



Neuroprotective Potential of a Lanosteryl Triterpene from
Protorhus longifolia Stem Bark (Benrh.) Engl in high fat diet fed
and STZ -induced diabetic neuropathy in rats

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February 2019

Neuroprotective Potential of a Lanosteryl Triterpene from *Protorhus longifolia* Stem Bark (Benrh.) Engl in high fat diet fed and STZ -induced diabetic neuropathy in rats

A thesis submitted in fulfillment of the requirement for Masters degree in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, South Africa.

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Declaration

I **Ndlovu Musawenkosi**, hereby affirm that the work entitled “neuroprotective potential of a lanosteryl triterpene from *Protorhus longifolia* in high-fat diet and STZ-induced diabetic rats” is my original work. I have not copied from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

Signature of Author

Date

Signature of Supervisor

Date

DEDICATION

This work is dedicated to the Almighty God who gave me strength day by day and the Ndlovu family with their greatest support and encouragement.

ACKNOWLEDGEMENTS

I have a pleasure in acknowledging Dr. R.A. Mosa and Prof. A.R. Opoku (Supervisors) for their support and advice throughout my research project. Your efforts and patience are highly appreciated, thank you.

I appreciate the support given by our Biochemistry research group. Their support has made my research easier. This research was financially supported by the University of Zululand Research Committee.

I am grateful to the National Research Foundation (NRF) for funding this research project

Finally, I wish to thank my family and friends; Mr. & Mrs. Ndlovu (Mom and Dad), Mabhida Sihle Ephraim, Majola Londiwe, TSK Mngomezulu and NS Nhleko for their special support in all challenges I came across during the study period.

“I am the vine; you are the branches. If you remain in me and I in you, you will bear much fruit; apart from me you can do nothing” **John 15 v 5**

Abstract

Chronic hyperglycemia, observed in the diabetic state, is responsible for various diabetes related complications including development of diabetic nephropathy. Diabetic neuropathy development and progression is linked to various hyperglycemia-induced cellular and tissue damaging factors such as advanced glycation end product (AGE) formation, oxidative stress and inflammation. Activation of these pathophysiological pathways alters the antioxidant defense of nerve cells and cause protein malfunction, resulting in loss of neurotrophic support. Currently, there is no specific treatment for neuropathy and the current antidiabetic drugs fail to prevent development of long-term diabetic complications. Therefore, researchers have renewed their interest in the exploration of natural products as alternative potential remedies for the disease treatment. The present study aimed at investigating the neuroprotective potential of lanosteryl triterpene (RA-3) from *Protorhus longifolia* stem bark in high fat diet (HFD) fed and streptozotocin (STZ) -induced diabetic neuropathy in rats.

The triterpene was isolated from the chloroform extract of *P. longifolia* stem bark using silica gel column chromatography. The chemical structure of RA-3 was confirmed by spectral data analysis. The *in vitro* anti-glycation activity of the triterpene was investigated using BSA and haemoglobin-fructose assays. The HFD fed and STZ- induced diabetic neuropathy in the rat model was used to evaluate the diabetic neuroprotective potential of RA-3. The animals were initially divided into two major groups, the normal fed and HFD fed rats for 28 days. The HFD group rats were then injected (intraperitoneally) with a low dose of STZ (30 mg/kg body weight) to induce diabetes. The diabetic rats were further randomly divided into three groups: diabetic control, positive control treated with metformin (100 mg/kg) and RA-3 (100 mg/kg) treated group. The drugs were orally administered daily for 28 days. The tail flick method was used to assess the responses of the rats to cold and hot allodynia following 28 days of treatment with the respective drugs. On the last day of the experimental period, all rats were euthanized and blood (serum), sciatic nerve and brain tissues were collected for analysis of biochemical parameters.

The *in vitro* results revealed the anti-protein glycation potential of RA-3, accompanied by reduced fructosamine content and enhanced protection of protein thiol groups. Elevated fasting plasma glucose and serum fructosamine levels, as well as a significant reduction in the sciatic nerve levels of nerve growth factor (NGF), were detected in the non-treated diabetic group. However, treatment of the diabetic rats with RA-3 effectively lowered the fasting blood glucose and serum fructosamine while increasing the NGF levels. Relatively reduced sensation to both hot and cold allodynia, along with reduced activity of acetylcholinesterase, were also detected in the RA-3-treated diabetic rats when compared to the increased parameters in the untreated diabetic group. Treatment of the diabetic rats with RA-3 further exhibited increased tissue (sciatic nerve) antioxidant status, superoxide dismutase and reduced glutathione content accompanied by reduction in malondialdehyde levels. Relatively lower levels of pro-inflammatory markers (cyclooxygenase-2, tumor necrosis factor alpha, interleukin-6 and transforming growth factor beta-1) were also observed in both the serum and sciatic nerve tissue of the RA-3 treated diabetic rats. The levels of these inflammatory markers were elevated in the diabetic control group. The results obtained indicate that RA-3 possesses anti-diabetic neuroprotective potential. Its mechanism of action could be linked to its antihyperglycemic, antioxidant and anti-inflammatory activities.

LIST OF ABBREVIATIONS

AGEs	Advanced glycation end products
ANOVA	One-way analysis of variance
AR	Aldose Reductase
BDNF	Brain-derived neurotrophic factor
BGL	Blood glucose levels
BW	Body weight
COX-2	Cyclooxygenase-2
DM	Diabetes mellitus
DN	Diabetic neuropathy
DPN	Diabetic peripheral neuropathy
ELISA	Enzyme-linked immunosorbent assay
FBG	Fasting blood glucose
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione Reduced
GLUT 2	Glucose 2 transporter
HFD	High Fat Diet
IDF	International Diabetes Federation
IGF	Insulin-like growth factor
IL-1 β	Interleukin beta 1
IL-6	Interleukin-6
IR	Infrared
MDA	Malondialdehyde

NF- κ B	Nuclear factor-kappa B
NGF	Nerve Growth Factor
NMR	Nuclear magnetic resonance
Nrf2	Nuclear factor (erythroid derived 2)-like 2
NTFs	Neurotrophic factors
PKC	Protein Kinase C
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
RAGE	Receptor of advanced glycation end products
SOD	Superoxide dismutase
SDH	Sorbitol dehydrogenase
STZ	Streptozotocin
TGF	Transforming growth factor
TNF- α	Tumor necrosis factor alpha
UZRC	University of Zululand Research Committee
WHO	World Health Organization

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CHAPTER 1

1.0. Introduction

Diabetic neuropathy (DN) is the commonest complication of diabetes diagnosed in half of the diabetic population worldwide (Iqbal *et al.*, 2018). The persistently elevated blood glucose levels, observed in the diabetic state, are responsible for various diabetic complications including development and progression of DN. The risk of developing DN increases with age and the period of uncontrolled diabetic state. Amongst diabetic neuropathies, diabetic peripheral neuropathy (DPN) is considered the leading and most painful complication of many diabetic patients. Pain is mostly felt in the periphery which involve the hands, toes and feet. Uncontrolled DPN, characterized by nerve dysfunction, increase the risk of diabetic foot ulcers and if untreated may lead, to leg or hand amputations, experienced by long term diabetic patients (Zangiabadi *et al.*, 2014; Javed *et al.*, 2015; Cox *et al.*, 2017).

Hyperglycemia-induced oxidative stress, common in the diabetic state, stimulates several glucose metabolic pathways that are associated with neuropathy progression. These include the polyol pathway, formation of advanced glycation end products (AGEs), decreased antioxidant defence and activation of chronic inflammatory processes (Zychowska *et al.*, 2013; Sandireddy *et al.*, 2014). Furthermore, loss of growth factors, reduction in neurotransmitter levels and consequent neurological disorders as well as cognitive impairment are critical in the pathology of diabetic neuropathy. It is imperative to understand the biochemical basis of the pathological pathways implicated in DN development could prove crucial in the development of anti-neuropathy remedies.

Currently, there is no specific treatment offered for diabetic neuropathy since its mechanism is still not fully understood. However, there are some conventional drugs used to manage neuropathic pain. Prolong use of the current conventional drugs is linked to various side effects and intolerance over time (Sun *et al.*, 2018). The search for alternative management or preventive strategies against diabetes and its associated complications is ongoing. Therefore, there is a renewed interest in the exploration of medicinal plants and their derived bioactive compounds as new remedies for the disease treatment.

Plant-derived triterpenes, due to their diverse and significant bioactivities, have become one of the targets in the development of a new generation of drugs against human metabolic disorders and their related complications. A lanosteryl triterpene (methyl-3 β -hydroxylanosta-9,24-dien-21-oate) commonly referred to as RA-3, from *Protorhus longifolia* stem bark, has been reported to have antihyperlipidemic (Machaba *et al.*, 2014), antihyperglycemic (Mosa *et al.*, 2015; Mabhida *et al.*, 2017) and cardioprotective (Mosa *et al.*, 2016; Sangweni *et al.*, 2018) effects. The reported antidiabetic properties of triterpene (RA-3) could be crucial in the prevention and/or treatment of diabetes related complications such as neuropathy. It was therefore interesting and important to ascertain the potential therapeutic effect of RA-3 against diabetic neuropathy and thus the focus of this study.

1.0. Structure of the Dissertation

The dissertation consists of six chapters and three appendices of which the contents and information thereof are described as follows:

Chapter 1: This chapter gives a brief background and motivation for the study. The structure of the dissertation is also provided.

Chapter 2: This chapter presents the literature review together with the aim and objectives of the study.

Chapter 3: The chapter gives a description of materials and methods used to conduct all the experiments in the study.

Chapter 4: All the results obtained from the study are presented in this chapter

Chapter 5: The chapter gives an overall discussion of the obtained results in context to literature and the research question.

Chapter 6: This chapter gives the conclusion drawn from the obtained results. Limitations and recommendation(s) for further studies are also provided.

Appendices: Presents all additional or supplementary information and/or documents

CHAPTER 2

2.0. Literature Review

Diabetes mellitus (DM), a metabolic disorder characterized by chronic hyperglycemia, is a global health concern rapidly reaching epidemic levels. According to IDF estimates, in 2017, 425 million people were living with diabetes of which the number is expected to rise to 628 million in 2040 provided no action is taken (IDF, 2017). Adaptation to a sedentary lifestyle and high energy diets, characterized by high fats and carbohydrates, such as fructose, are some of the common factors responsible for the continuous increase of diabetes cases worldwide. Both high fat and high fructose diets have a major role in the development of peripheral cellular insulin resistance that leads to increased postprandial blood glucose (Mamikutty *et al.*, 2014; DiNicolantonio *et al.*, 2015; Kolderup and Svihus, 2015). Since insulin is considered a “metabolic switch” that controls cellular utilization of lipids and glucose as major metabolic fuels, insulin resistance steers metabolic disorders and pathophysiologies. Insulin resistance results in chronic hyperglycemia and abnormally elevated levels of circulating blood lipids “hyperlipidemia” observed in type 2 diabetic patients. Hyperlipidemia and hyperglycemia are considered to be the main contributors to type 2 diabetes and associated complications (WHO, 2016; IDF, 2017). Insulin resistance observed in type 2 diabetes is reported to account for majority of diabetes cases (Cantley and Ashcroft, 2015). An uncontrolled diabetic condition is a risk factor for various pathophysiologies including retinopathy, nephropathy and neuropathy (Cade, 2008, WHO, 2016). Diabetic neuropathy, being one of the commonest complications of diabetes, is the subject of investigation in this study.

2.1. Diabetic Neuropathy

Neuropathy, a nerve condition characterised by pain in all parts of the body, is a debilitating complex disease, causing damage to the nervous system and affecting motor, sensory and autonomic nerves (Callaghan *et al.*, 2012; Themistocleous *et al.*, 2014). It is strongly hypothesized that damage to the sciatic nerve, the longest nerve in humans, plays a significant role in the development of neuropathy (Glat *et al.*, 2016). Sciatic nerve injury or damage causes loss of sensory and motor functions (major symptoms of

neuropathy) (Türedi *et al.*, 2018). Neuropathy is classified into different types which have distinct syndromes in different nerve types. These neuropathies include peripheral, autonomic, focal and multifocal neuropathies (Albers and Pop-Busui, 2014; Juster-Switlyk and Smith, 2016). Chronic hyperglycemia detected in diabetic patients is considered the main contributor to development and progression of painful diabetic neuropathy (Garcia *et al.*, 2012).

Diabetic neuropathy (DN) is a symmetrical and length-dependent sensory-motor polyneuropathy that may involve motor, sensory and autonomic nerves due to chronic hyperglycemia and vascular risk factors. About 60 to 70% of people living with either type 1 or type 2 diabetes develop some neuropathic pain (Erbaş *et al.*, 2016). Nerve damage or dysfunction is recognized as a major contributor to morbidity in diabetic patients who have suffered the disease for a long period (Javed *et al.*, 2015). Sensory and peripheral motor nerves are the most commonly affected nerves, resulting in reduction in muscle strength (Verge *et al.*, 2014), loss of protective sensation and foot deformity (Tiaka *et al.*, 2011).

The commonest type of DN is diabetic peripheral neuropathy (DPN), also known as distal symmetrical polyneuropathy (DSPN), which accounts for 75% of diabetic neuropathy that affects peripheral nerves of both type 1 and type 2 diabetic patients. Literature reports that about 54% of 100 000 people per year show symptoms of DPN (Battula *et al.*, 2017). Patients diagnosed with DPN suffer from various painful and non-painful clinical signs and symptoms such as numbness, burning and tingling sensation (Sandireddy *et al.*, 2014; Fatani *et al.*, 2015). Progression of DPN can lead to impaired balance, loss of postural control and poor physical function. Major complications associated with DPN include foot ulcers, infections and non-traumatic limb amputations (Zangiabadi *et al.*, 2014; Cox *et al.*, 2017). Population based studies report that type 2 diabetic patients (13.46%) are at a greater risk when compared to type 1 diabetic patients (8.54%) (Battula *et al.*, 2017). Clinical data show that type 2 diabetic patients are at a life time risk of 15-25 % to develop foot ulcers related to neuropathy (Khawaja *et al.*, 2018). The prevalence of DN is further exacerbated by the fact that about 39% of patients with DN never receive

treatment and about 13% of those patients do not report their symptoms (Hébert *et al.*, 2017). Diabetic neuropathies also seem to be more common in people who are overweight, with high levels of blood lipids (Zangiabadi *et al.*, 2014). Furthermore, lack of neurotrophic factors and increased activity of acetylcholinesterase have been reported to play a vital role in the pathology of diabetic neuropathy (Yagihashi *et al.*, 2011; Garcia *et al.*, 2012; Eslami *et al.*, 2016). Understanding the biochemical pathways involved in the pathophysiology of DN could prove crucial in the development of anti-neuropathy therapeutic remedies.

2.2. Role of neurotrophic factors in DPN

Neurotrophic factors (NTFs) are a family of growth factors that structurally and functionally support growth, development and maintain neuron survival in different tissues (Liu *et al.*, 2018). NTFs are further involved in nerve rejuvenation, control of neuronal plasticity and support in repairing injured nerves (Zhou *et al.*, 2016). Mammalian cells consist of four different neurotrophins which comprise neurotrophin 3 (NT-3), neurotrophin 4, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) commonly known as NT-4 or NT-4/5 (Khan and Smith, 2015). This group of NTFs contribute in cell regulation processes, cell proliferation, maturation and differentiation, and control survival of cells (Sebben *et al.*, 2011). Current research shows that neurotrophins have a potential therapeutic effect against peripheral nerve injury (Glat *et al.*, 2016; Tezcan *et al.*, 2017).

Nerve growth factor (NGF) is the most studied neurotrophin in DPN. NGF is a highly soluble protein, compounded by several cells in the endocrine, immune and nervous system, stimulating the growth of axon (Gao *et al.*, 2017). NGF is released in response to inflammation or tissue injury from a range of cells including lymphocytes, keratinocytes macrophages and mast cells (Minnone *et al.*, 2017). Functions of NGF are not distinct from the other NTFs since it supports growth, differentiation, rejuvenation of nerves and further aids in repair of injured nerves. Apart from its physiological functions, NGF has been reported to possess anti-apoptotic activities with pro-differentiation activities of neurons and plays a crucial role in embryonic development of the nervous system

(Bradshaw *et al.*, 2015). Experimental evidence has also shown that the neuroprotective effect of NGF is its ability to ameliorate endoplasmic reticulum stress which is related to the activation of phosphatidylinositol 3-kinase/ Akt (PI3K/Akt) and extracellular signal-related kinase (ERK)1/2 pathways (Li *et al.*, 2013).

With strong evidence, it is hypothesized that reduced production of NGF plays a vital role in the pathology of diabetic neuropathy (Kornblatt *et al.*, 2016). Decreased expression of NGF protein, together with its receptors, positively correlates with neuron apoptosis in clinical diabetes confirming its association with the development of DPN (Sun *et al.*, 2018). Gao *et al.* (2017) reported down-regulation of NGF in dorsal horn and dorsal root ganglion (DRG) neurons one week after STZ-induction of diabetic neuropathy in rats. In a similar model of DN, Eslam *et al.* (2016) also showed decreased regulation of NGF in sciatic nerves and spinal motor segment of diabetic neuropathy induced rats.

2.3. Polyol Pathway and Advanced glycation end products formation

Glucose (C₆H₁₂O₆) is an essential metabolite for cell survival when blood levels are strictly maintained within narrow limits of 4.0 to 6.0 mmol/L. Abnormally high blood glucose levels are toxic, activating pathophysiological pathways such as the polyol pathway and consequent formation of advanced glycation end products (AGEs). In the polyol pathway, elevated glucose levels activate aldose reductase (AR), the enzyme which reduces glucose to sorbitol in the first step of the pathway (Figure 2.1). The second step of the polyol pathway involves oxidation of sorbitol to fructose catalyzed by sorbitol dehydrogenase (SDH), which requires nicotinamide adenine dinucleotide (NAD⁺) as a coenzyme. Sorbitol is highly hydrophilic making it unable to diffuse through cell membranes, therefore accumulating intracellularly (Mathebula, 2015). Accumulation of both fructose and sorbitol leads to increased water content in the nerve tissues (Khan *et al.*, 2011) resulting in damage to cells due to the generated osmotic stress (Tarr *et al.*, 2013; Zychowska *et al.*, 2013; Mathebula, 2015).

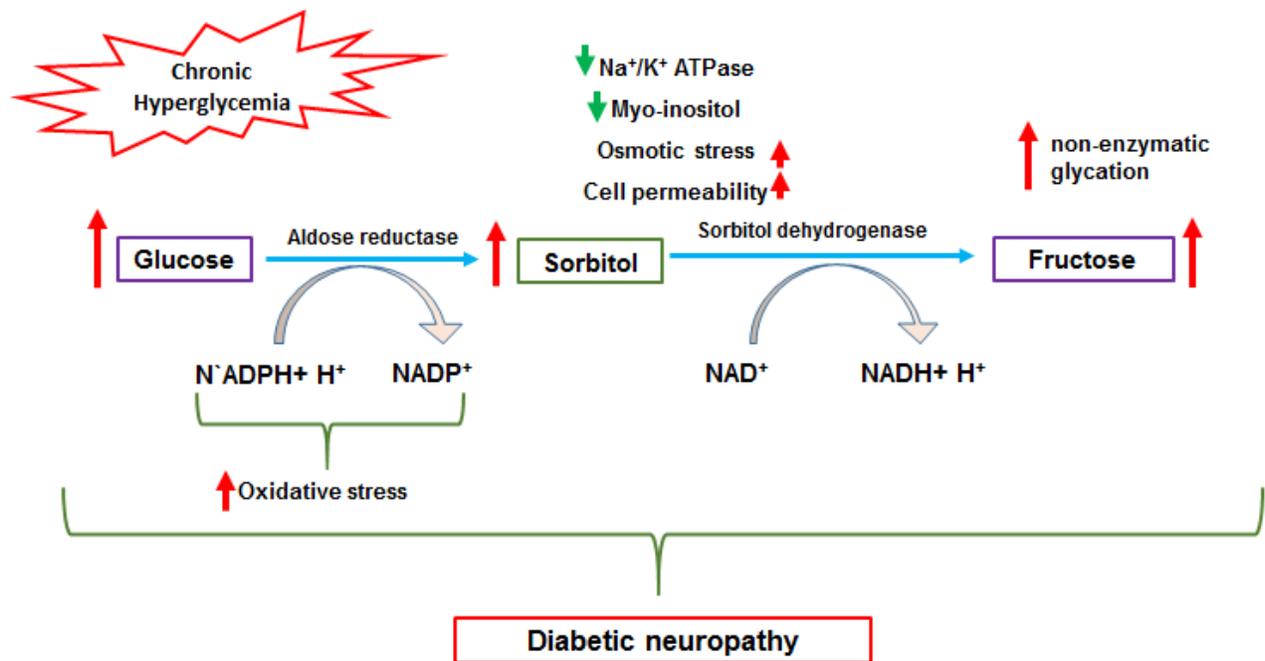


Figure 2.1: Hyperglycemia-induced activation of polyol pathway. Elevated blood glucose levels activate aldose reductase which catalyzes the conversion of glucose to sorbitol. Sorbitol is further converted to fructose under the catalysis of sorbitol reductase with NAD^+ as a cofactor (Adapted from: Zychowska *et al.*, 2013).

Activation of the polyol pathway further increases the production of intracellular dicarbonyl AGEs precursors such as methylglyoxal and 3-deoxyglucosone, which may lead to proteins modification later forming AGEs (Sugimoto *et al.*, 2008). AGEs are generated as a result of non-enzymatic reactions between the primary group of amino acids and the carbonyl end of carbohydrates, yielding a Schiff base through the Millard reaction (Figure 2.2). The Schiff base undergoes acid/base catalysis and rearrangement to form unstable compounds known as Amadori products, such as fructosamine (which is clinically used to measure short-term glycemic control) (Muniz *et al.*, 2018; Snelson and Coughlan, 2019). Amadori products undergo further reactions that lead to the production of advanced glycation endproducts (AGEs) (Nowotny *et al.*, 2015). Increased generation of reactive oxygen species (ROS) during glycation and glycooxidation has been reported to cause oxidative damage to amino acids. This causes residues of proteins form carbonyl derivatives which diminish the antioxidative defense of proteins by destroying the thiol

groups (Nowoty *et al.*, 2015). Prevention of fructosamine generation and protection of protein thiol groups mainly inhibit production of AGEs.

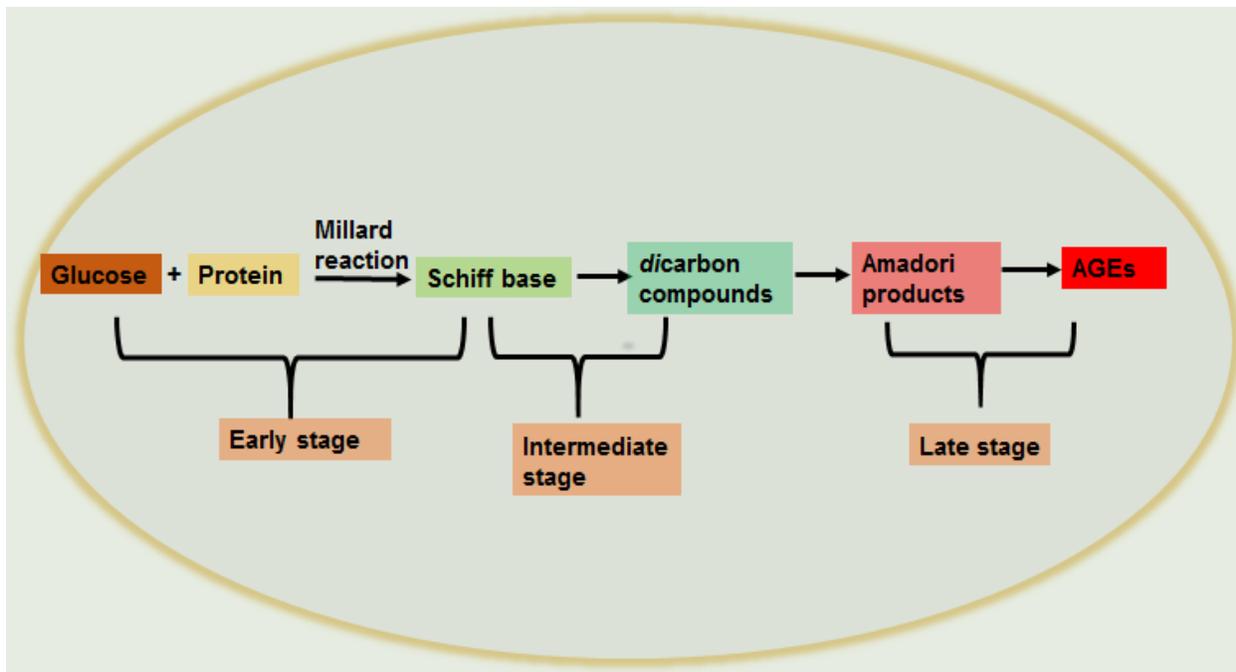


Figure 2.2: A three stage schematic reaction showing some chemical processes involved in the formation of AGEs.

Formation of AGEs in the body is a normal process that occurs at a slow but constant rate and these products accumulate with age. However, chronic hyperglycemia characterising diabetes mellitus significantly accelerates their formation (Peppia *et al.*, 2003; Sharma *et al.*, 2012; Guo *et al.*, 2018). Literature has suggested that glycation of the peripheral nerve extracellular matrix caused by diabetes causes defects in peripheral nerve regeneration. Moreover, hyperglycemia induced-AGEs causes demyelination of peripheral nerves, increasing their susceptibility to phagocytosis by macrophages.

AGEs mediate cellular damage through direct change of protein structures and functions, but most commonly cause damage via binding to the receptor of advanced glycation end products (RAGE) (Nowotny *et al.*, 2015). RAGE have been found to be highly expressed

in endothelial cells of both perineural and endoneural vessels of the peripheral nerves of diabetic rats (Singh *et al.*, 2014, Fournet *et al.*, 2018). AGE-RAGE interaction activates oxidative stress and nuclear factor kappa B (NF- κ B) which enhances nerve cell apoptosis through activation of pathophysiological pathways, such as chronic inflammation and oxidative stress (Oyenihi *et al.*, 2015). Inhibitors of AGEs such as aminoguanidine and thiazolidine derivative have been shown to improve nerve conduction (Sugimoto *et al.*, 2008).

2.4. Oxidative stress and inflammation in DN

Hyperglycemia-induced oxidative stress and inflammation are the major factors that usually mediate the cellular and tissue damaging effects of diabetes (Asmat *et al.*, 2016; Snelson and Coughlan, 2019). Oxidants contribute to the activation of inflammatory processes leading to the expression of proinflammatory markers. Similarly, inflammatory cells liberate several reactive species that can result in oxidative stress (Biswas, 2016). Increased production of reactive species and inflammation are closely related to pathophysiological events including diabetic neuropathy. Oxidative stress alters the antioxidant defense system of a cell, leading to increased oxidative impairment to the cellular components. Besides decreasing antioxidant enzymes activity, oxidative stress also causes neuropathy through oxidation and nitration of nerve proteins, DNA damage and lipid peroxidation on membranes of nerve cells.

To ameliorate oxidative stress, human cells are equipped with antioxidant (endogenous or exogenous) defense systems. These include antioxidant enzymes (catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD) and non-enzymatic antioxidants (reduced glutathione (GSH), ascorbic acid, carotenoids and α -tocopherol). However, in pathophysiological events such as neuropathy, the antioxidant defense system is altered in a manner that decreases its activity, thus increasing chances of oxidative damage to nerve cell components. Fatani and co-workers (2015) reported a decrease in anti-oxidant enzymes and a relative increase in lipid peroxidation in the sciatic nerve of STZ-induced diabetic rats (Fatani *et al.*, 2015). Similarly, Koneri *et al.* (2014)

reported decreased activity of SOD and catalase, and increased levels of MDA (a measure of lipid peroxidation) in STZ-induced diabetic neuropathy in rats.

Elevated intracellular glucose in nerve cells promotes production of diacylglycerol that activate protein kinase C (PKC) (Yagihashi *et al.*, 2011; Hosseini and Abdollahi, 2013). Activated PKC initiates an intracellular signaling cascades including overexpression of NF- κ B and transforming growth factor beta-1 (TGF- β 1) and increases the production of the extracellular matrix and cytokines (Hosseini and Abdollahi, 2013) (Figure 2.3). TGF- β 1 induces neuronal cell injury and reduces neurite growth, which are features implicated in the pathogenesis of DPN (Anjaneyulu *et al.*, 2008). Activation of NF- κ B initiates the release of proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α), TGF- β 1, interleukins (IL-1 β , IL-6, and IFN- γ) and prostaglandin endoperoxide synthase 2 (COX-2) (Sandireddy *et al.*, 2014; Fukuoka *et al.*, 2017).

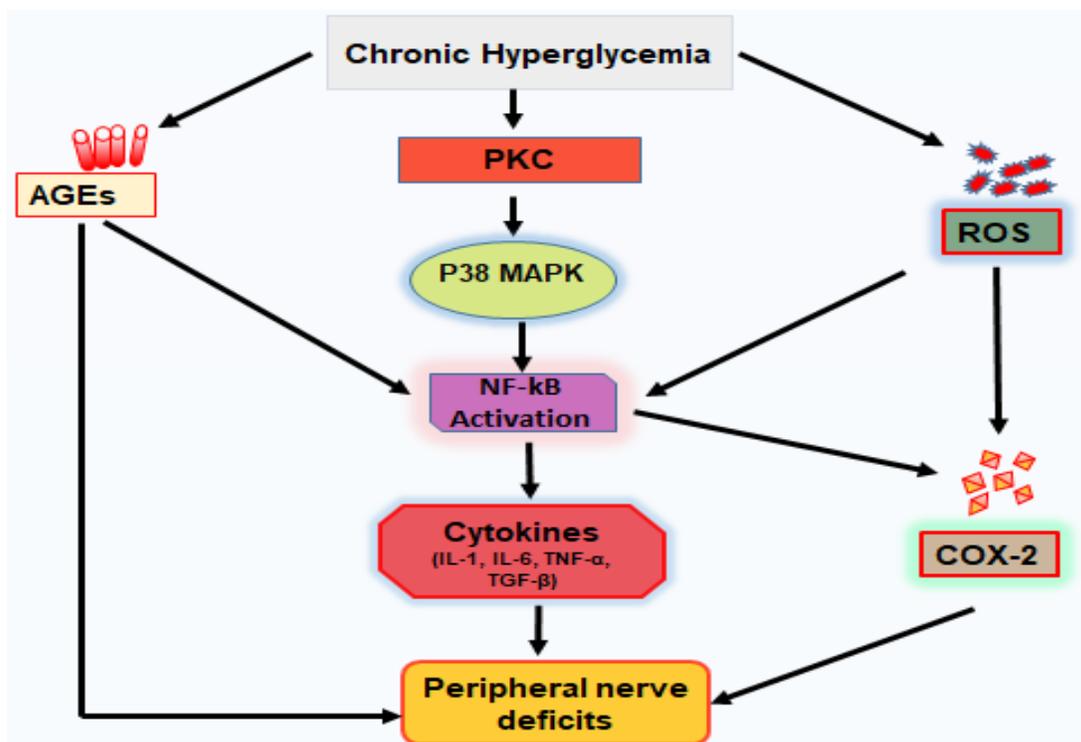


Figure 2.3: A schematic diagram demonstrating role of AGE-RAGE interaction and PKC pathway activation in the secretion of proinflammatory markers. ROS and activation of the P38 MAPK lead

to activation of NF- κ B which further enhances secretion of proinflammatory cytokines that mediate nerve damage (Luevano-Contreras and Chapman-Novakofski, 2010).

In the diabetic state, increased expression of NF- κ B accelerates apoptosis through release of the pro-inflammatory mediators such as TNF- α , TGF- β 1 and IL-6 (Tabur *et al.*, 2015; Li *et al.*, 2018). Increased levels of IL-6 and TNF- α in the STZ model of diabetic neuropathy in rats have been reported (Yang *et al.*, 2015). Kumar *et al.* (2011) also reported elevated signs of inflammation in the peripheral nerves of diabetic patients (Kumar *et al.*, 2011). While TNF- α is known to enhance expression of adhesion molecules and impaired neurotropic support, increased COX-2 activity activates inflammatory processes that can worsen neuronal degeneration and impairment (Kang *et al.*, 2017). Over-expression of COX-2 is associated with uncontrolled cell proliferation, apoptosis and growth (Gandhi *et al.*, 2017). Furthermore, NF- κ B activation is also known to suppress the antioxidant system of cells through downregulation of the nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway weakening the cellular antioxidant defense (Sandireddy *et al.*, 2014). Literature reports that agents that can inhibit activation and expression of pro-inflammatory mediators could be beneficial in the prevention and management of diabetic neuropathy (Oh *et al.*, 2016).

2.5. Biological role of acetylcholine in DN

Acetylcholine (ACh) is a versatile neurotransmitter found in all autonomic ganglia, neuromuscular junction and in many synapses in the central nervous system. The neurotransmitter ACh has been known for its involvement in cortical plasticity, learning, attention, memory and in cognitive functioning (Wallace and Bertrand, 2013). In the peripheral nervous system, ACh functions at the neuromuscular junction between the motor nerves and skeletal muscle (Colovic *et al.*, 2013). Acetylcholine also play a major role in the growth of nerve cells (Ahmed *et al.*, 2012). However, impulse transmission at the cholinergic synapse is terminated by rapid hydrolysis of ACh by acetylcholinesterase (AChE) at neuromuscular junctions. AChE hydrolyses ACh to choline and acetate (Figure 2.4).

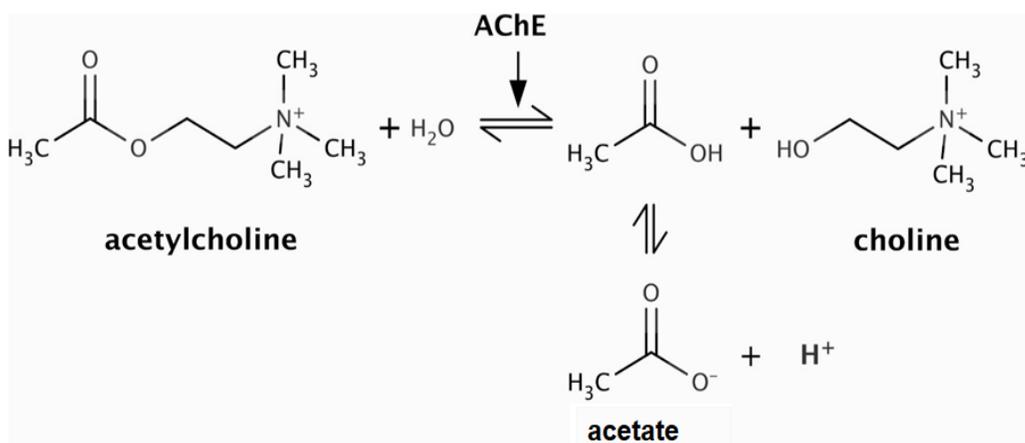


Figure 2.4: Hydrolysis of acetylcholine by acetylcholinesterase to acetate and choline.

The hydrolysis of ACh leads to termination of the neurotransmitter physiological actions (Sachdeva *et al.*, 2015). Reduction of acetylcholine levels below normal range contributes to progressive cognitive impairment, and causes neurological dysfunctions and neurodegenerative diseases (Sathya, 2013). Diabetes favours acetylcholine reduction in the nervous system leading to advanced extensor and flexor muscles weakness (Garcia *et al.*, 2012). Brain and sciatic nerves of acrylamide induced diabetic neuropathy in rats have shown increased AChE activity (Sathya, 20113). Therefore, tight regulation or inhibition of AChE could be essential in maintaining cognitive function and performance. Various exogenous compounds such as donepezil, galantamine and rivastigmine have been used to inhibit AChE in medical practice (Petrov *et al.*, 2013; Owokotomo *et al.*, 2015).

2.6. Current treatment of DN

Since the mechanism of DN is still not fully understood, there is currently no absolute treatment offered for diabetic neuropathy. Literature reports that the first line of treatment for DN is glycemic control (Javed *et al.*, 2015). Antidiabetic drugs with hypoglycaemic effect have the potential to manage DPN at its earlier stages. Common antidiabetic drugs, metformin and rosiglitazone (thiazolidinedione), have been reported to have neuroprotective effects (Melemedjian *et al.*, 2013; Mao-Ying *et al.*, 2014; Wang *et al.*,

2016). There are also some conventional drugs used to manage neuropathic pain. These include drugs such as tricyclic antidepressants, analgesics, anticonvulsants, anesthetics, opioids, topical capsaicin, neurontin, gabapentin, anti-inflammatory drugs and pregabalin (Galuppo *et al.*, 2014; Adeyi and Nneji, 2015). However, the prolonged use of the current conventional drugs is linked with various side effects such as muscle pain, itching, headache, vomiting, renal toxicity and diarrhea (Romano *et al.*, 2012). Furthermore, some of these drugs are costly, especially in developing countries where a large population is still economically challenged.

Despite the availability of current clinical treatment or management for diabetic peripheral neuropathy, the disease progresses with epidemic levels affecting the quality of life of diabetic patients. Due to the limitations associated with the currently available drugs, there is a continuous search for alternative remedies for the treatment of DPN. Researchers have turned their attention to medicinal plants and/or their derived bioactive compounds as alternatives for the development of new anti-neuropathic drugs that could target primary cause and/or secondary complications of DPN.

2.7. Medicinal plants and their derivatives in human health promotion

Medicinal plants have been used as integral part of traditional medicine since ancient times and they continue to be viable sources of pharmacologically active drugs against various diseases. In the past 20 years, about a quarter of Food and Drug Administration (FDA)-approved drugs were based on ingredients of natural products origin (Newman and Cragg, 2012; Thomford *et al.*, 2018). Examples of plant-derived drugs currently in clinical use include morphine (from *Papaver somniferum* commonly known as the opium poppy), aspirin (acetylsalicylic, from *Salix alba* commonly known as the willow tree), artemisinin (from *Artemisia annua*) and paclitaxel, a terpene from *Taxus baccata*.

When compared to their synthetic counterparts, medicinal plants and/or their derivatives are usually considered to be medicinally safe due to their low toxicity and limited risk of side effects (Prasad, 2014). The advantages posed by medicinal plants over their

synthetic counterparts encourage continuous search and development of new pharmacological drugs from plants. However, challenges and difficulties, such as isolation of pure pharmacologically active compounds, lack of standardization procedures, poor documentation in clinical trials according to standards and poor description of biological mechanism, hinder or slow acceptance and approval of natural products (Thomford *et al.*, 2018).

In a continuous search for new pharmacologically active neuroprotective agents, the neuroprotective effect of various medicinal plant extracts or their derivatives has been evaluated. Extracts of *Toona sinensis* seeds (Wang *et al.*, 2016) and *Lagerstroemia speciosa* L (Bhokare and Upaganlawa, 2015) have been reported to manage diabetic neuropathy. Antioxidant activity has been described as the basis of their neuroprotective potential. Fatani *et al.* (2015) reported the antidiabetic neuropathy properties of *Gymnema sylvestre* extracts which showed anti-inflammatory activities and the potential to regulate growth factors in the sciatic nerves of diabetic neuropathy rats. An antidiabetic plant derived compound, Luteolin, has also been reported to have neuroprotective potential against diabetes induced neuropathy (Oh *et al.*, 2016).

Scientific evidence also supports plant-derived triterpenes as new targets for drug development against diabetes and its related complications such as neuropathy. Some plant-derived triterpenoids with antidiabetic properties have been reported to exhibit neuroprotective effect (Yu *et al.*, 2006; Wang *et al.*, 2016). Since diabetes is a risk factor of various pathophysiologies such as micro- and macro-vascular ailments and neuropathies (Cade, 2008, WHO, 2016), the antihyperglycemic activity of triterpenes could be crucial in the prevention of such complications. In addition to antihyperglycemic activity, triterpenes are commonly known to exert their therapeutic activities through other various mechanisms such as antioxidant and anti-inflammatory activities (Sporn *et al.*, 2011). The antioxidant properties of triterpenes may be attributed to their ability to upregulate nuclear factor-E2-related factor (Nrf2) which regulates both enzymatic and non-enzymatic antioxidants in cells (Pareek *et al.*, 2011).

In our laboratory, lanosteryl triterpenes including methyl-3 β -hydroxylanosta-9, 24-dien-21-oate (**RA-3**, Figure 2.5) from the stem bark of *Protorhus longifolia* (Benrh.) Engl. (Anacardiaceae), have been reported to possess antihyperglycemic activity (Mosa *et al.*, 2015; Mabhida *et al.*, 2017). In addition to effective glycemetic control, the therapeutic effect of RA-3 has also been associated with the ability to enhance antioxidant defense while suppressing inflammation mediators and thus protecting diabetic tissue damage. The cardioprotective (Mosa *et al.*, 2016; Sangweni *et al.*, 2018) and anti-hyperlipidemic (Machaba *et al.*, 2014; Mosa *et al.*, 2014) effects of RA-3 with weak cytotoxic effect (Mosa *et al.*, 2014) have also been documented.

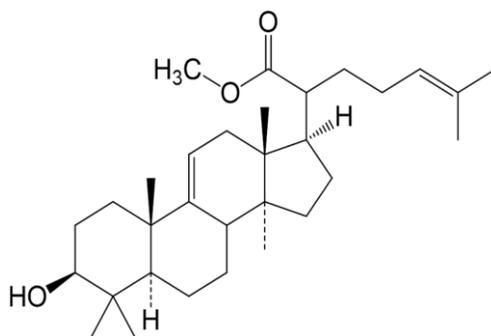


Figure 2.5: Chemical structure of **RA-3** (Methyl-3 β -hydroxylanosta-9, 24-dien-21-oate)

The reported significant antidiabetic properties of the lanosteryl triterpene (RA-3) could be crucial in the prevention and/or treatment of diabetes related complications such as neuropathy. It was therefore important to also ascertain the potential therapeutic effect of RA-3 against diabetic neuropathy.

2.8. Common experimental models of DN

Animal models remain favorable for studying DN that can present human behavior and facilitate a lead to the development of therapeutic agents against DN. Different animal models including genetically or spontaneous derived models such as non-obese diabetic

Akita mice, Zucker diabetic fatty rats, type 1 insulinopenic BB/Wor rats, leptin-receptor-deficient mice and type 2 hyperinsulinemic diabetic BBZDR/Wor rats have been used in studying DN (Gao and Zheng, 2014). Also, a combination of dietary factor and/or chemical effect such as in high fat diet (HFD) or high fructose (HF) fed and streptozotocin (STZ) induced diabetic neuropathy rat models are commonly used experimental models (Islam, 2013; Gao and Zheng, 2014). Recent studies (Dupas *et al.*, 2016; Barrière *et al.*, 2018) have utilized HFD/HF or HFD/STZ combinations to induce DN in rats.

However, it has been argued that even though all the models develop some neuropathic symptoms, some of those symptoms are not critical as those observed in human diabetic neuropathic patients (Islam, 2013; Biessels *et al.*, 2014). Literature reports that depending on the study period the STZ model (with different doses), which is currently the most commonly used model, shows symptoms similar to those clinically observed in diabetic patients (Gao and Zheng, 2014; Biessels *et al.*, 2014; Madias, 2016). Loss of sensation, reduced nerve fiber density, impaired locomotor activity which are some of the clinical signs commonly observed on diabetic patients have been reported in the STZ-induced DN rat model (Gao *et al.*, 2014; Nasiry *et al.*, 2017).

The toxicity of STZ is related to the glucose moiety in its chemical structure which enables its entry to beta cell via glucose 2 transporter (GLUT 2) in the plasma membrane (Eleazu *et al.*, 2013). Moreover, other cells that express GLUT 2 transporter such as those in liver, kidney, intestine, as well as in the central nervous system (CNS) are also susceptible to STZ toxicity (Eleazu *et al.*, 2013; Thorens *et al.*, 2015). The mechanism of STZ cellular damage is associated with increased oxidative stress, endothelial dysfunction, and inflammation which are cellular events reported in diabetic neuropathy. STZ-induced DN in Wistar albino rats exhibited a decrease in antioxidant status of sciatic nerves, which was accompanied with increased inflammatory mediators and reduced pain threshold from the paw pressure analgesia and tail flick test (Fatani *et al.*, 2015). The HFD and STZ-induced diabetic rat model is also a well characterized system to study complications

associated with type 2 diabetes (Kuang et al., 2012). Thus in this study, the HFD fed and STZ induced DN rat model was adapted.

2.9 Aim and objectives

2.9.1 Aim

To investigate the neuroprotective potential of a lanosteryl triterpene (RA-3) from *Protorhus longifolia* stem bark in HFD fed and STZ -induced diabetic neuropathy in rats.

2.9.2 Objectives

- a) To isolate and characterize the triterpene (RA-3) from the chloroform extract of the plant material.
- b) To evaluate the *in vitro* antiglycation activity of the triterpene
- c) To investigate the neuroprotective potential of the triterpene in HFD fed and STZ-induced diabetic neuropathy in rats.

CHAPTER 3

3.0. Materials and Methodology

This chapter gives a description of materials and methods used to extract and isolate the target compound (RA-3) from the plant material. It also describes the materials and methods used to evaluate the neuroprotective potential of the triterpene. Details on the preparation of some reagents are presented in Appendix A

3.1 Materials

3.1.1 Chemicals and Reagents

Sigma-Aldrich, St Louis, MO, USA: Glutathione (GSH) assay kit - catalog number CS0260, Superoxide dismutase (SOD) assay kit - catalog number 19160, Antioxidant assay kit – catalog number CS0790, Malondialdehyde (MDA) assay kit - catalog number MAK085, Interleukin-6 (IL-6) ELISA kit - catalog number RAB0314, tumor necrosis factor alpha (TNF- α) ELISA kit – catalog number – RAB 0477, beta Nerve growth factor (β -NGF) ELISA kit – catalog number- RAB 1119, nitro-blue tetrazolium, 1-deoxy-1-morpholino-fructose, L-cysteine, acetylthiocholine iodide, 5,5'-Dithiobis (2-nitrobenzoic acid), bovine serum albumin (BSA), haemoglobin, aminoguanidine metformin and streptozotocin (STZ).

Elabscience Biotechnology Co, Ltd, USA: Cyclooxygenase-2 (PTFS2/COX-2) ELISA kit – catalog number - E-EL-M0959, Transforming growth factor beta 1 (TGF- β 1) ELISA kit – catalog number - E-EL-R0084.

Merck, Darmstadt, Germany: hexane, ethyl acetate, methanol, ethanol, sea sand, chloroform, and silica gel 60 (70-230 mesh ASTM).

The chemicals and solvents used in the study were of analytical grade.

3.2. Methodology

3.2.1 Extraction and Isolation of the compound

The fresh stem bark of *Protorhus longifolia* was collected from KwaHlabisa, KwaZulu Natal. The plant material (voucher specimen number: RAUZ01) was duly confirmed by a botanist in the Botany Department, University of Zululand. The lanosteryl triterpene (RA-3) was extracted and isolated from the chloroform extract of the plant material (Figure 3.1), following a well-established method described by Mosa *et al.* (2014). The triterpene was routinely isolated and purified over silica gel chromatography. The structure of RA-3 (methyl-3 β -hydroxylanosta-9,24-dien-21-oate) was confirmed based on spectral (NMR, IR) data analysis and by comparison with literature values (Mosa *et al.*, 2014, Machaba *et al.*, 2014).

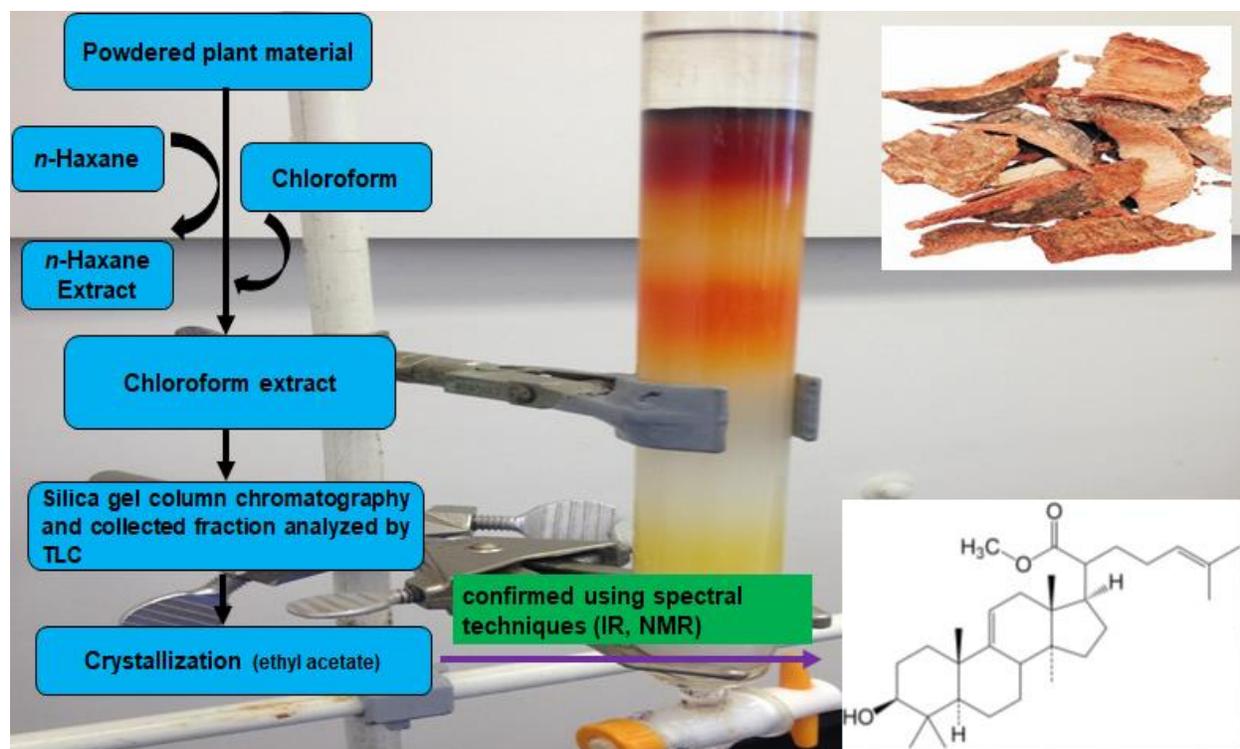


Figure 3.1: Schematic representation of RA-3 extraction and isolation from the plant material. Spectral techniques were used for confirmation of the compound structure.

3.2.2 *In vitro* anti-protein glycation activity

Unless stated otherwise, all the experiments were replicated three times and mean values of the results reported

3.2.2.1. Bovine serum albumin glycation

The antiglycation activity of RA-3 was determined using the Bovine Serum Albumin (BSA)-fructose assay as described by Deo *et al.* (2016). A reaction mixture (2 mL) containing BSA (10 mg/ml), fructose (100 mM), RA-3 (0-5 mg/ml) and 0.02% sodium azide was incubated at 37 °C for 15 days. Aminoguanidine was used as standard. The amount of glycated protein (AGE) was determined by measuring fluorescence at excitation of 370 nm and emission of 440 nm. The anti-glycation activity was expressed as:

Percentage inhibition of fluorescent AGE (%) = $[(F_{\text{control}} - F_{\text{Tsample}}) / F_{\text{control}}] \times 100$.

Where F_{control} represents fluorescence of the control sample and F_{Tsample} represents fluorescence of the tested samples.

3.2.2.2. Haemoglobin glycation

The anti-protein glycation effect of RA-3 was evaluated on haemoglobin following the method of Madhuri *et al.* (2016). The reaction mixture consisted of 500 μL of RA-3 (0-0.25 mg/mL), haemoglobin (0.06%), 0.02% ciprofloxacin and 2% fructose. All components of the reaction mixture were prepared in 0.01 M phosphate buffer (pH 7.4). Gallic acid was used as a standard for this experiment. The reaction mixture was incubated in the dark for 72 h. Absorbance was read at 443 nm. The experiment was replicated three times and the percentage (%) inhibition of haemoglobin glycation was calculated using the formula

% Inhibition = $(A_0 - A_1) / A_0 \times 100$

Where, A_0 is the absorbance of the control sample and A_1 is the absorbance of the sample in the presence of inhibitor. The concentration of RA-3 providing 50 % inhibition (IC_{50}) was determined from the graph of % inhibition against RA-3 concentrations.

3.2.2.3. Determination of fructosamine

Following the evaluation of the anti-protein (BSA and haemoglobin) glycation, the concentration of fructosamine in the samples was determined using nitro-blue tetrazolium (NBT) assay, as previously described by Adisakwattana *et al.* (2012). The glycated BSA or haemoglobin was incubated with 0.5 mM NBT in 0.1 M sodium carbonate buffer (pH 10.4) at 37°C for 15 min. The amount of fructosamine in the glycated protein was determined by measuring absorbance at the wavelength of 590 nm. The standard curve of 1-deoxy-1-morpholino-fructose (1-DMF) was used to calculate the concentration of fructosamine (mg/ml).

3.2.2.4. Determination of protein thiol group

The Ellman's assay was used to determine the level of protein thiol groups following the proteins' incubation with fructose (Adisakwattana *et al.*, 2012). The glycated samples were incubated with 5 mM 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) solution for 15 min, followed by measuring the absorbance at the wavelength of 410 nm. The concentration of the free thiol groups of the samples was determined from the standard curve of L-cysteine ($\mu\text{g/mL}$).

3.2.3. Experimental Animals

Ethical clearance (UZREC 17110-030 PGM 2017/481) for the use of animals and all experimental procedures was obtained from the University of Zululand Research Ethics Committee (UZREC). Sprague Dawley rats (of either sex) weighing 100-120 g were obtained from the animal unit of the Department of Biochemistry and Microbiology, University of Zululand. The animals were housed under standard conditions, in a room with a 12:12 h light/dark cycle and controlled temperature (23 °C). They were allowed free access to standard rat chow and drinking water. The animals were left to acclimatize for five days before the experiment commenced.

3.2.4 Diabetes induction

The rats were divided into two respective groups; normal diet (ND) and high-fat diet groups. The high-fat diet (HFD) was composed of 55% normal rat chow, 40% beef fat and 5% cholesterol (Oosterveer *et al.*, 2009). The animals were placed on their respective diets for 28 days. Hyperlipidemia in the HFD fed animals was confirmed by measuring total blood cholesterol and triglyceride levels from the rat's tail tip using Accutrend Plus CTG meter (Roche Products, Johannesburg). After 28 days of high fat diet feeding, the rats were overnight fasted, followed by intraperitoneal injection of a low dose (30 mg/kg body weight) of freshly prepared STZ solution (in 0.1 M cold citrate buffer pH 4.5) to induce diabetes. Plasma glucose levels were measured Five days after STZ injection using the Accutrend CTG meter. The rats presenting with fasting blood glucose level \geq 7.5 mmol/L were considered diabetic and were used for this study.

3.2.5 *In vivo* evaluation of the neuroprotective potential of the triterpene

The neuroprotective potential of the triterpene (RA-3) was investigated in diabetes induced peripheral neuropathy in rats. After a week of STZ induction, the diabetic group was further divided into four groups of five rats per group (Figure 3.2). The rats in the experimental groups received a single daily dose of RA-3 at 100 mg/kg orally (daily for 28 days), while negative and positive control groups received 2% Tween 20 (vehicle) and metformin (100 mg/kg) orally. The animals in the ND fed group received an equivalent volume of distilled water.

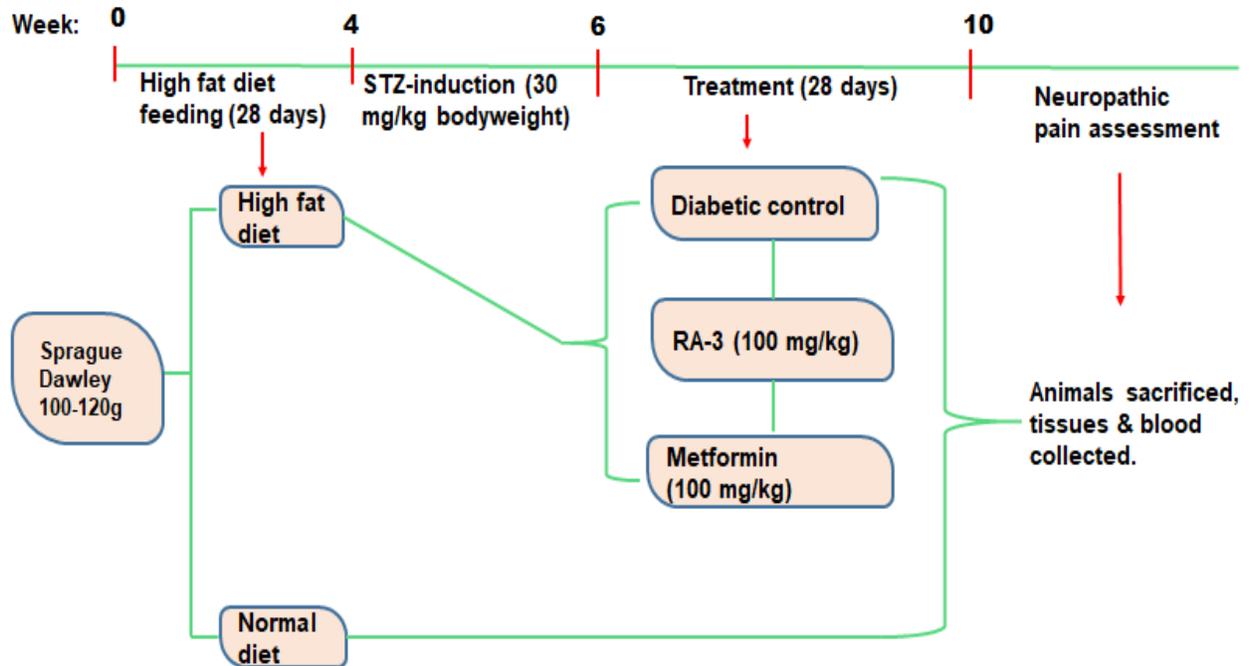


Figure 3.2: Experimental design for the investigation of diabetic neuropathy protective potential of RA-3.

Blood glucose, total cholesterol and triglycerides levels were measured weekly (7 days intervals) until the last day of the treatment. Changes in body weight were also monitored weekly. At the end of the experimental period, the animals' responses to pain were assessed. Appropriate restraining methods as recommended by the National Society for the Prevention of Cruelty to Animals (NSPCA) were used to hold the animals for operating procedures.

3.2.5.1 Cold allodynia

The method described by Bhokar and Upananlawar (2015) with modifications was followed to determine the sensitivity of the rats to cold (cold allodynia). The rat's tail was dipped into a fixed volume of ice-cold water and its level of sensitivity to cold was determined by a tail flick. For measurement of the tail flick latency, the cut-off time was 15 seconds.

3.2.5.2 Thermal allodynia

The thermal hyperalgesia of the rats was determined as described by Bhokar and Upaganlawar, (2015) with some modifications. The rat's tail was immersed in hot water maintained at the maximum temperature of 55 °C. Time taken by the rat to flick its tail was recorded and used as a guide of pain response, the cut-off time was 15 seconds in order to avoid harm to the tail.

3.2.5.3 Biochemical analysis

On the last day of the experimental period, all the animals were fasted for 8 h and euthanized under anaesthesia. The loss of sensation was confirmed by the pedal withdrawal reflex and assessed by pinching the tails and the metacarpal region of the hind foot between the index finger and the thumb, prior to blood collection. Blood was immediately collected by cardiac puncture and the sciatic nerve and brain were removed for analysis of important biochemical parameters and histopathology, respectively.

The blood was allowed to clot, and serum was collected. Sciatic nerves and brains were weighed and homogenized in 0.1 M phosphate buffered saline (pH 7.2) and 0.1 M phosphate (pH 8.0), respectively. While the brains were used for determination of acetylcholinesterase activity, some sciatic nerves were preserved in neutral buffered formalin (10% v/v) for histological analysis. The serum and sciatic nerves homogenate were kept at -80°C until required. These samples were used for biochemical estimation of some antioxidants (SOD, GSH) and MDA content, β -NGF, TGF- β 1 and inflammation markers (TNF- α , COX-2 & IL-6). Respective commercial assay kits and enzyme-linked immunosorbent assay (ELISA) kits were used to analyze for the biochemical parameters following manufacturers' instructions.

3.2.5.4 Estimation of serum fructosamine concentration

Fructosamine concentration in the serum of the rats was determined using nitro-blue tetrazolium (NBT) assay as previously described by Adisakwattana *et al.* (2012). Briefly,

serum samples from the different groups were incubated with 0.5 mM NBT in 0.1 M sodium carbonate buffer (pH 10.4) at 37°C for 15 min. The absorbance was measured at the wavelength of 590 nm. The standard curve of 1-deoxy-1-morpholino-fructose (1-DMF) was used to calculate the concentration of fructosamine (mg/ml).

3.2.5.5 Estimation of acetylcholinesterase activity in the brain tissue

The colorimetric method of Ellman *et al.* (1961), as described by Khan *et al.* (2012), was followed to determine the effect of the triterpene (RA-3) on acetylcholinesterase activity in the brain tissue of the experimental rats. The brain homogenate (source of the enzyme) was diluted (x10) with 0.1 M phosphate buffer (pH 8.0). The reaction mixture consisted of 100 µL of the homogenate, 140 µL of 0.1 M phosphate buffer (pH8.0), and 25 µL of DNTB reagent. The reaction was initiated by adding 5 µL of acetylthiocholine iodide and absorbance of the yellow color produced was measured at 412 nm for 5 min, at 60 second intervals. The enzyme activity was calculated, and the final reading of enzyme activity was expressed as µ moles/minute/mg tissue.

$$\text{Acetylcholinesterase activity (M/ml)} = \frac{A/\text{min} \times V_t}{\epsilon \times b \times V_s}$$

Where, A/min = Change in absorbance per min, $\epsilon = 1.361 \times M^{-1}cm^{-1}$, b= 96 well path length (0.05 cm), V_t = Total volume and V_s = sample volume.

3.2.6 Statistical analysis

Unless otherwise stated, the results obtained were expressed as mean \pm standard error of the mean (SEM). Statistical comparisons between the groups were performed by one-way analysis of variance (ANOVA) followed by Dunnett *post hoc* test and two-way ANOVA, followed by Tukey's multiple comparisons test using Graph Pad Prism version 6 (V₆). The statistical difference was considered significant where $p < 0.05$.

CHAPTER 4

4.0 Results

4.1. Isolation and confirmation of the compound (RA-3)

RA-3 was routinely isolated and purified from the chloroform extract of *P. longifolia* stem bark using chromatographic techniques. The physical (white crystals, mp 204-205°C) and spectral data (IR (KBr) ν_{\max} = 3469, 1683 cm^{-1} , molecular formula $\text{C}_{31}\text{H}_{50}\text{O}_3$) of this compound correlated with previous reports from our laboratory (Machaba *et al.*, 2014; Mosa *et al.*, 2014). The chemical structure of RA-3 was confirmed as methyl-3 β -hydroxylanosta-9, 24-dien-21-oate (Figure 2.5). The IR, ^1H -NMR and ^{13}C -NMR spectral data are presented in Appendix B.

4.2. *In vitro* antiglycation activity of the triterpene

4.2.1 Haemoglobin and BSA glycation

The haemoglobin and BSA-Fructose assays were used to investigate the anti-protein glycation activity of RA-3 and the results are presented in Figure 4.1. The results demonstrated that RA-3 exhibited a concentration dependent inhibitory effect against haemoglobin and BSA glycation with the IC_{50} values of 1.53 and 1.04 mg/mL, respectively. The inhibitory effect of the triterpene was comparable to those of gallic acid and aminoguanidine which were used as standards.

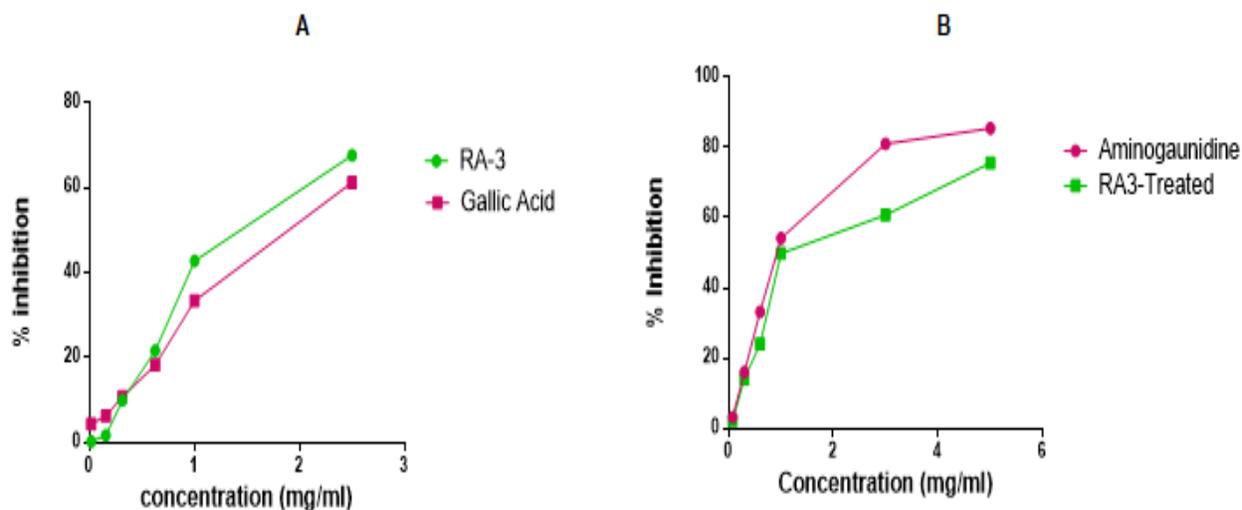


Figure 4.1: Inhibitory effect (%) of RA-3 on haemoglobin (A) and BSA (B) glycation. IC₅₀ values of RA-3 were 1.53, 1.04 mg/mL against haemoglobin and BSA glycation, respectively. While gallic acid exhibited the IC₅₀ of 1.90 mg/mL against haemoglobin, aminoguanidine showed the IC₅₀ of 0.82 mg/mL against BSA glycation respectively.

4.2.2 Fructosamine and thiol group content

The triterpene, in a concentration dependent manner, significantly ($p \leq 0.0001$) decreased the levels of fructosamine in both BSA and haemoglobin glycated samples (Figure 4.2a & 4.2b). The antiglycation property of RA-3 was further proven by the significantly ($p \leq 0.0001$) increased concentration of protein thiol groups in both BSA and haemoglobin glycated samples (Figure 4.2c & 4.2d).

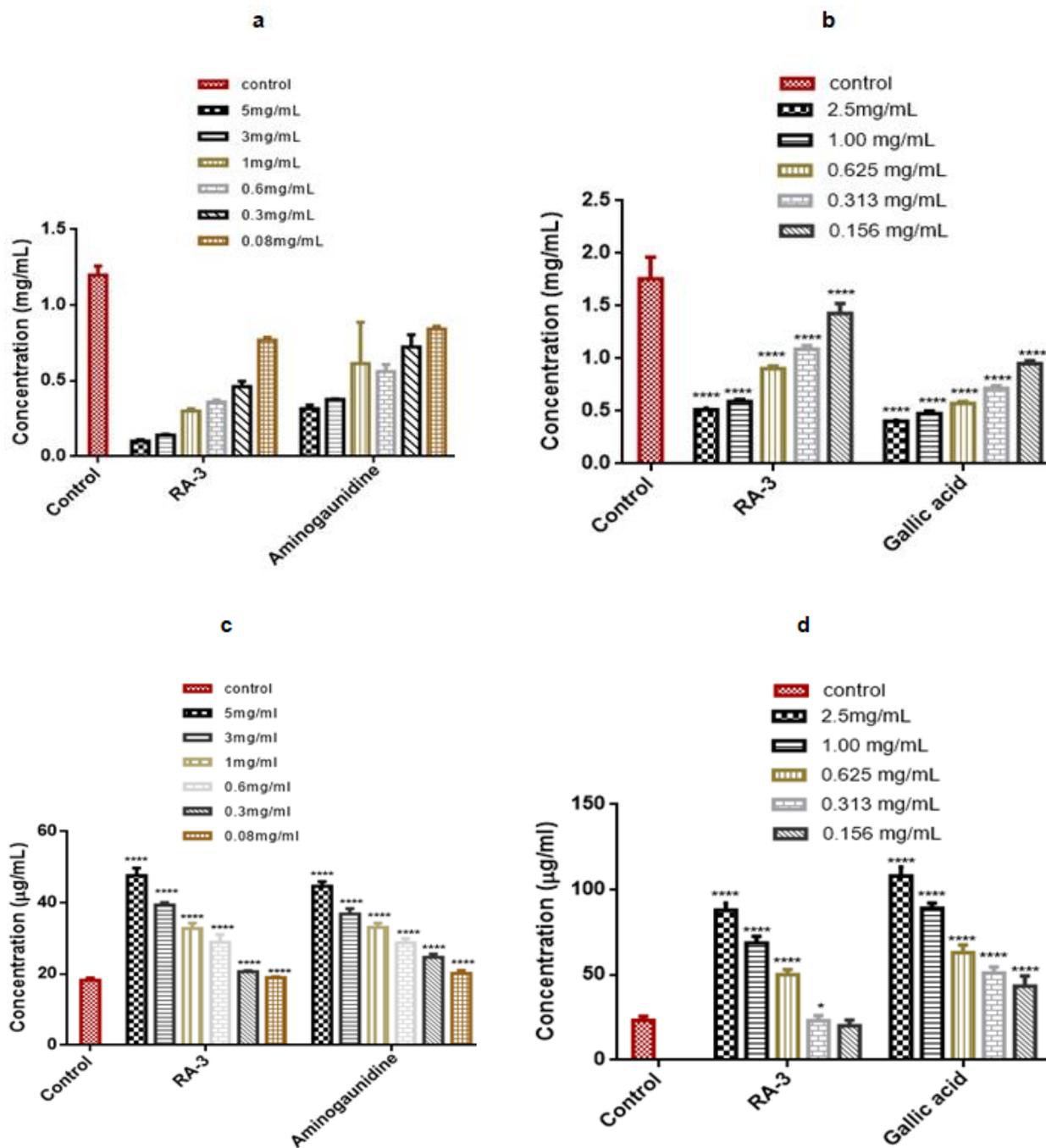


Figure 4.2: Effect of RA-3 on levels of fructosamine and protein thiol groups in BSA/fructose (a & c) and haemoglobin/fructose systems (b & d). Results are expressed as mean \pm SD (n=3), #### $p \leq 0.0001$ vs. control group.

4.3. Bodyweight changes in the diabetic neuropathy induced rats

Body weight changes (ΔBW) in the diabetic animals after 28 days of treatment period are presented in Table 4.1. A significant increase ($p \leq 0.05$) in bodyweight was observed in the normal animals in comparison to the non-treated diabetic animals. The non-treated diabetic group displayed a decrease in bodyweight on the last day of the study period. However, an increase in bodyweight was recorded in the rats administered with RA-3 or metformin.

Table 4.1: Body weight changes following diabetic neuropathy induction and treatment of the rats with RA-3 and metformin.

Group	Initial BW (g)	Final BW (g)	ΔBW
Normal control	140.5 \pm 1.88	167.4 \pm 11.70	+26.9
Diabetic control	172.6 \pm 25.98	159.9 \pm 19.39	-12.7*
Diabetic + RA-3	133.6 \pm 8.78	136.5 \pm 7.90	+2.9
Diabetic + Metformin	158.1 \pm 4.18	176.8 \pm 4.80	+18.7

Data expressed as the mean \pm SEM, $n = 5$. * $p \leq 0.05$ vs. normal control. **Initial BW** = Bodyweight after STZ administration

4.4. Changes in fasting blood levels of glucose, serum HDL and fructosamine of the experimental rats

The effect of RA-3 on blood glucose levels of the diabetic rats following 28 days of treatment is presented in Table 4.2. While the diabetic control rats showed persistently higher fasting blood glucose levels, a remarkable decrease ($p \leq 0.0001$) in the fasting blood glucose was observed after 28 days administration of RA-3 or metformin in the diabetic rats. The observed hyperglycemic state in the diabetic control group was accompanied by elevated serum levels of fructosamine and similarly, lower serum levels of the fructosamine were observed in diabetic rats treated with RA-3 or metformin (Figure

4.3). Furthermore, increased serum levels of HDL were also observed in the diabetic group treated with RA-3 when compared with the decreased levels of the lipoprotein (HDL) in the untreated diabetic group (Table 4.2).

Table 4.2: Effect of RA-3 on FBG and HDL levels of the experimental rats

Group	FBG Day 0 (mmol/L)	FBG 28 (mmol/L)	HDL (mmol/L)
Normal control	5.30 ± 0.300	5.36 ± 0.467	0.67 ± 0.018
Diabetic control	12.77 ± 0.561**	20.67 ± 0.163****	0.30 ± 0.335***
Diabetic + RA-3	16.37 ± 0.536***	6.63 ± 0.353####	0.44 ± 0.027#
Diabetic + Metformin	17.17 ± 0.276****	8.93 ± 0.384####	0.56 ± 0.052##

Data expressed as mean ± SEM and each group consisted of five rats. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ vs. normal control, #### $p \leq 0.0001$ vs. diabetic control. **FBG Day 0** = first day of drug administration to the diabetic rats.

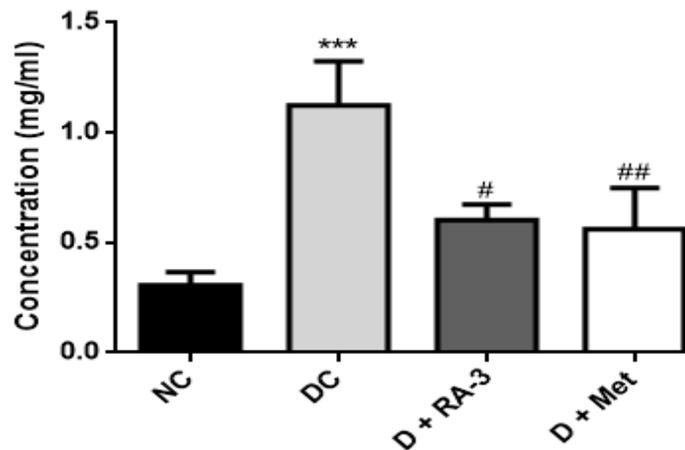


Figure 4.3: Effect of RA-3 on serum levels of fructosamine in the diabetic neuropathy-induced rats. RA-3 was administered to the experimental group animals for 28 days. Metformin was used as a standard. Results are expressed as the mean ± SEM, $n=5$. **NC**= Normal control, **DC**= Diabetic control, **D + RA-3** = Diabetic + RA-3, **D + Met** = Diabetic + Metformin. *** $p \leq 0.001$ vs normal control, ## $p \leq 0.01$, # $p \leq 0.05$ vs diabetic control.

4.5. Response of the diabetes induced animals (rats) to hot and cold allodynia

The results of the rats' responses to hot and cold allodynia are shown in Tables 4.3a and 4.3b, respectively. The diabetic animals exhibited a significantly ($p \leq 0.0001$) delayed tail withdrawal latency in both hot and cold-water tail immersion test when compared to the normal control animals (Table 4.3a and 4.3b). A decrease in tail flick latency on hot water tail immersion test was observed after treatment of the rats with RA-3 and metformin for 28 days when compared to the untreated group (Table 4.3a). Similar results were observed for the cold-water tail flick test, whereas the RA-3 treated diabetic rats demonstrated an improvement in tail flick latency compared to the untreated group (Table 4.3b). The effect of RA-3 was comparable to that of metformin.

Table 4.3a: Effects of RA-3 on the responses of rats to thermal allodynia in diabetic neuropathy state

Group	Tail Withdrawal Latency (s)	
	Day 0	Day 28
Normal control	6.64 ± 0.384	6.17 ± 0.328
Diabetic control	11.1 ± 0.244****	11.7 ± 0.244****
Diabetic + RA-3	11.4 ± 0.261****	9.70 ± 0.458###
Diabetic + Metformin	11.5 ± 0.184****	8.05 ± 0.358####

Results are expressed as the mean ± SEM, (n=5). **** $p \leq 0.0001$ vs. normal control, #### $p \leq 0.0001$, ### $p \leq 0.001$ vs. diabetic control. **Day 0** = first day of drug administration to the diabetic rats.

Table 4.3b: Effects of RA-3 on the responses of rats to cold allodynia in diabetic neuropathy state

Group	Tail Withdrawal Latency (s)	
	Day 0	Day 28
Normal control	5.87 ± 0.480	5.87 ± 0.480
Diabetic control	11.8 ± 0.266****	12.0 ± 0.203****
Diabetic + RA-3	11.7 ± 0.116****	8.09 ± 0.521###
Diabetic + Metformin	11.7 ± 0.276****	7.95 ± 0.358####

Results are expressed as the mean ± SEM, (n=5). **** $p \leq 0.0001$ vs. normal control, #### $p \leq 0.0001$, ### $p \leq 0.001$ vs. diabetic control. **Day 0** = first day of drug administration to the diabetic rats.

4.6. Effect of RA-3 on tissue antioxidant status

Table 4.4 presents the results of the effect of RA-3 on the tissue (sciatic nerve) antioxidant levels in the rats. Significantly lower GSH, SOD and total antioxidant status levels, along with a relatively higher MDA level, were observed in the diabetic control animals when compared to the normal control group. However, treatment of the diabetic rats with either RA-3 or metformin displayed a significantly ($p \leq 0.0001$) increased tissue GSH, SOD and total antioxidant status in comparison to the diabetic control animals. This was accompanied by a significant ($p \leq 0.0001$) decrease in the MDA levels. RA-3 and metformin treatment produced comparable results.

Table 4.4: Effects of RA-3 on tissue (sciatic nerve) antioxidants levels of the diabetic neuropathy -induced rats

Group	GSH (nmol/mL)	SOD (inhibition rate %)	MDA levels (nmol/ μ L)	Total Antioxidant (mM)
Normal control	8.93 \pm 0.216	53.4 \pm 1.60	0.03 \pm 0.016	1.62 \pm 0.113
Diabetic control	4.65 \pm 0.217*	21.9 \pm 1.14****	0.40 \pm 0.037 ****	0.49 \pm 0.035****
Diabetic + RA-3	8.19 \pm 0.146	41.6 \pm 1.91#### **	0.05 \pm 0.014####	0.99 \pm 0.090*** ####
Diabetic + Metformin	8.88 \pm 0.189	41.8 \pm 1.12#### *	0.04 \pm 0.011 ####	0.90 \pm 0.043*** ####

Results are expressed as the mean \pm SEM, (n=5). **** p \leq 0.0001, *** p \leq 0.001, * p \leq 0.05 vs. normal control, #### p \leq 0.0001, ### p \leq 0.001 vs. diabetic control.

4.7. The effect of RA-3 on serum and tissue IL-6, TNF- α , TGF- β 1 and COX-2

Levels of inflammation markers such as IL-6, TNF- α , TGF- β 1 and COX-2 were analyzed in both serum and tissue (sciatic nerve) of the neuropathy induced rats. Significantly higher IL-6 (91.1 \pm 5.88, 74.5 \pm 2.917), TNF- α (498.3 \pm 11.80, 293.1 \pm 13.56) and COX-2 (0.813 \pm 0.098, 0.600 \pm 0.042) levels in both serum and tissue, as an indicator of inflammation, were observed in the diabetic control group when compared to their normal control counterparts (Figure 4.4a). A significant decrease in the levels of these inflammation markers (IL-6 38.47 \pm 4.16, 44.7 \pm 1.71), TNF- α (362.7 \pm 7.97, 217.9 \pm 6.94) and COX-2 (0.309 \pm 0.026, 0.347 \pm 0.031) were observed following the treatment of the rats with either RA-3 or metformin. A similar pattern was also observed in the levels of TGF- β 1 (Figure 4.4b) in the animals treated with either RA-3 or metformin when compared to the untreated diabetic control group.

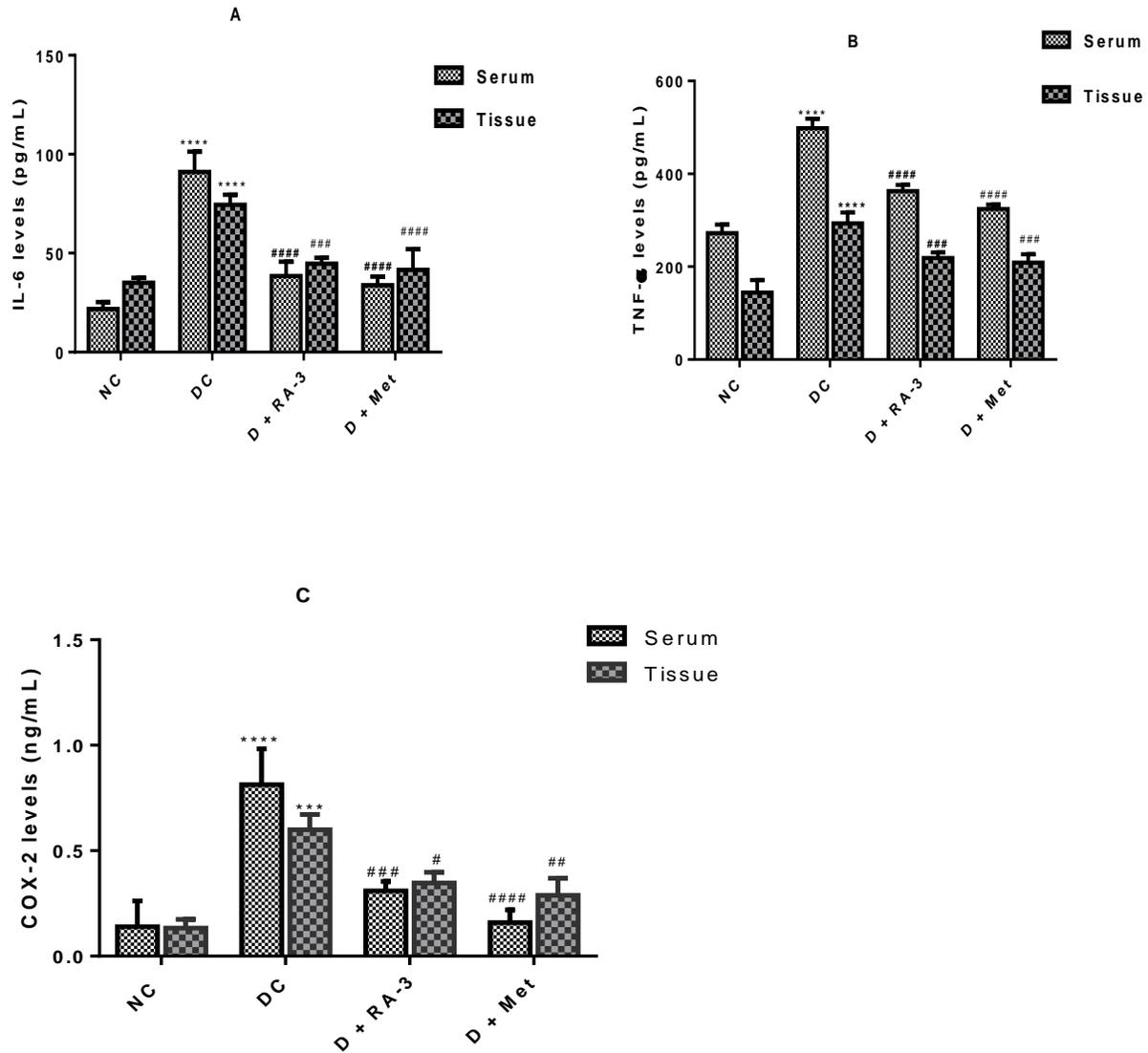


Figure 4.4a: Serum and tissue levels of IL-6 (A), TNF- α (B) and COX-2 (C) in the diabetic neuropathy-induced rats. The diabetic neuropathy experimental group rats were treated with RA-3 for 28 days. Metformin was used as a standard. Results are expressed as the mean \pm SEM, $n=5$. **NC**= Normal control, **DC**= Diabetic control, **D + RA-3** = Diabetic + RA-3, **D + Met** = Diabetic + Metformin. **** $p \leq 0.0001$, *** $p \leq 0.001$ vs normal control, ##### $p \leq 0.0001$, ### $p \leq 0.001$, ## $p \leq 0.01$ vs diabetic control.

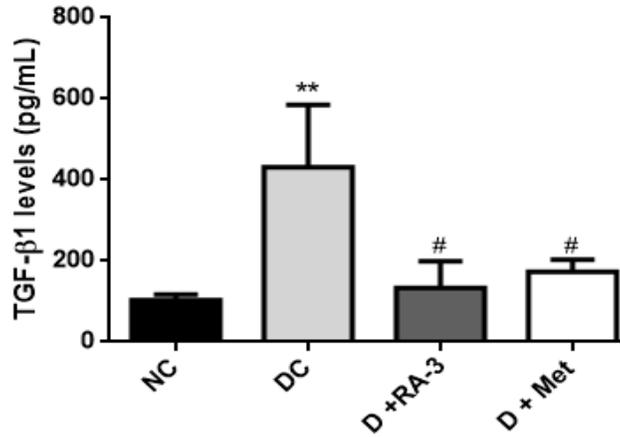


Figure 4.4b: Effect of RA-3 on serum TGF-β1 levels in the diabetic-neuropathy induced rats. The diabetic neuropathy experimental group rats were treated with RA-3 for 28 days. Metformin was used as a standard. Results are expressed as the mean \pm SEM, $n=5$. **NC**= Normal control, **DC**= Diabetic control, **D + RA-3** = Diabetic + RA-3, **D + Met** = Diabetic + Metformin. ** $p \leq 0.01$ vs normal control, # $p \leq 0.05$ vs diabetic control.

4.8. RA-3 effect on nerve growth factor (NGF) levels

The serum and tissue (sciatic nerve) levels of neurotrophic factors such as nerve growth factor (NGF) were determined and the results are shown in Figure 4.5. Even though there was no significant difference on the serum levels of NGF between the groups, higher and significant differences were observed on the tissue levels of the protein. The levels of NGF were significantly ($p \leq 0.001$) reduced in the untreated diabetic animals when compared to their normal counterparts. However, administration of RA-3 to the diabetic animals for 28 days exhibited a significant ($p \leq 0.01$) increase in their NGF levels when compared to the non-treated diabetic group. The effect of RA-3 was comparable to that of metformin (0.10 ± 0.043 and 0.08 ± 0.011 , respectively).

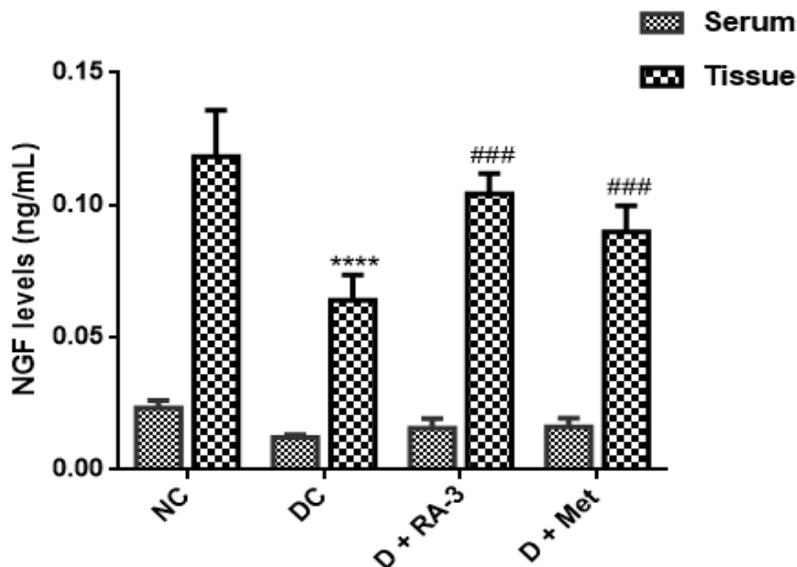


Figure 4.5: Effect of RA-3 on NGF levels from the serum and sciatic nerves of the diabetic neuropathy-induced rats. The diabetic neuropathy experimental group rats were treated with RA-3 for 28 days. Metformin was used as a standard. Results are expressed as the mean \pm SEM, $n=5$. **NC**= Normal control, **DC**= Diabetic control, **D + RA-3** = Diabetic + RA-3, **D + Met** = Diabetic + Metformin. ^{****} $p \leq 0.001$ vs normal control, ^{###} $p \leq 0.001$ vs diabetic control.

4.9. Acetylcholinesterase activity

The acetylcholinesterase (AChE) activity in the brains of the rats induced with diabetic-neuropathy was also determined. The results of the effect of RA-3 on the brain AChE activity are presented in Figure 4.6. A significantly ($p \leq 0.01$) higher enzyme activity was observed in the brain tissue of the diabetic control rats when compared with the normal control group. However, administration of RA-3 or metformin to the diabetic animals for 28 days significantly ($p \leq 0.001$ & $p \leq 0.05$) decreased the enzyme activity when compared to the non-treated diabetic group.

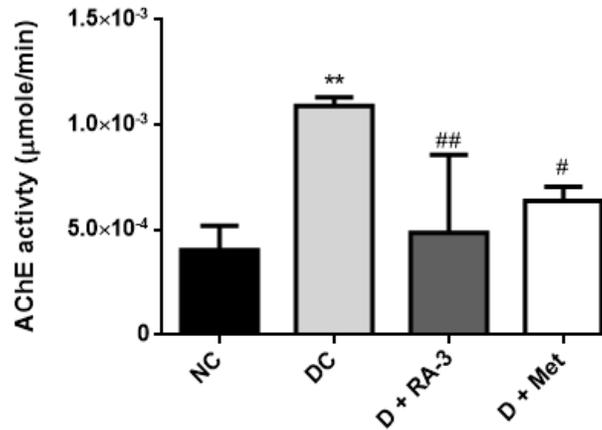


Figure 4.6: Brain acetylcholinesterase activity in the diabetic-neuropathy induced rats. The diabetic neuropathy experimental group rats were treated with RA-3 for 28 days. Metformin was used as a standard antidiabetic drug. Results are expressed as the mean \pm SEM, $n=5$. **NC**= Normal control, **DC**= Diabetic control, **D + RA-3** = Diabetic + RA-3, **D + Met** = Diabetic + Metformin. ** $p \leq 0.01$ vs normal control, ## $p \leq 0.001$, # $p \leq 0.05$ vs diabetic control.

CHAPTER 5

5.0. Discussion

Diabetic peripheral neuropathy (DPN) is the most common complication of diabetes mellitus diagnosed in half of the diabetic patient population worldwide (Erbaş *et al.*, 2016; Iqbal *et al.*, 2018). Despite high prevalence of the complication, the direct mechanism leading to its onset remains a subject of extensive research. However, literature has suggested glucose toxicity, a characteristic of chronic hyperglycemia, as the main initiator to DPN development and progression. Researchers have renewed interest in medicinal plants and their derived bioactive compounds including triterpenes, as potential remedies for the disease treatment. Several bioactivities, including antihyperglycemic activity (Mosa *et al.*, 2015; Mabhida *et al.*, 2017) of RA-3, a lanosteryl triterpene from *P. longifolia*, have been reported from our laboratory. The current study evaluated the potential therapeutic effect of the triterpene against DPN.

Chronic hyperglycemia causes biochemical changes leading to the development of DPN and other complications through activation of various biochemical pathways such as polyol pathway, the formation of AGEs, inflammation and oxidative stress (Sandireddy *et al.*, 2014; Guo *et al.*, 2018). Abnormally high fasting blood glucose levels (with diminished glucose tolerance) and decreased expression of neurotrophic factors, such as NGF, have been observed in animal models of diabetic peripheral neuropathy (Fatani *et al.*, 2015; Kelany *et al.*, 2016; Kaur *et al.*, 2017). In the current study, the high fasting blood glucose and serum fructosamine levels, accompanied by a significant reduction in the sciatic nerve levels of NGF in the diabetic rats as well as reduction in body weight of the rats, indicated induction of diabetic neuropathy. Moreover, the relatively reduced sensation of the diabetic animals to both hot and cold allodynia further confirmed the induction of diabetic neuropathy in the rats. Decreases in the fasting blood glucose levels and serum fructosamine, which is used as a measure of short-term glycemic management in diabetic patients (Muniz *et al.*, 2018) along with the observed increase in body weight of the triterpene-treated diabetic rats, proved the antihyperglycemic activity of RA-3 previously reported by Mosa *et al.* (2015) and Mabhida *et al.* (2017). The increased serum HDL

levels in the triterpene treated animals were also in agreement with the antihyperlipidemic effect of RA-3 reported by Machaba *et al.* (2014). Since glycemic control is one of the current management strategies against DPN (Kavya, 2017), the hypoglycemic and hyperlipidemic potential of RA-3 could play a crucial role in the prevention and management of diabetic peripheral neuropathy.

NGF, a member of neurotrophic factors, is involved in nerve rejuvenation, control of neuronal plasticity, axonal growth and support in repairing injured nerves (Zhou *et al.*, 2016). Decreased expression of NGF (together with its receptors) is strongly associated with neuronal apoptosis and impaired axonal transport in DPN (Sun *et al.*, 2018). The observed increase in sciatic nerve NGF levels in the diabetic rats treated with RA-3 indicates the potential of the triterpene to prevent loss of neuronal integrity and apoptosis and thus alleviate DPN. Plant-derived triterpenoids with antidiabetic properties have been reported to exhibit neuroprotective effect (Yu *et al.*, 2006; Wang *et al.*, 2016). Wang and colleagues (2016) have reported that treatment of HFD/STZ-induced diabetic rats with methanolic extract of *Toona sinensis* seeds increased expression of NGF in the serum of diabetic rats (Wang *et al.*, 2016). Folic acid has also been reported to increase sciatic nerve NGF levels of STZ-induced diabetic rats (Yilmaz *et al.*, 2013). Since neuropathy is also associated with sensory disturbances with the loss of pain sensation leading to amputations and disabilities (Koneri *et al.*, 2014; Wang *et al.*, 2016), the ability of RA-3 to improve the rats' sensitivity to heat and cold stimuli further indicates its neuroprotective potential. The obtained results were consistent with Koneri and colleagues, (2014) who demonstrated that a triterpene saponin decreased tail immersion latency time as a sign of pain sensation in STZ-induced diabetic neuropathy rats (Koneri *et al.*, 2014).

In addition to neurotrophic factors, acetylcholine plays a significant part in maintaining cholinergic neurons and cognitive function. However, increased activity of AChE observed in the diabetic state (Sathya, 2013) stimulates hydrolysis of the neurotransmitter and thus terminates its physiological actions (Garcia *et al.*, 2012; Sachdeva *et al.*, 2015). Therefore, tight regulation or inhibition of AChE could be essential in maintaining cognitive

function and performance, thus preventing neurological disorders. In the present study, the observed decrease in AChE activity in the RA-3 treated group reveals the potential of RA-3 to inhibit AChE thus preventing cholinergic dysfunctions. Some other plant-derived triterpenes have been reported to have AChE inhibitory properties (Kekkar *et al.*, 2013; Culhaoglu *et al.*, 2015). Rosmarinic acid, a natural compound found in different plants, has been shown to exert AChE inhibitory effects in the STZ-induced diabetes rats (Mushtaq *et al.*, 2013).

The diabetic neuropathy development and progression is linked to various hyperglycemia induced cellular and tissue damaging factors such as AGEs formation and oxidative stress (Sandireddy *et al.*, 2014; Guo *et al.*, 2018). Moreover, hyperglycemia induced-AGEs causes demyelination of peripheral nerves, increasing their susceptibility to phagocytosis by macrophages. Literature supports that inhibition of AGEs could also serve as therapeutic strategy in the management of DPN (Iqbal *et al.*, 2018). Inhibitors of AGEs, such as aminoguanidine and a thiazolidine derivative, have been reported to improve nerve conduction (Mushtaq *et al.*, 2013). The ability of RA-3 to effectively inhibit glycation of BSA and haemoglobin *in vitro*, accompanied by reducing the serum fructosamine concentration in diabetic rats, indicated its potential *in vivo* inhibition of AGEs formation. Oleanolic acid (pentacyclic triterpene) (oleanolic acid) also exhibit anti-protein glycation activity in *in vivo* experiments (Wang *et al.*, 2011). Increased generation of ROS during glycation and glycooxidation has been reported to generate oxidative damage to amino acids remnants of proteins to form carbonyl derivatives, which impair the oxidative defense of proteins by destroying the thiol groups (Nowoty *et al.*, 2015). The antiglycation mechanism of RA-3 might be related to its ability to prevent production of fructosamine, accompanied by the protection of protein thiol groups. The results obtained in the current study are similar to those obtained by Xi *et al.* (2010), where they reported the antiglycation activity of triterpenoids saponin from *Aralia taibaiensis* extracts in BSA/Haemoglobin-Glucose assays.

Furthermore, oxidative stress is interrelated with chronic tissue inflammation in the pathological mechanism of diabetes and its complications, particularly peripheral neuropathy. Improving tissue antioxidant capacity is imperative in prevention and control of diabetes mellitus, thus preventing neuropathy development. Data obtained from the present study demonstrated a potential increase in antioxidant status of injured sciatic nerve tissue after treatment with RA-3. The increased antioxidant status accompanied by decreased MDA levels (a measure of lipid peroxidation) in the RA-3 treated diabetic group indicated the potential of the compound to improve the tissues' antioxidant defense system. Previously conducted studies (Mosa *et al.*, 2015; Mabhida *et al.*, 2018) have also displayed the ability of RA-3 to improve the tissue antioxidant status of diabetic rats. The diabetic neuroprotective potential of the triterpene could partly be associated with its tissue antioxidant defense boosting nature.

The protective effect of RA-3 against hyperglycemic induced cellular and tissue damage was further indicated by the reduced serum and tissue levels of inflammatory mediators (TGF- β 1, IL-6, TNF- α , and COX-2) in the diabetic animals treated with the triterpene. The ability of RA-3 to reduce levels of inflammatory mediators and enhance antioxidant status shows its great potential to inhibit NF- κ B activation and potentially reduce its expression. NF- κ B is amongst the inducible transcription factors that significantly regulates immune and expression of pro-inflammatory genes (Dludla *et al.*, 2016; Lui *et al.*, 2018). NF- κ B activation also suppresses the antioxidant system of cells through suppressing the nuclear factor erythroid 2-related factor 2 (Nrf-2) actions, thus weakening the antioxidant defense (Sandireddy *et al.*, 2014).

In the diabetic state, increased expression of NF- κ B accelerates apoptosis through the release of inflammatory mediators such as TGF- β 1, TNF- α and IL-6 (Tabur *et al.*, 2015; Li *et al.*, 2018). While TNF- α is known to enhance expression of adhesion molecules and impaired neurotropic support, increased COX-2 activity triggers pro-inflammatory processes that can worsen neuronal degeneration and impairment (Kang *et al.*, 2017). Over-expression of COX-2 is associated with uncontrolled cell apoptosis, proliferation and

growth (Gandhi *et al.*, 2017). Furthermore, the cytokine TGF- β 1 induces neuronal cell injury and reduces neurite growth, which are features involved in the pathogenesis of DPN (Anjaneyulu *et al.*, 2008). The ability of RA-3 to reduce inflammatory markers strongly suggests the anti-inflammatory effect the triterpene, thus a potential to attenuate diabetic neuropathy. Literature data report that agents that can inhibit activation and expression of pro-inflammatory mediators could also serve as a therapeutic strategy in the prevention and management of diabetic neuropathy (Oh *et al.*, 2016). Ethanolic extract of *Gymnema sylvestre* decreased serum and sciatic nerve content of inflammatory cytokines (IL-1 β , TNF- α and IL-6) against STZ-induced diabetic neuropathy rats (Fatani *et al.*, 2015).

Chronic hyperglycemia contributes to the development of DPN by activating various pathways such as the polyol pathway, the formation of AGEs, oxidative stress and inflammation (Sandireddy *et al.*, 2014; Guo *et al.*, 2018). Considering the shift to natural products in health promotion, natural therapeutic agents with antihyperglycemic, antioxidant and anti-inflammatory activities could be beneficial in the prevention DPN. Some other plant derived triterpenes, exerting antihyperglycemic, antioxidant and anti-inflammatory activities, have been reported to show neuroprotective potential in animal models of diabetic neuropathy (Furtado *et al.*, 2017, Meeran *et al.*, 2018). The diabetic neuroprotective potential of RA-3 could also be linked to its antihyperglycemic, anti-inflammatory and antioxidant properties.

CHAPTER 6

6.0. Conclusion

Chronic hyperglycaemia observed in diabetic patients is considered responsible for the development and progression of painful diabetic neuropathy. The antidiabetic properties of RA-3, a lanosteryl triterpene from *P. longifolia*, have previously been well established (Mosa *et al.*, 2015; Mabhida *et al.*, 2017; Mabhida *et al.*, 2018). The present study investigated the diabetic neuroprotective effect of the triterpene. While chronic hyperglycemia initiates and accelerates events leading to diabetic neuropathy development and progression (Figure 6.1), the ability of RA-3 to reverse these contributing factors and subsequently increase tissue NGF levels indicate its neuroprotective potential.

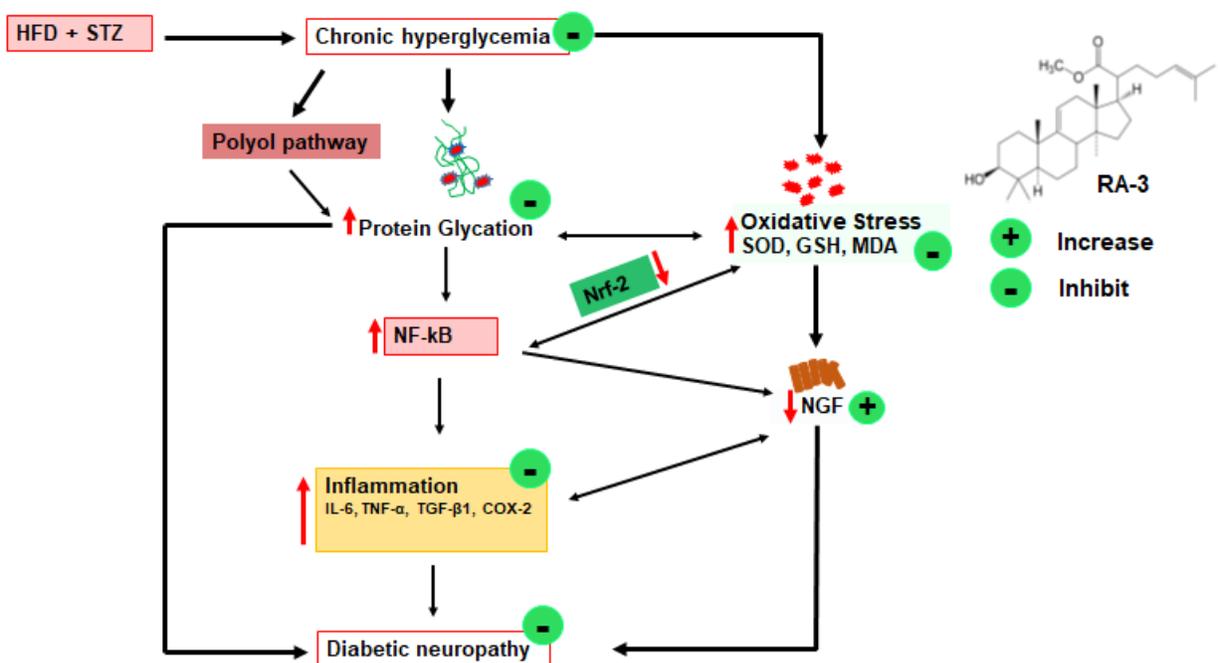


Figure 6.1: A schematic overview of events leading to diabetic neuropathy and possible mechanism(s) through which RA-3 could be exerting its diabetic neuroprotective effect. Chronic hyperglycemia observed in diabetic state activates polyol pathway, AGEs formation, oxidative stress and inflammation activation with the latter two directly causing decreases in tissue levels of NGF and consequent DN development.

In conclusion, the results obtained in the present study demonstrate that RA-3 possesses diabetic neuroprotective properties. The neuroprotective potential of RA-3 was supported by the observed increase in tissue NGF levels, improved sensitivity of the animals to heat and cold stimuli as well as the decreased AChE activity in the triterpene treated diabetic rats. In addition to its antihyperglycemic effects, the mechanism through which the triterpene exerts its neuroprotective effect could be associated with its antioxidant and anti-inflammatory activities. The diverse bioactive properties of the triterpene encourage the potential use of this compound, directly or as a structural template, in the development of new pharmacologically active neuroprotective agents.

6.1. Limitations

Due to time limitation and budget, protein expression to further confirm the molecular mechanism of RA-3 on its neuroprotective effect as well as histological analysis of the sciatic nerve to confirm the prevention of nerve cell death or degeneration could not be performed. However, the biochemical analysis conducted provided substantial evidence for the neuroprotective potential of RA-3

6.2. Recommendation for future studies

- i. Histopathological analysis of the sciatic nerves from the diabetic neuropathy induced rats, is recommended to confirm the protective effect of RA-3.
- ii. It is necessary to evaluate effect of RA-3 on expression of proteins and genes involved in diabetic neuropathy development and progression.

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APPENDIX A

A0. Details of preparation of some reagents

A1. 0.1 M phosphate buffer, pH 8.0

Phosphate buffer (0.1 M) was prepared by dissolving 0.01 M di-sodium hydrogen orthophosphate (Na_2HPO_4) and 0.01 M sodium di-hydrogen orthophosphate (NaH_2PO_4) in 200 ml distilled water and the pH was adjusted with sodium hydroxide (NaOH) and hydrochloric acid (HCl) to pH 8.0.

A2. 2 M sodium carbonate buffer pH 10.4

Sodium carbonate buffer (2 M) was prepared by dissolving 2 M (4.24 g) sodium carbonate and 2 M (3.36 g) sodium bicarbonate in 20 mL distilled water, the pH was adjusted to pH 10.4 with sodium hydroxide and hydrochloric acid.

A3. 0.1 M Citrate buffer pH 4.5

Citrate buffer (0.1M) was prepared by dissolving 0.1 M (1.47 g) tri-sodium citrate and 0.1 M (1.05 g) citric acid in 50 mL distilled water and the pH was adjusted to pH 4.5 with sodium hydroxide and hydrochloric acid.

A4. Composition of High fat diet

The ingredients (55 g normal rat chow, 40 g beef fat, and 5 g cholesterol) were thoroughly mixed together and well dried prior feeding.

APPENDIX B

B0. Details of Methodology

B1. Extraction

Fresh stem bark of *Protorhus longifolia* was washed with tap water, chopped into smaller pieces, and air-dried. The air-dried plant material was extracted sequentially with hexane and chloroform at room temperature (25°C). The plant material was first extracted (1:5 w/v) with hexane (for 48 h) to remove fats and other fatty plant components. This was followed by filtration of the extract using whatman no.1 filter paper. While the filtrate was collected for solvent recovery, the residue was subsequently subjected to chloroform extraction (for 48 h) and then the extract also filtered. Both the hexane and chloroform filtrates were separately concentrated *in vacuo* at $37 \pm 2^\circ\text{C}$, using Heidolph rotary evaporator, to recover the solvents and obtain the crude extracts. The crude chloroform extract was used for the isolation of the lanosteryl triterpene.

B2. Isolation and purification

The lanosteryl triterpene (RA-3) was isolated from the chloroform extract of the plant material following a well-established method described by Mosa *et al.* (2014). Silica gel (Silica gel 60; 0.063-0.2 mm, 70-230 mesh ASTM) was prepared into slurry and this slurry was added to the glass column as the stationary phase. The gel in the column was allowed to reach a constant level before it could be sealed with a thin layer of acid purified sea sand. The initial solvent system (n-hexane: ethyl acetate, 9:1) was run through the column to ensure a tight packing as well as to remove any air bubbles. The crude extract was loaded on to the column. The column was then again sealed with a thin layer of acid purified sand. The column was then steadily run with the initial solvent system until the front coloured area of the extract has reached three quarters of the column length. A series of 40 ml fractions were collected in beakers to analyse the eluents. The column

was step wisely eluted with solvent system of increased polarity (1:9 →7:3). Thin layer chromatography (TLC) was used to analyse the small collected fractions. The TLC plates were developed using a 10% H₂SO₄ spray reagent and then heated (110°C). The fractions with similar profile were combined and separately concentrated *in vacuo*. The targeted compound (RA-3) was obtained as white crystals after recrystallization in ethyl acetate. The chemical structure of RA-3 was confirmed using spectroscopic techniques (NMR, IR) as well as by comparison with literature data (Machaba *et al.*, 2014; Mosa *et al.*, 2014).

APPENDIX C

Table C1: ^1H and ^{13}C NMR data of RA-3

Position	δ_{C}	Type	δ_{H}
1	30.4	CH ₂	
2	23.9	CH ₂	
3	74.3	CH	4.25 (1H,s,OH)
4	37.4	C	
5	44.4	CH	
6	17.3	CH ₂	
7	25.8	CH ₂	
8	49.6	C	
9	145.9	C	
10	34.7	C	
11	118.3	CH	5.11 (1H,t)
12	28.6	CH ₂	
13	43.3	C	
14	51.0	C	
15	31.3	CH ₂	
16	27.0	CH ₂	
17	47.7	CH	
18	13.3	CH ₃	
19	21.7	CH ₃	
20	48.4	CH	
21	177.3	C	
22	34.7	CH ₂	
23	25.5	CH ₂	
24	125.6	CH	5.21 (1H,t)
25	136.4	C	
26	17.4	CH ₃	1.61 (3H,s)
27	25.8	CH ₃	1.65 (3H,s)
28	21.9	CH ₃	1.21 (3H,s)
29	27.4	CH ₃	0.90 (3H,s)
30	22.1	CH ₃	1.16 (3H,s)
-OCH ₃	59.8		3.85 (3H,s)

APPENDIX D

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RESEARCH ETHICS COMMITTEE**
(Reg No: UZREC 171110-030)



RESEARCH & INNOVATION

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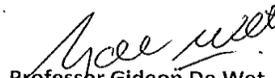
ETHICAL CLEARANCE CERTIFICATE

Certificate Number	UZREC 171110-030 PGM 2017/481						
Project Title	Neuroprotective potential of a lanosteryl triterpene from protorhus longifolia stem bark in high fat and fructose diet-induced diabetic neuropathy in rats.						
Principal Researcher/ Investigator	M Ndlovu						
Supervisor and Co-supervisor	Dr RA Mosa			Prof AR Opoku			
Department	Biochemistry and Microbiology						
Faculty	SCIENCE AND AGRICULTURE						
Type of Risk	Low Risk– Data collection from animals						
Nature of Project	Honours/4 th Year		Master's	x	Doctoral		Departmental

The University of Zululand’s Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project. The Researcher may therefore commence with data collection as from the date of this Certificate, using the certificate number indicated above.

- Special conditions:**
- (1) This certificate is valid for 3 years from the date of issue.
 - (2) Principal researcher must provide an annual report to the UZREC in the prescribed format [due date-30 April 2018]
 - (3) Principal researcher must submit a report at the end of project in respect of ethical compliance.
 - (4) The UZREC must be informed immediately of any material change in the conditions or undertakings mentioned in the documents that were presented to the meeting.

The UZREC wishes the researcher well in conducting research.


Professor Gideon De Wet
 Chairperson: University Research Ethics Committee
 Deputy Vice-Chancellor: Research & Innovation
 27 March 2018

CHAIRPERSON UNIVERSITY OF ZULULAND RESEARCH ETHICS COMMITTEE (UZREC) REG NO: UZREC 171110-30 28-03-2018 RESEARCH & INNOVATION OFFICE
