oxygen species and reactive nitrogen species cause unfavorable effects to all cells in the body, especially the neurons in the brain (Multhaup *et al.,* 1997). The brain does not regenerate as other parts of the body do, and it is very active metabolically, therefore very susceptible to injury by oxidative stress (Andersen, 2004). Many of the reactive oxygen species are made in the mitochondria. These species usually include "hydroxide and superoxide radicals and hydrogen peroxide" (Rahman *et al.,* 2012). Nitric oxide (NO) is made by the N-methyl-D-aspartate glutamate receptors in the nervous system. When these receptors are overly activated, this initiates a "calcium influx" making nitric oxide and other reactive oxygen species (Rahman *et al.,* 2012). Usually in the healthy brain a normal amount of reactive oxygen species is not harmful, but in fact it is needed. Reactive oxygen species are involved in cell signaling in small amounts (Calabrese *et al.,* 2007). However, in the diseased aging brain, reactive oxygen species accumulate and cause protein accumulation and aggregation. In addition to the increased production of reactive oxygen species, there is a decreased production of antioxidant agents such as the enzymes "superoxide dismutase, glutathione peroxidase, and glutathione reductase" (Andersen, 2004) in the brains of persons with neurodegeneration (Benzi and Moretti, 1995). The ROS and NOS are able to directly cause protein accumulation by destroying chaperone and proteasomal processes. The function of the molecular chaperone is to find misfolded proteins and help to fold them properly. The ubiquitin-proteasome-system (UPS) functions to find and degrade proteins that are misfolded. Without these two processes, misfolded proteins would accumulate, and eventually aggregate. A number of studies have shown that NO seems to have destructive effects on the chaperones and UPS proteins (Nakamura and Lipton, 2007). Antioxidant functions are impaired at this point in a neurodegenerating cell body so there is no defense against this cycle as it takes over the brain

(Rahman *et al.,* 2012). Damage to the mitochondria will cause a depletion of ATP, an influx in calcium, and in most cases the opening of the mitochondrial permeability transition pore leading to apoptosis

Reactive oxygen species not only cause direct protein accumulation and mitochondrial dysfunction, but can also cause glial cell activation and inflammation. (Salminen *et al.,* 2012) When glial cells detect damage induced by reactive oxygen species, the microglia are stimulated to activate genes that are involved in the release of cytokines, NO, and NADPH oxidase (Andersen 2004). The cytokines include proinflammatory interleukins-1, and -6, and tumor necrosis factor-α (TNF-α). These things, in turn, cause damage to the neurons by over-inflammation, tissue damage, and the production of more reactive oxygen species. NO has detrimental effects on the cell and once NO is mixed with the superoxide anion reactive oxygen species, peroxynitrite ($ONOO^-$) is formed which can nitrosylate proteins causing them to become deformed and accumulated. (Zhang *et al.,* 2010). This form of reactive nitrogen species is thought to be a major part of the β-amyloid protein toxicity in Alzheimer's disease (Qin *et al.,* 2002; Andersen, 2004).

Certain glial cells known as astrocytes have a specific function in neurodegeneration as well. Astrocytes are the cells responsible for glutamate, an excitatory factor of the central nervous system. Over activation of the astrocytes causes altered glutamate handling, leading to abnormal excitatory processes and calcium signalling wave. (Malta *et al.,* 2012). Increased activation of astrocytes leads to increased chances of death of dopaminergic neurons as seen in the pathogenesis of Parkinson's disease (Andersen, 2004).

The reactive oxygen species created throughout many processes in neurodegeneration have deleterious effects on the central nervous system. The ROS are an initiating factor in the process of apoptosis. These molecules can cause direct damage to open the mitochondrial permeability

transition pore which activates transduction pathways, kinases and releasing cytochrome c. ROS can also have damaging effects on things like RNA and DNA (Krantic *et al.,* 2005). The damage done to RNA and DNA can then force a cell into programmed cell death. Reactive oxygen species activate astrocytes to cause glutamate excitotoxicity. With this comes a cascade of events causing programmed cell death through the activation of NMDA (N-Methyl-D-Aspartate) receptors. This causes an influx of calcium, production of nitric oxide (NO), and depolarization of the mitochondrial membrane. This then causes the mitochondria to produce more ROS to create more damage, especially to the DNA. An enzyme named poly-ADP-ribose polymerase-1 then comes in to help relocate the damaged DNA. If the enzyme is not present as in many cases of neurodegeneration, a translocation to the nucleus cannot occur, therefore the death caspases are activated, and causing apoptosis to be initiated (Krantic *et al.,* 2005). This is an especially dominant process in motor neurons in the cortex, brain stem, and spinal cord in Amyotrophic Lateral Sclerosis and in the substantia nigra in Parkinson's disease (Fontbonne *et al.,* 2001).

Reactive oxygen species cause damage to the mitochondria, which is in the most part, where they are produced. They cause damage to the mtDNA itself to produce a vicious cyc le of the mitochondria that is never ending. When ROS start to accumulate, they activate the glial cells, more specifically, the microglia and astrocytes, to cause neuroinflammation and glutamate excitotoxicity (Ramalingam and Kim, 2012)

2.18 Curcumin

Curcumin, commonly called diferuloyl methane, is a hydrophobic polyphenol derived from the rhizome (turmeric) of the herb Curcuma longa (Priyagarsini, 2013). Turmeric has been used

traditionally for many ailments because of its wide spectrum of pharmacological activities. Curcumin has been identified as the active principle of turmeric; chemically, it is a bis-a, b-unsaturated b-diketone that exhibits keto-enol tautomerism. Scientific research spanning over more than four decades has confirmed the diverse pharmacological effects of curcumin and established its ability to act as a chemopreventive agent as well as a potential therapeutic agent against several chronic diseases (Esatbeyoglu *et al.*, 2012; Priyagarsini, 2013). Curcumin, having nearly a two centuries old scientific history, is still attracting researchers from all over the world (Shishodia *et al.,* 2005). Curcumin has been shown to exhibit antioxidant, anti-inflammatory, antimicrobial, and anticarcinogenic activities. It also has hepatoprotective and nephroprotective activities, suppresses thrombosis, protects against myocardial infarction, and has hypoglycemic and antirheumatic properties (Jagetia and Aggarwal, 2007). Moreover, curcumin has been shown in various animal models and human studies to be extremely safe even at very high doses (Aggarwal *et al.*, 2007; Kunnumakkara *et al.,* 2008). Inspite of its efficacy and safety, curcumin has not yet been approved as a therapeutic agent (Anand *et al.*, 2007). The poor aqueous solubility, relatively low bioavailability, and intense staining color of curcumin have been highlighted as major problems.

Table 2.1. Local names of curcumin (U.A. Garkuwa (Personal table, February, 2 2017))

Language	English	Hausa	Yoruba	Arabic
Local name	Curcumin	Kurkur	Atale ile pupa	Kurkum

Plate 1: Curcumin powder (U. A. Garkuwa (Personal photograph, February 2, 2017))

2.19 Analogues and derivatives

Curcumin is a member of the linear diarylheptanoid class of natural products in which two oxy-substituted aryl moieties are linked together through a seven-carbon chain. The C7 chain of linear diarylheptanoids is known to have unsaturation, oxo functions, enone moiety, and a 1,3-diketone group. Except for the oxo and hydroxy functions, the C7 chain is generally unsubstituted. This unsaturation in the linker unit has an E-configuration (trans C C bonds). The aryl rings may be symmetrically or unsymmetrically substituted; the most prevalent natural substituents are of the oxy type, such as hydroxy or methoxy elements (Shishodia *et al.*, 2007) **2.19.1 Natural analogues from turmeric**

Turmeric contains three important analogues, curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC). Collectively called curcuminoids, the three compounds differ in methoxy substitution on the aromatic ring. While curcumin has two symmetric o-methoxy phenols linked through the a,b-unsaturated b-diketone moiety, BDMC, also symmetric, is deficient in two o-methoxy substitutions, and DMC has an asymmetric structure with one of the phenyl rings having o-methoxy substitution. Of the three curcuminoids, curcumin is the most abundant in turmeric, followed by DMC and BDMC. Commercially available curcumin mixture contain 77% curcumin, 17% DMC, and 3% BDMC. A lesser known curcuminoid from turmeric is cyclocurcumin, first isolated and characterized (Anand *et al.*, 2008).

2.20 Structure-Activity Correlation

Although curcumin, DMC, and BDMC differ in their chemical structures only with regard to methoxy substitution, they exhibit significantly different antioxidant, antitumor, and anti-inflammatory activities (Anand *et al.*, 2008). To date there has been no systematic study that clearly correlates the physicochemical and molecular properties of the three curcuminoids with

their biological activities. However, the existing literature provides some clues to understanding which group is actually responsible for a given biological activity of the curcuminoids since many reports suggest that curcumin has better radical scavenging and antioxidant ability than the other two, and that DMC is superior to BDMC in this activity (Dairam *et al.,* 2007).

The ability of curcuminoids to act as antioxidants or prooxidants in the presence of metals such as Cu(II), Fe(II), or Pb(II) arises mainly from their chelating power (Dairam *et al.,* 2007). Although transition metal-chelation by curcumin can take place through either the diketone moiety or the o-methoxy phenol moiety, in most cases chelation is observed only through the diketo group. Since the three curcuminoids possess similar diketone moieties, their effects on metal-induced toxicity should be similar. The reasons and the actual mechanism of the antitumor activities of the curcuminoids are still far from understood. It is still not known why the o-methoxy-deficient BDMC is a more potent ROS inducer and the o-methoxysubstituted curcumin is a more potent suppressor of NF-kB activation (Sandur *et al.,* 2007). The effect of change in the lipophilicity of the curcuminoids with methoxy substitution in influencing some of these activities also cannot be ignored. Hydrogenation of the heptadiene moiety in curcumin to produce THC markedly increased the antioxidant activity but significantly reduced the antitumor and anti-inflammatory abilities. It is clear that the o-methoxy phenol groups, when not linked through conjugation with the b-diketone moiety, make the molecule a better antioxidant (Sandur *et al.,* 2007)

2.21 Antioxidant Activity

The antioxidant activities of curcumin and related compounds have been investigated by a variety of assay systems, in both in vitro and in vivo conditions. The disparity in assay conditions makes exact comparisons rather difficult. In one of the early papers on the antioxidant activity of curcumin and its derivatives, it was observed that the phenolic hydroxyl groups are needed for

antioxidant activity and that the presence of more than one of these groups, as in the curcumin derivative bis (3,4-dihydroxycinnamoyl) methane, confers better activity than that of curcumin itself (Sharma *et al.,* 1976). The mechanistic aspects of curcumin antioxidant activity have been more recently investigated at length, and the studies seem to suggest that the phenolic OH groups are important in the antioxidant activity (Chen *et al.,* 2006). A possible role for the diketone moiety was suggested based on their observations using dimethyltetrahydrocurcumin and further advocated by the work of Jovanovic *et al*. (1999). The presence of an ortho alkoxy group seems to potentiate the antioxidant activity (Ligeret *et al.,* 2006), as does an additional hydroxy group as in bis (3,4-dihydroxy) cinnamoylmethane (Chen *et al.,* 2006; Wei *et al.,* 2006). The effect of the position of the hydroxyl group has been investigated under in vivo conditions, and that the 2-hydroxyphenyl group, as seen in bis (2-hydroxycinnmoyl) methane, yields better antioxidant activity than the 4-hydro-xyphenyl group, as present in curcumin (Weber *et al.,* 2006). The desirability of the bdiketo unit has been studied by Sardijiman *et al*. (1997) using bis (4-hydroxybenzylidene) acetones, 2,6-bis-benzylidenecyclohexanones, and cyclopentanones having a C5 linker. These workers report that the 4-hydroxyphenyl group confers potent antioxidant activity, which is much enhanced by one, or two, methoxy substituents ortho to the hydroxy group. These C5- linked bis (4-hydroxyphenyl) -1,4-pentadien-3-ones showed greater antioxidant activity than curcumin. In a similar observation among 2,6-bis-benzylidenepiperidones, cycloheptanones and acetones, Youssef *et al.* (2004) demonstrated greater antioxidant activity in those examples that bear a 3-alkoxy-4- hydroxyphenyl unit.

2.22 Anti-inflammatory Activity

Saturation of the alkene and reduction of the carbonyl functions in the C7 linker of curcumin appear to reduce its anti-inflammatory activity by suppressing activation of NF-kB through inhibition of IkB kinase activity (Mohammadi *et al.,* 2005). An early study pointed to the fact that the hydroxyphenyl unit in curcumin confers anti-inflammatory activity since acylation and alkylation of the phenolic hydroxy group of curcumin were found to drastically reduce its anti-inflammatory activity. It was suggested that the presence of a 4-hydroxyphenyl unit is required for anti-inflammatory activity and that this activity seems to increase if additional small-sized alkyl or methoxy groups are present on the adjacent 3- and 5-positions on the phenyl ring (Nurfina *et al.,* 1997)

2.23 Anti-cancer and Anti-carcinogenic Activity

The anti-carcinogenic properties recognized by Talalay *et al.,* (2001) have been demonstrated in curcumin, and it has been suggested that the presence of a hydroxyphenyl group in compounds analogous to curcumin, especially in the 2-position, is supportive of the chemoprotective activity through the ability to induce Phase II detoxification enzymes. The necessity of the ‘‘ene-[1,3-dioxo]-ene’’ C7 linker, however, could not be firmly established; Markaverich *et al.* (1992) suggests that the 2,6-bis(3,4- dihydroxy or 4-hydroxy-3-methoxybenzylidene) cyclohexanones, having only a ‘‘ene-oxo-ene’’ motif, could inhibit cancer cell proliferation in vitro and in vivo.

2.24 Alloxan Monohydrate

Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing cells in the pancreas (that is beta cells) when administered to rodents and many other animal species. This causes an insulin-dependent diabetes mellitus (called "Alloxan Diabetes") in these animals, with characteristics similar to type 1 diabetes in humans (Tyberg *et al.,* 2001).

Alloxan is selectively toxic to insulin-producing pancreatic beta cells because it preferentially accumulates in beta cells through uptake via the GLUT2 glucose transporter. Alloxan, in the presence of intracellular thiols, generates reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid. The beta cell toxic action of alloxan is initiated by free radicals formed in this redox reaction. One study suggests that alloxan does not cause diabetes in humans (Mrozikiewicz *et al.,* 1994). Others found a significant difference in alloxan plasma levels in children with and without diabetes Type 1 (Mrozikiewicz *et al.,* 1994).

2.25 Impact upon Beta Cells

Because it selectively kills the insulin-producing beta-cells found in the pancreas, alloxan is used to induce diabetes in laboratory animals. This occurs most likely because of selective uptake of the compound due to its structural similarity to glucose as well as the beta-cell's highly efficient uptake mechanism (GLUT2) (Tyberg *et al*., 2001). Alloxan is not toxic to the human beta-cell, even in very high doses, probably because of differing glucose uptake mechanisms in humans and rodents (Tyberg *et al*., 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

A total of twenty (20) Swiss Albino Mice of both sexes weighing (20 – 30) grams were used for the study. The animals were housed in plastic cages under standard laboratory conditions with free access to food and water. Animals were allowed for two weeks to acclimatization to the laboratory environment before the commencement of the experiments.

3.1.2 Drugs and reagents

All drugs and reagents were obtained commercially and are of analytical grades.

Curcumin was purchased from Arkure Health Center (Haryana, India). Alloxan was purchased from (Sigma chemical Company St. Louis U.S.A.). A digital glucometer (Accu-Chek Advantage, Roche Diagnostic, Germany) was used for the determination of the blood glucose levels of the animals.

3.2 Methods

3.2.1 Induction of diabetes

The animals were fasted for 12-16 h with free access to water prior to the induction of diabetes. Diabetes was induced by single intraperitoneal injection of Alloxan monohydrate (Sigma St. Louis, U.S.A.) at a dose of 150 mg/kg b w dissolved in 0.9% cold normal saline (Katsumata *et al*., 1999). The mice were then kept for the next 24 h on 5% glucose solution bottles in their cages to prevent hypoglycemic (Dhandapani *et al*., 2002). Hyperglycemia was defined by fasting blood glucose level > 200 mg/dl (Matteucci and Giampietro, 2008)

3.2.2 Experimental design

Twenty Swiss albino mice were used for the study, the animals were divided into five (5) groups of four (4) each. All administration were done orally for 21 days. The animals were grouped as follows:

Group I: Normal (normoglycemic control), received distilled water orally for 21 days

Group II: Diabetic (diabetic control) received olive oil 1 ml/kg orally for 21 days

Group III: Diabetic, received glibenclamide 1 mg/kg (Leon-Reyes *et al.,* 2008) orally for 21 days

Group IV: Diabetic, received curcumin 50 mg/kg orally for 21 days

Group V: Diabetic, received curcumin 100 mg/kg orally for 21 days

3.3 Estimation of Blood Glucose

The blood samples were obtained from the animal tail vein. A glucometer was used to measure the blood glucose levels using glucose oxidase principle (Beach and Turner, 1958) using the digital glucometer (Accu-Check Advantage, Roche Diagnostic, Germany), and results were obtained as mg/dL (Rheney and Kirk, 2000).

3.3.1. Glucose oxidase principle

It involves glucose oxidase enzyme which catalyse the oxidation of beta D-glucose present in the plasma to D- glucono -1, 5- lactone leading to the formation of hydrogen peroxide; it is followed by the slow hydrolysis of the lactone to D- gluconic acid. The hydrogen peroxide produced is then broken down to oxygen and water by a peroxidase enzyme.

3.4 Assessment of Neurobehavioral Deficit

The experimental procedure basically include the Y-maze and the Novel Object Recognition Task assessed before and after treatment.

3.4.1 Y-maze

The Y-maze is composed of three equally spaced arms (at 120°, arm's length 50 cm, width 10 cm, and wall height 20 cm). The floor of each arm is made of Perspex. Y-maze is a quick and useful initial test for general cognitive function. This test is based on the innate preference of animals to explore an arm that has not been previously explored (Hughes, 2004). Y-maze function is sensitive to damage in areas concerned with learning and memory functions such as the hippocampus, and is also disrupted by drugs that cause memory loss (Hughes, 2004).

Spontaneous alternation version: In this version each mouse was placed in the Y-maze for 6-8 min and the number of arms entered as well as the sequence of entries is recorded and a score is calculated to determine alternation rate. An alternation is defined as entry into all three arms consecutively (Hughes, 2004), for instance if the animal makes the following arm entries; AC , CA,B,C,A,CAB,C,A, in this example, the animal made 13 arm entries 8 of which are correct alternations. The number of maximum spontaneous alternations is then the total number of arms entered minus two, and the percentage alternation is calculated as ((actual alternations /maximum alternations) x 100). A high alternation rate is indicative of sustained spatial working memory as the animals must remember which arm was entered last to not re-enter it (Hughes, 2004).

Plate 3.1. Y maze test (U. A. Garkuwa (Personal photograph, Feb. 2, 2017))

3.4.2 Novel object recognition task:

The Novel object memory task is an open field assessment of the natural tendency of mice to investigate a novel object instead of a familiar one. The choice to explore the novel object as well as the reactivation of exploration after object displacement reflects the use of learning and (recognition) memory processes.

This task comprised a sample phase and a test phase separated by a 24 hours delay. In the sample phase, the mice were presented with two same objects. These objects were placed in the corners of an arena 15 cm from each adjacent wall. Each rat were placed in the center of the arena and allowed to explore the objects for 5 min. between the sample and test phases, all of the objects were cleaned with alcohol to remove olfactory cues. In the test phase, one of the objects was changed, and the mice were allowed to explore the objects for 5 min. The time spent exploring the two objects that had changed was compared with the time spent exploring the other object (spatial memory, recognition and discrimination ability). If spatial memory and discrimination ability are intact, the rat will spend more time exploring the two objects that are in different locations compared with the two objects that are in the same locations. The discrimination ratio was calculated as the difference in time spent by each animal exploring object(s) that changed position compared with the object(s) that remained in the same position divided by the total time spent exploring all objects (Barker *et al.,* 2007; Baxter, 2010; Antunes and Biala, 2012).

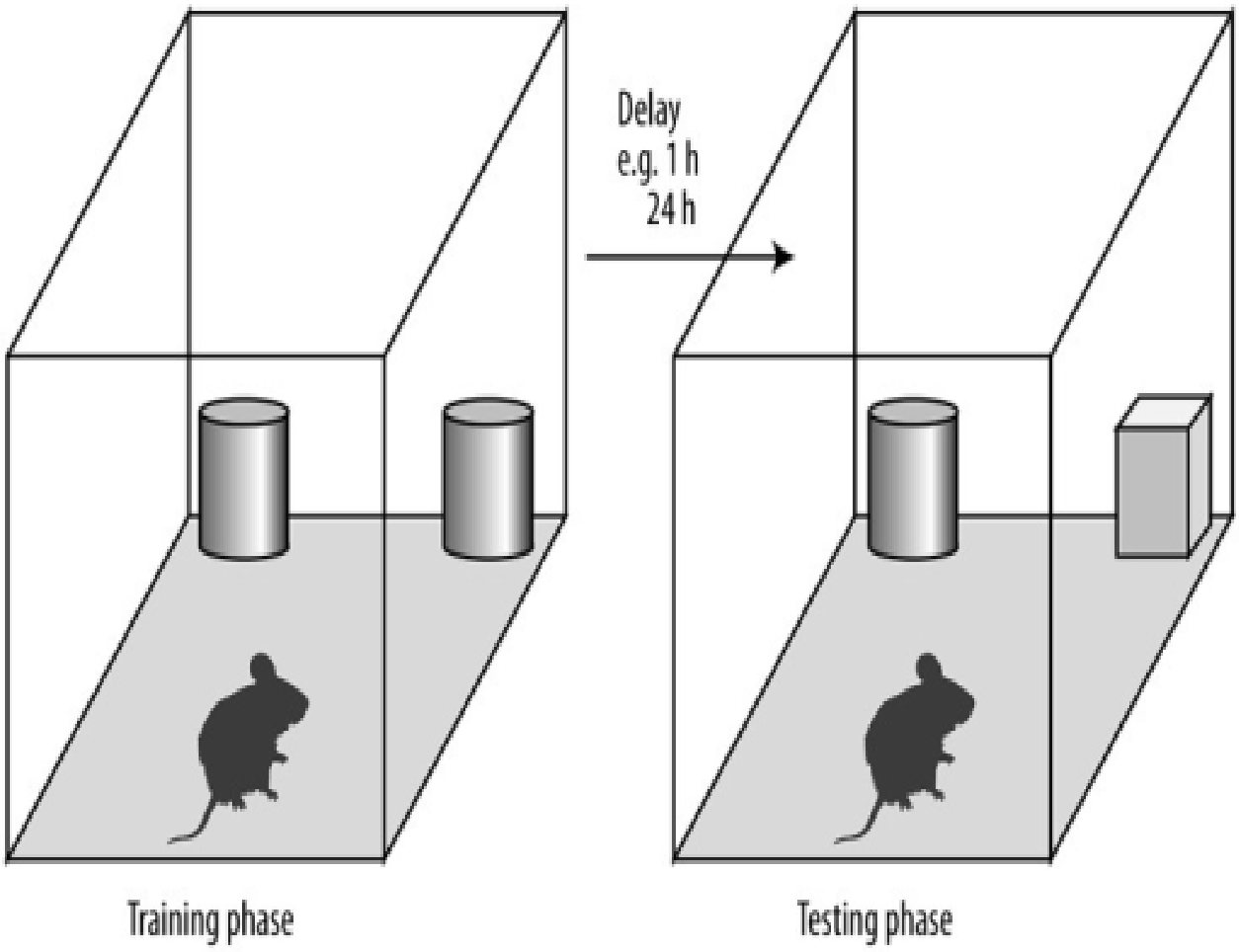

Figure 3.2. Novel object recognition test (Brodziak *et al.*, 2014)

3.5 Estimation of Oxidation Stress Biomarkers

3.5.1 Catalase activity

The serum catalase (CAT) activity was determined using mice catalase ELISA kit in accordance to the manufacturers manual. **Principle:** the kit uses enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay mice CAT. **Procedure:** the standard solution of mice Catalase (CAT) was added to wells that are pre-coated with Catalase (CAT) monoclonal antibody and then incubated. After incubation, the anti Catalase (CAT) antibodies labelled with biotin to unite with Streptavidin-HRP was added, which forms the immune complex. The unbound enzymes after incubation and washing was removed, then

substrate A and B was added. The solution turned blue and change to yellow with the effect of acid. The shades of solution and the concentration of Rat Catalase (CAT) were positively correlated. A microplate reader was then used to read the absorbance of all wells and was recorded. A graph of concentration of standards was plotted against their absorbance. The absorbance and concentration was obtained from the corresponding axis and recorded

3.5.2 Superoxide dismutase activity

The superoxide dismutase (SOD) activity was determined using mice superoxide dismutase ELISA kit in accordance to the manufacturers manual. **Principle:** the kit uses enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay mice SOD. **Procedure:** the standard solution of mice SOD was added to wells that are pre-coated with SOD monoclonal antibody and then incubated. After incubation, the anti-SOD antibodies labelled with biotin to unite with Streptavidin-HRP was added, which forms the immune complex. The unbound enzymes after incubation and washing was removed, then substrate A and B was added. The solution turned blue and change to yellow with the effect of acid. The shades of solution and the concentration of mice superoxide dismutase (SOD) were positively correlated. A microplate reader was then used to read the absorbance of all wells and was recorded. A graph of concentration of standards was plotted against their absorbance. The absorbance and concentration was obtained from the corresponding axis and recorded

3.5.3 Glutathione peroxidase activity

The glutathione peroxidase (GPx) activity was determined using mice glutathione peroxidase ELISA kit in accordance to the manufacturers manual. **Principle:** the kit uses enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay mice GPx. **Procedure:** the standard solution of mice GPx was added to wells that are pre-coated

with GPx monoclonal antibody and then incubated. After incubation, the anti-GPx antibodies labelled with biotin to unite with Streptavidin-HRP was added, which forms the immune complex. The unbound enzymes after incubation and washing was removed, then substrate A and B was added. The solution turned blue and change to yellow with the effect of acid. The shades of solution and the concentration of mice glutathione peroxidase (GPx) were positively correlated. A microplate reader was then used to read the absorbance of all wells and was recorded. A graph of concentration of standards was plotted against their absorbance. The absorbance and concentration was obtained from the corresponding axis and recorded

2.5 Statistical Analysis

All data obtained were presented as Mean ± standard error of mean (SEM). The data were analysed using one way mixed analysis of variance (ANOVA) followed by *Tukey's post hoc* test to determine the level of significance using SPSS (version 22). Values of $p < 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Blood Glucose Levels of Curcumin Treated Diabetic Swiss Albino Mice

Table 4.1 shows the results of the effects of Curcumin (50 mg/kg and 100 mg/kg) on blood glucose level of alloxan-induced diabetic Swiss albino mice. The Curcumin treated groups showed significant ($P < 0.05$) decrease in the fasting blood glucose levels after 21 days of administration, when compared to diabetic control group (group II) treated with olive oil with values of 108.25 ± 16.01 mg/dl and 114.75 ± 5.56 mg/dl (with percentage glycemic change of 59.73% and 61.00%) for 50 mg/kg and 100 mg/kg compared to 221.50 ± 6.12 mg/dl (8.00%) respectively

Table 4.1: Effect of Curcumin on Fasting Blood Glucose Levels of Alloxan-induced Diabetic Swiss Albino Mice.

Groups	**day 0 (mg/dl)**	**day 21 (mg/dl)**	**PGC (%)**
Normoglycemic control	96.25 ± 6.79^{a}	94.00 ± 6.12^{a}	2.34
Diabetic control	240.75 ± 5.85^{b}	221.5 ± 14.03^{b}	8.00
Glib 1 mg/kg	272.75 ± 14.53^{b}	126.5 ± 11.32^{a}	53.62
Cur 50 mg/kg	267.50 ± 11.53^{b}	108.25 ± 16.01^{a}	59.73
Cur 100 mg/kg	294.25 ± 31.15^{b}	114.75 ± 5.56^{a}	61.00

Values having superscript letter a is statistically significant ($p < 0.05$) compared with b; PGC = percentage glycemic change, Cur = curcumin and Glib = Glibenclimide

4.2 Percentage Alternation of Curcumin Treated Diabetic Swiss Albino Mice

Figure 4.1 Bar chart showing the results of the effects of curcumin (50 mg/kg and 100 mg/kg) on spatial working memory of alloxan-induced diabetic Swiss albino mice. The 100 mg/kg dose of curcumin showed significant ($P < 0.05$) increase in the percentage spontaneous alternation after 21 days of administration, when compared to the diabetic control group treated with olive oil with values of 74.39 ± 8.06 % compared to 47.50 ± 13.65 % respectively

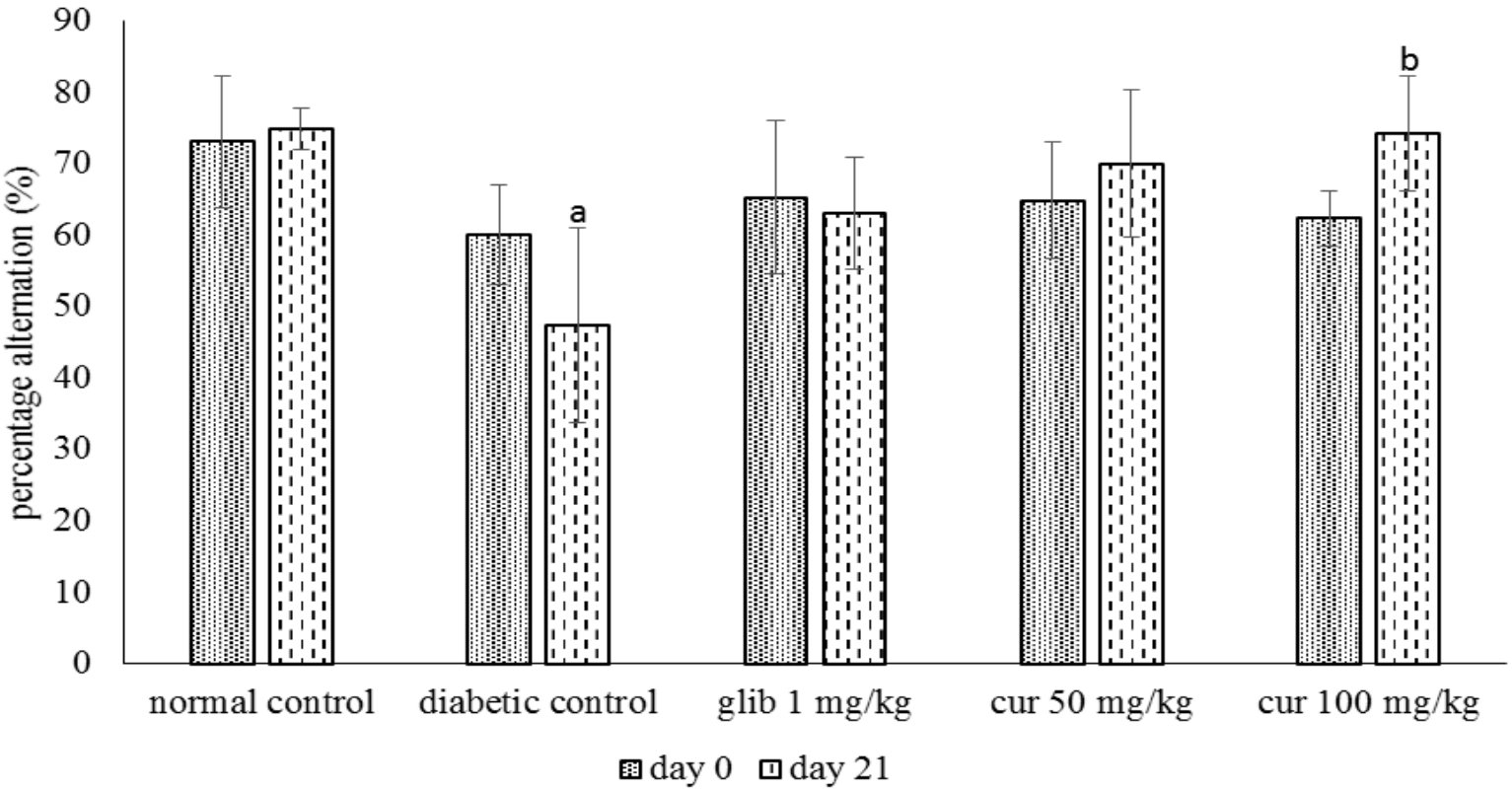

Figure 4.1: Effect of Curcumin on Spatial Working Memory using Y-maze test in Alloxan-Induced Diabetic Swiss Albino Mice.

Values with error bars having different superscripts letters a,b are significant ($p < 0.05$); a = compared with normal and b compared with control. Cur = curcumin and Glib = Glibenclimide

4.3 Short Term Memory of Curcumin Treated Diabetic Swiss Albino Mice

Figure 4.2: Bar chart showing the results of the effects of curcumin (50 mg/kg and 100 mg/kg) on short term memory of alloxan-induced diabetic Swiss albino mice. The curcumin treated groups showed significant ($P < 0.05$) increase in the time spent exploring the novel object after 21 days of administration, when compared to the pre-treatment (day 0) with values of 11.66 ± 9.39 seconds and 14.85 ± 6.46 seconds compared to the -11.20 ± 0.55s and -10.90 ± 6.46 seconds respectively.

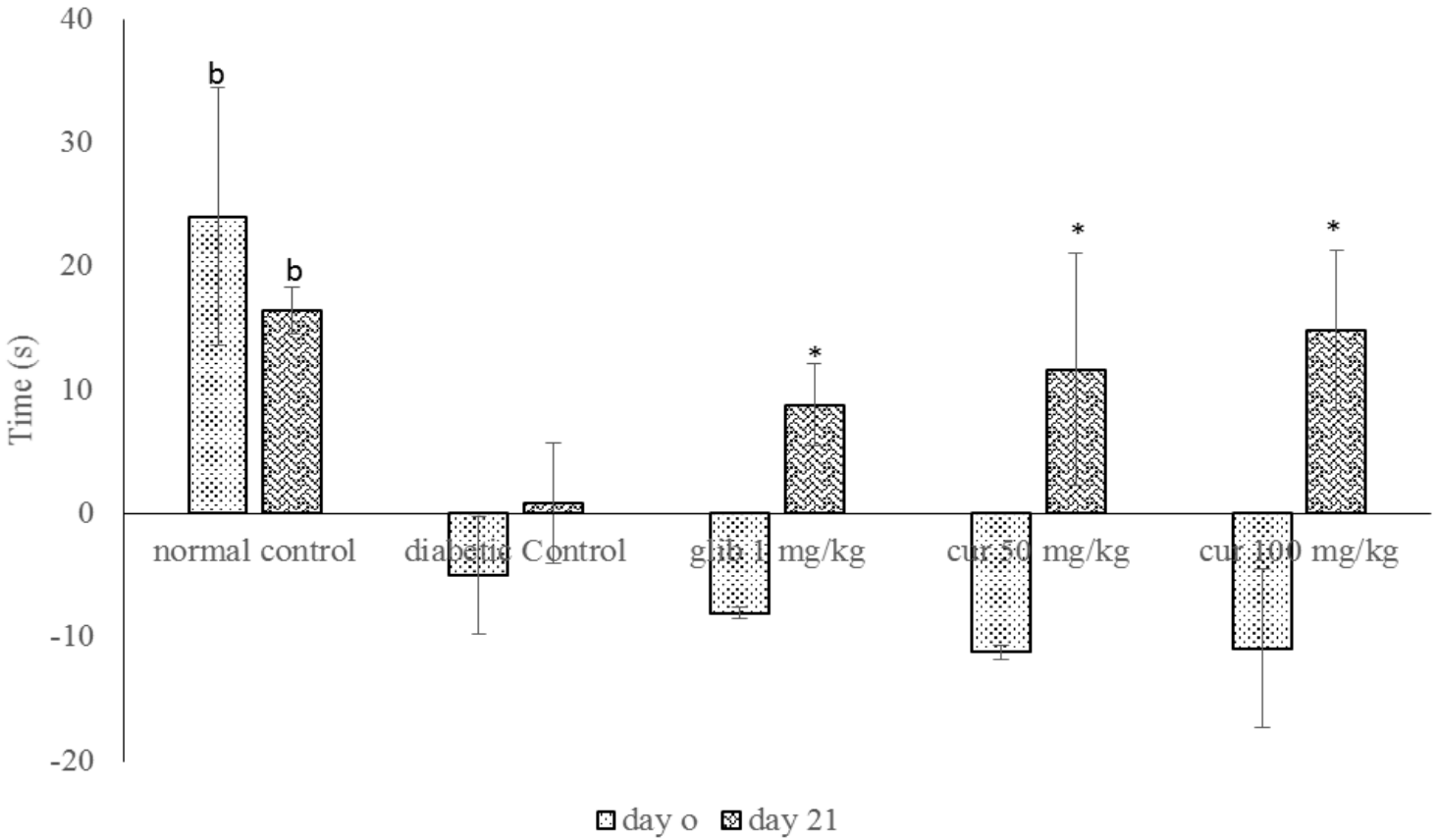

Figure 4.2: Effect of Curcumin on the Difference using NORT in Alloxan-Induced Diabetic Swiss Albino Mice. Values with error bars having different superscripts letters b,* are significant ($p < 0.05$); b = compared with control and * = with day 0. Cur = curcumin and Glib = Glibenclimide

4.4 Discrimination Index of Curcumin Treated Diabetic Swiss Albino Mice

Figure 4.3 Bar chart showing the results of the effects of curcumin (50 mg/kg and 100 mg/kg) on discrimination index level of alloxan-induced diabetic Swiss albino mice. The curcumin treated groups showed significant ($P < 0.05$) increase in discrimination after 21 days of administration, when compared to the pre-treatment (day 0) with values of 0.39 ± 0.04 and 0.24 ± 0.21 seconds compared to the -0.44 ± 0.03 and -0.27 ± 0.11 seconds respectively

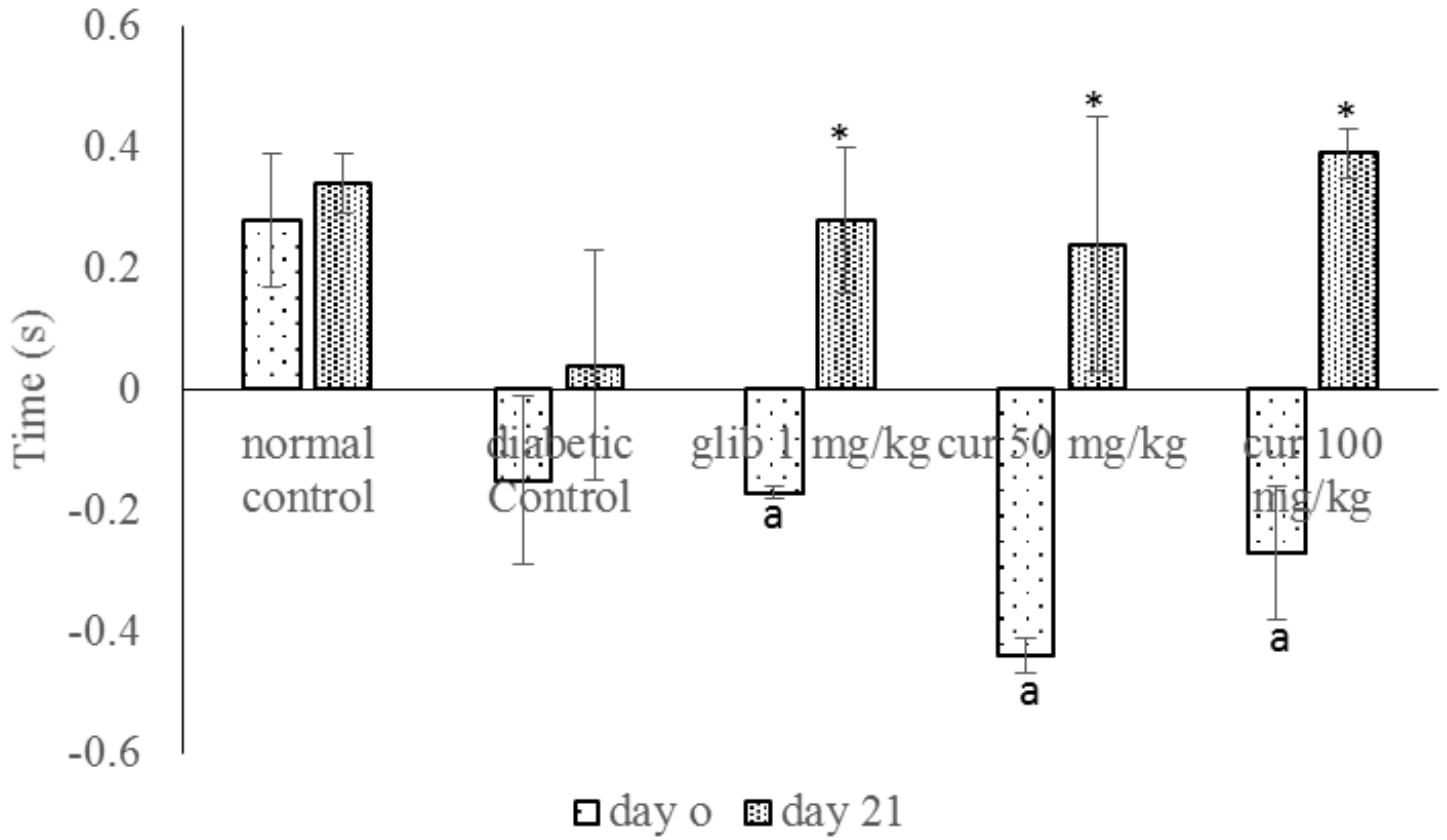

Figure 4.3: Effect of Curcumin on Discriminatory Memory using NORT in Alloxan-Induced Diabetic Swiss Albino Mice. Values with error bars having different superscripts letters a,* are significant ($p < 0.05$). Cur = curcumin and Glib = Glibenclimide

4.5 Recognition Index of Curcumin Treated Diabetic Swiss Albino Mice

Figure 4.4 Bar chart showing the results of the effects of curcumin (50 mg/kg and 100 mg/kg) on recognition index of alloxan-induced diabetic Swiss albino mice. The curcumin treated groups showed significant ($P < 0.05$) increase in the recognition after 21 days of administration (post-treatment), when compared to the pre-treatment (day 0) with values of 63.71 ± 2.95 and 58.17 ± 2.55 seconds compared to the 36.04 ± 8.19 and 38.20 ± 4.73 seconds respectively

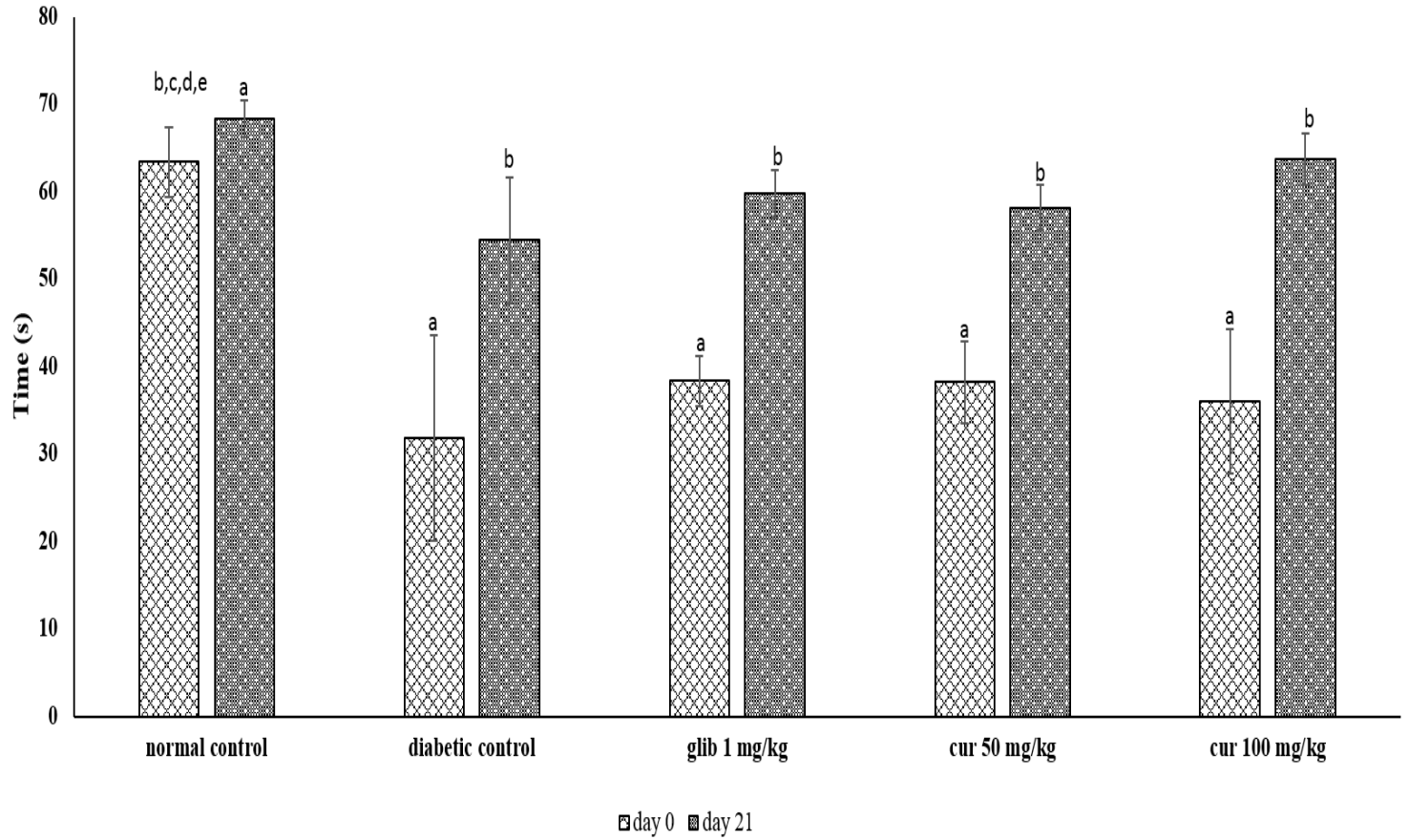

Figure 4.4: Effect of Curcumin on Recognitive Index using NORT in Alloxan-Induced Diabetic Swiss Albino Mice. Values with error bars having different superscripts letters a,b,c,d,e and * are significant ($p < 0.05$); a,b,c,d,e and * = compared to normal, diabetic, glib, cur 50, cur 100 and with day 0 respectively. Cur = curcumin and Glib = Glibenclimide

4.6 Serum Catalase Levels of Curcumin Treated Diabetic Swiss Albino Mice

Figure 4.5 Bar chart showing the results of the effects of curcumin (50 mg/kg and 100 mg/kg) on serum catalase level of alloxan-induced diabetic Swiss albino mice. The curcumin treated groups showed significant ($P < 0.05$) increase in the serum catalase level after 21 days of administration, when compared to control group treated with olive oil with values of 78.92 ± 3.94 IU/L and 85.05 ± 3.23 compared to the 62.27 ± 7.07 IU/L respectively

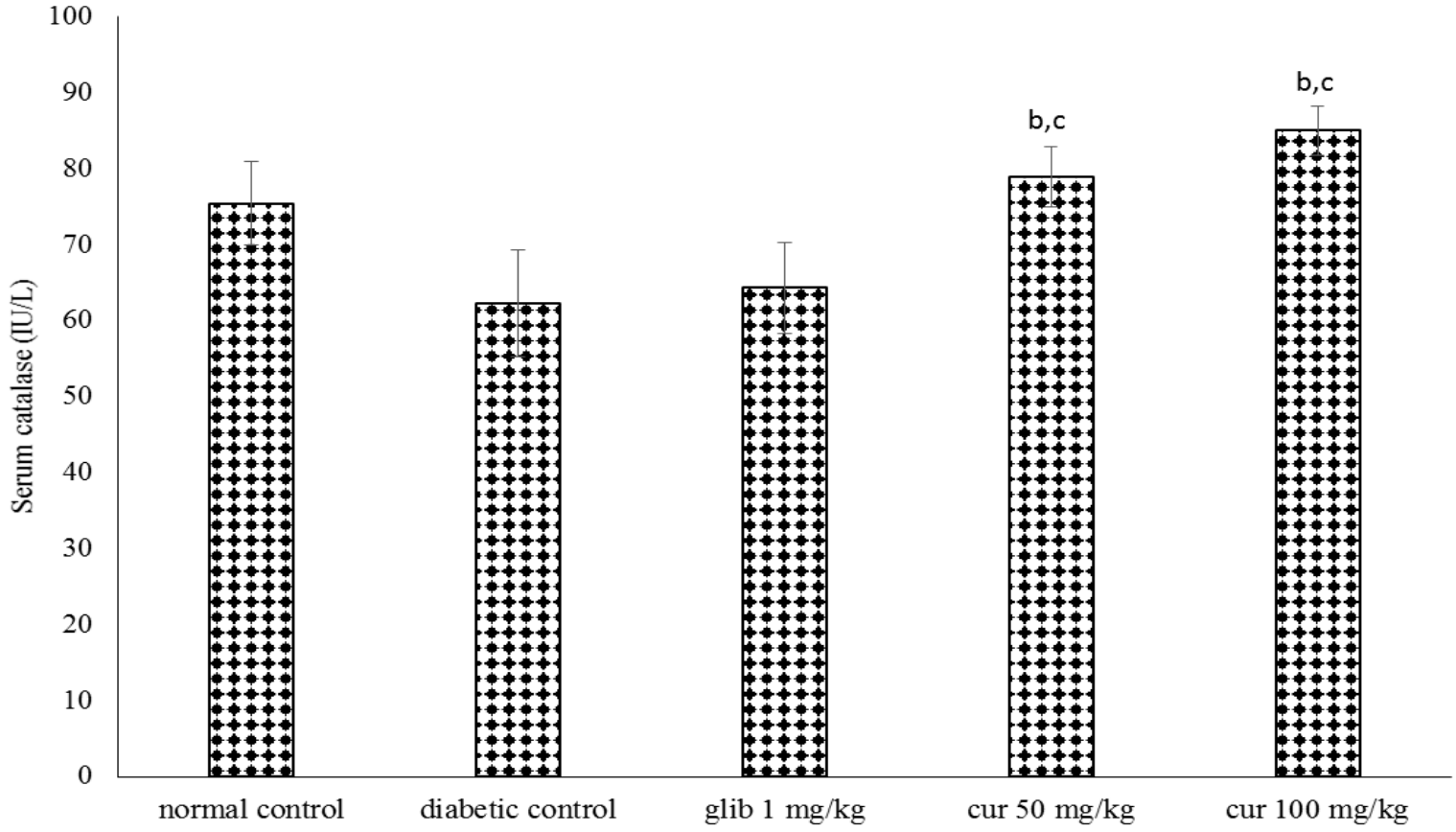

Figure 4.5: Effect of Curcumin on Serum Catalase Level in Alloxan-Induced Diabetic Swiss Albino Mice. Values with error bars having different superscripts letters b,c are significant ($p < 0.05$); b = compared with control (a) and c compared with glib. Cur = curcumin and Glib = Glibenclimide

4.7 Serum Superoxide Dismutase Levels of Curcumin Treated Diabetic Swiss Albino Mice

Figure 4.6 Bar chart showing the results of the effects of curcumin (50 mg/kg and 100 mg/kg) on serum superoxide dismutase level of alloxan-induced diabetic Swiss albino mice. The curcumin treated groups showed significant ($P < 0.05$) increase in level of SOD after 21 days of administration, when compared to control group treated with olive oil with values of 8.94 ± 1.16 IU/L and 12.84 ± 0.84 IU/L compared to the 5.75 ± 0.96 IU/L respectively

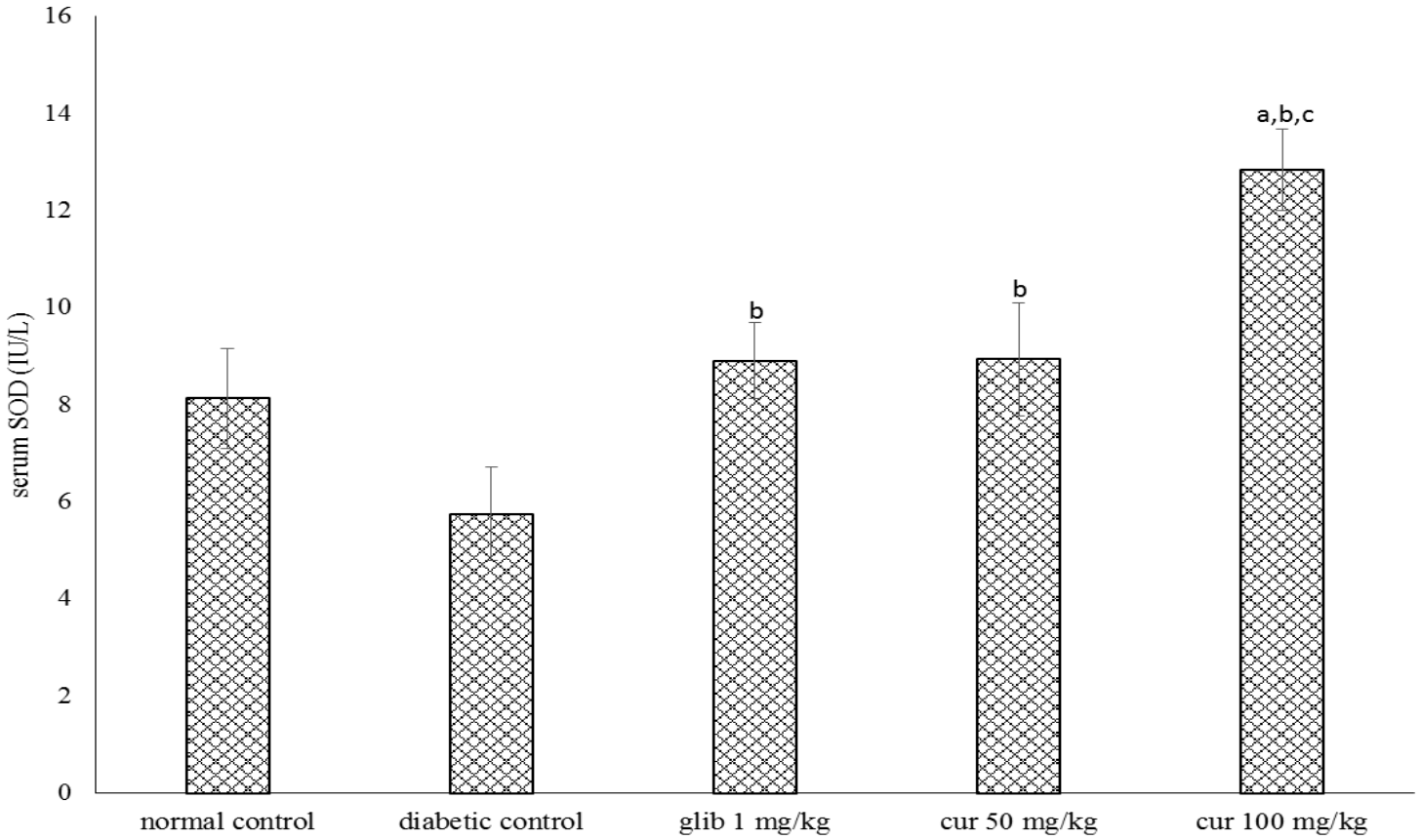

Figure 4.6: Effect of Curcumin on Superoxide Dismutase Level in Alloxan-Induced Diabetic Swiss Albino Mice. Values with error bars having different superscripts letters a,b and c are significant ($p < 0.05$); a = compared with normal, b compared with control and c = compared with cur. Cur = curcumin and Glib = Glibenclimide

4.8 Serum Glutathione Peroxidase Level of Curcumin Treated Diabetic Swiss Albino Mice

Figure 4.7 Bar chart showing the results of the effects of curcumin (50 mg/kg and 100 mg/kg) on serum glutathione peroxidase level of alloxan-induced diabetic Swiss albino mice. The curcumin treated groups showed no significant ($P < 0.05$) increase in the serum GPx level after 21 days of administration, when compared to control group treated with olive oil.

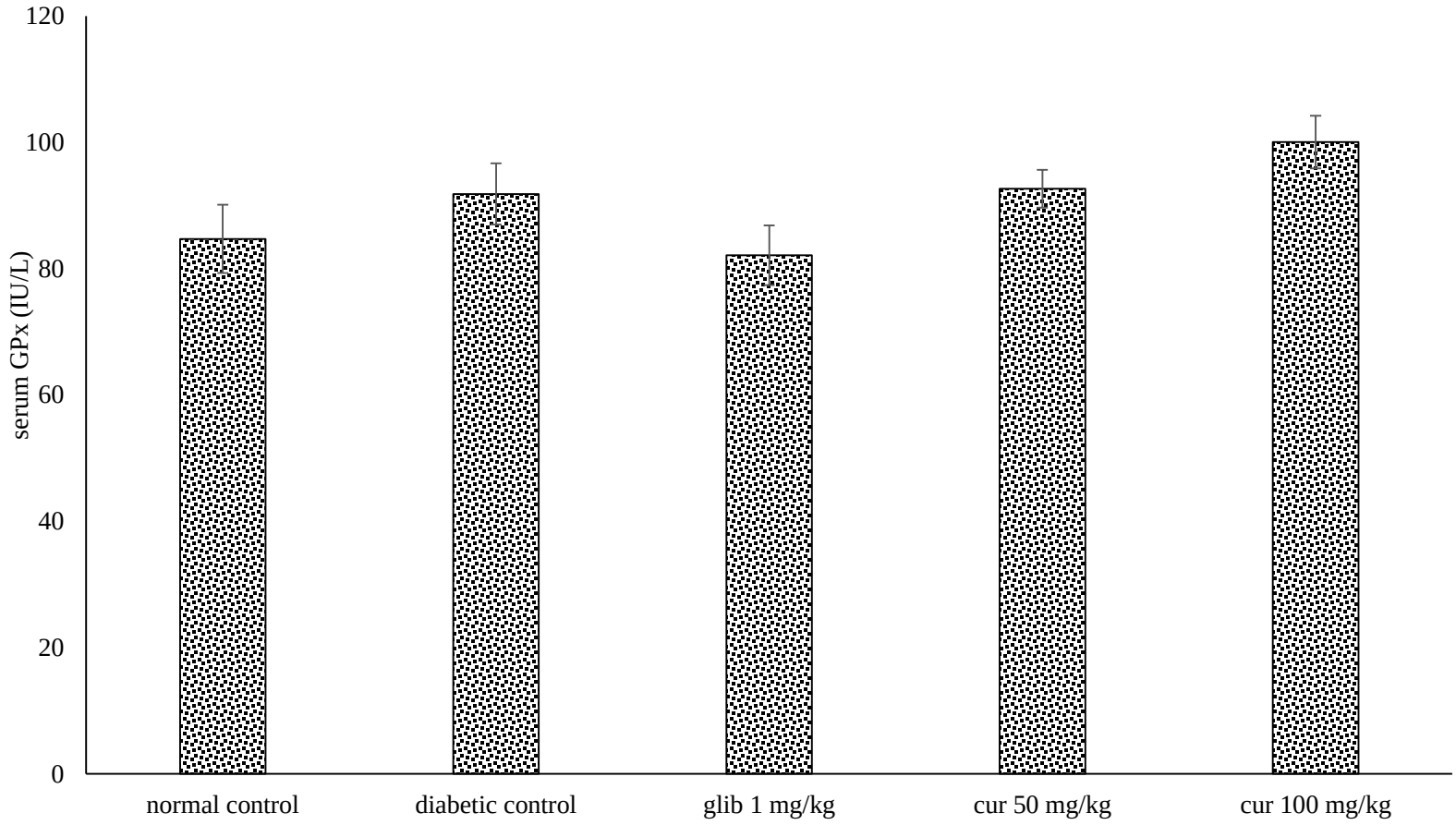

Figure 4.7: Effect of Curcumin on Serum Glutathione Peroxidase Level in Alloxan-Induced Diabetic Swiss Albino Mice. Cur = curcumin and Glib = Glibenclimide

CHAPTER FIVE

5.0 DISCUSSION

Diabetic mellitus is a metabolic disease associated with impaired glucose metabolism which in effect alters intermediary metabolism of lipids and proteins adversely (Onakpa and Ajagbonna, 2012). Alloxan, a beta cytotoxin, destroys pancreatic β-cells of islets of Langerhans resulting in a decrease in endogenous insulin secretion and paves ways for the decreased utilization of glucose by body tissues leading to elevation of blood glucose level, decreased protein content, increased levels of cholesterol and triglycerides (Lenzen, 2008). Turmeric has been used traditionally for many ailments because of its wide spectrum of pharmacological activities. Scientific research spanning over more than four decades has confirmed the diverse pharmacological effects of curcumin and established its ability to act as a chemopreventive agent as well as a potential therapeutic agent against several chronic diseases (Esatbeyoglu *et al.*, 2012; Priyagarsini, 2013)

The result obtained in this study showed that curcumin at both doses significantly ($p < 0.05$) decrease the fasting blood glucose level when compared to the control group. The decrease in the fasting blood glucose level observed in the present study suggest that daily administration of curcumin has improved the hyperglycemic condition in Swiss albino mice indicating that curcumin has strong antihyperglycemic effect. The result also indicated that the two doses of curcumin and standard anti-diabetic drug, glibenclamide, does not show any significant difference after 21 days treatment. With curcumin even showing higher percentage glycemic change (PGC) compared to the standard anti-diabetic drug. Also, there is no any statistical difference in all the diabetic groups on day 0. Also, the treatment group and normoglycemic control group showed no significant difference, hence suggesting that curcumin might have led to the recovery of the pancreatic beta cells and or increase peripheral insulin utilization by increasing tissue sensitivity to insulin. The

result of the present study agrees with the findings of Sharma *et al.* (2014) who also demonstrated that curcumin alleviates fluoride induced hyperglycemia in rats

It is evident that hyperglycemia is associated with memory impairment as observed in all the groups that were diabetic (day 0) in this study. This was further confirmed by the result obtained in the control group which show further impairment in the spatial working memory after 21 days. Suggesting that the effect of the hyperglycemia, reactive oxygen species formation might be responsible for the further impairment in spatial working memory in diabetes. Hyperglycemia, ROS and inflammation have been implicated in the pathogenesis of cognitive impairment in diabetes (Seto *et al.,* 2015). The results obtained in the high dose of curcumin treated group showed a significant ($p < 0.05$) increase in percentage spontaneous alternation in Y maze test when compared to the control group. This is an indication that curcumin at high dose ameliorate the spatial working memory impairment induced by hyperglycemia. The group that received standard antidiabetic drug does not show any significant increase ($p < 0.05$) in spatial working memory compared to the diabetic control group also the low dose of curcumin shows improvement which was not significant, compared to the diabetic control group. Comparing between day zero (pre-treatment) and day twenty one (post-treatment), there was signifincant increase in the percentage spontaneous alternation in both doses of curcumin and the standard anti-diabetic drug group. These indicate that twenty one days administarion of curcumin ameliorated the spatial working memory impairment induced by diabetes. These effect might be as a result of the ability of curcumin to reverse the hyperglycemia state induced by alloxan. Hyperglycemia and reactive oxygen specie are the leading causes of dementia and cognitive deficits (Seto *et al.,* 2015). Hyperglycemia is one of the leading cause of neurotoxicity and cognitive impairment through increase generation of ROS, activation of polyol pathway and advanced glycation end products and glucose shunting into

hexosamine pathway which lead to end organ damage and neuronal death (Comin *et al.,* 2010; Valente *et al.,* 2010)

The short term memory version was assessed using the NORT. It is the difference between the time spent exploring novel object and time spent exploring the familiar object. From the results obtained in this study, there is no significant ($p < 0.05$) difference between the curcumin treated groups and the diabetic control group after treatment. This may be because the mice were able to adapt to the condition and surrounding environment. Also, the result from the curcumin treated groups indicates clearly that hyperglycemia is associated with short term memory impairment and that the curcumin at both doses significantly ($p < 0.05$) improve the short term memory of the mice considering the pre-treatment (day 0) and post-treatment (day 21) respectively. This may be associated with the antihyperglycemic effect of curcumin and its antioxidant properties (Tokac *et al.,* 2014). Hyperglycemia and reactive oxygen specie are the leading causes of dementia and cognitive deficits. Hyperglycemia is one of the leading cause of neurotoxicity and cognitive impairment through increase generation of ROS, activation of polyol pathway and advanced glycation end products and glucose shunting into hexosamine pathway which lead to end organ damage and neuronal death (Comin *et al.,* 2010; Valente *et al.,* 2010). This finding agree with the report of Jithendra and Talasila, (2012) who reported improvement in short term memory of curcumin treated experimental animals in statin induced short term memory loss in rats.

The discriminatory index was assessed using the NORT. The findings of this study does not show any significant ($p < 0.05$) when compared to the diabetic control group. Although the difference in the discrimination ability of the experimental animals between pre-treatment (day 0) and post-treatment (day 21) in the control group is not significant, the animals show some degree of improvement which may be associated with some degree of learning or the effect of olive oil.

Also, from the result observed in the curcumin treated group, it is evident that hyperglycemia is associated with impairment of discrimination ability in the experimental animals which was significantly ($p < 0.05$) improved by the daily administration of curcumin as seen in the groups treated with different doses of curcumin. There was no statistically significant ($p < 0.05$) difference between the curcumin treated groups and the standard anti-diabetic drug treated groups.

The findings for recognitive index of this study does not show any significant ($p < 0.05$) change when compared to the diabetic control group. Although the difference in the recognition ability of the experimental animals on day 0 and 21 in the control group is not significant, the animals show some degree of improvement which may be associated with some degree of learning or the effect of olive oil on retention. Also, from the result observed in the same group, it is evident that hyperglycemia impair retention ability in the experimental animals which was significantly ($p < 0.05$) improved by the daily administration of curcumin as seen in the groups treated with different doses of curcumin on day 0 and 21 respectively. This improvement may be associated with the antihyperglycemic together with the effect of curcumin on inflammation as reported by Al Rubaei et al. (2014). Inflammation has been implicated in the pathogenesis and progression of CI through the numerous proinflammatory markers and cytokines, such as C-reactive protein (CRP), tumour necrosis factor- (TNF-) α, interleukin- (IL-) 1β, and IL-6, have been shown to be upregulated in both T1DM and T2DM (Seto *et al.*, 2006).

The result showed a significant ($P < 0.05$) increase in some antioxidant enzymes activities in the curcumin treated group, when compared to the control group. This finding indicate that curcumin at both doses possess antioxidant effect by elevating the level of the antioxidant enzymes. The increase in antioxidant enzyme activities in the curcumin treated groups may be due to increase generation of ROS, occurring in oxidative stress associated with hyperglycemia.

The antioxidant enzymes play a crucial role in the cellular defence against ROS (Bernabucci *et al.*, 2002). The SOD offers the first line of defence against ROS by scavenging and catalyzing dismutation of superoxide, produced by cellular metabolism, into hydrogen peroxide (H_2O_2) and oxygen (O_2) (Lin *et al.,* 2005; Das, 2011). CAT and GPx are involved in the reduction of H_2O_2 into H_2O and O_2. The observed increase in SOD and CAT in curcumin treated groups indicates that curcumin was able to scavenge free radical by sparring the endogenous antioxidant or the endogenous antioxidant enzymes has been used up as a result of scavenging free radical. This result disagree with the finding of Al Rubaei *at al.* (2014) who reported decrease in antioxidant activity in curcumin treated rats in vivo whereas the results of the present study agree with that of Tokac *et al.* (2013) who reported an increase in the antioxidant activity in curcumin treated groups.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

In conclusion, the results obtained from the present study demonstrate that hyperglycemia induces spatial working memory deficit, short term memory deficit and impairment in recognition and discrimination indices, oxidative stress, curcumin administration for twenty one days dcreases the

fasting blood glucose level, improve the spatial working and short term memories, improve the discriminatory and recognitive indices and increase antioxidant activity.

6.2 RECOMMENDATIONS

Based on the findings from this study, the following recommendations should be considered

1. The use of curry powder which is rich in curcumin might be considered
2. Furthermore, other models which assess other memory domains of executive functions such as attention deficits, reasoning, memory should be considered
3. The need to investigate the exact mechanism of action via which curcumin protects against diabetes induced cognitive impairment is recommended

6.3 CONTRIBUTIONS TO KNOWLEDGE

1. Curcumin at 100 mg/kg administration has improved spatial working memory (74.39 ± 8.06%) using Y-maze test of diabetic Swiss albino mice compared to (47.50 ± 13.65%) at $p < 0.05$
2. Both doses of curcumin (50 mg/kg and 100 mg/kg) ameliorated diabetes induced social memory impairment using NORT at 58.57 ± 2.55 and 63.71 ± 2.95 compared to 38.20 ± 4.73 and 36.04 ± 8.19 respectively at $p < 0.05$
3. Curcumin administration ameliorated oxidative stress induced by hyperglycemia by upregulating antioxidant enzymes; serum catalase (85.05 ± 3.23) and serum superoxide dismutase (12.84 ± 0.84) compared to 62.27 ± 7.07 and 5.75 ± 0.96 respectively

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