UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES
SCHOOL OF PHARMACY

ANALGESIC EFFECTS OF *SYNEDRELLA NODIFLORA* (L) GAERTN WHOLE PLANT EXTRACT IN STREPTOZOTOCIN- INDUCED DIABETIC NEUROPATHY IN RATS.

BY

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JULY, 2018

DECLARATION

DECLARATION BY THE CANDIDATE

I hereby declare that this is the product of my own research undertaken under supervision and has neither been presented in whole nor in part for another degree elsewhere. I am solely responsible for any residual flaws in the work.

Signature…….. Date……/……/…….

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DECLARATION BY SUPERVISORS

We hereby declare that the principal work and presentation of the thesis were supervised by us in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

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Co-supervisor

Signature…… Date……/……/……

(Dr. Kennedy E. Kukuia)

DEDICATION

To my family for the support and to all who supported and encouraged me on this journey I say "thank you". Your advice has been useful as examples to me on this journey and I owe it to the instructions that you communicated.

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To my friends Martin Akandawigne, Frederick Alexander Koomson, Joseph Torbi, Awo Efua Koomson, Richard Nelson Lartey and to all post-graduate colleagues who made school worthwhile I say “thank you”.

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ABREVIATIONS

ALT	Alanine aminotransferase
AST	Aspartate transaminase
ALP	Alkaline phosphatase
CNS	Central Nervous System
DN	Diabetic neuropathy
DPN	Diabetic Peripheral Neuropathy
DSP	Diabetic sensorimotor polyneuropathy
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
HDL	High density lipoproteins
LDL	Low density lipoproteins
NO	Nitric oxide
PKC	Protein kinase c
ROS	Reactive oxygen species
ED_{50}	Effective dose for 50%
MDA	Malondialdehyde
PGB	Pregabalin
SNRI	Serotonin-Norepinephrine reuptake inhibitors
STZ	Streptozotocin
T1DM	Type 1Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
UDP	Uridine diphosphate

ABSTRACT

Background: The ethanolic extract of *Synedrella nodiflora* has demonstrated analgesic activity in acute and chemotherapy-induced neuropathic pain in rodents. The present study evaluates the analgesic effect in hyperalgesia and allodynia associated with streptozotocin–induced diabetic neuropathy in rats and also elucidates the antioxidant activity of the extract.

Method: Diabetic neuropathy was induced by intraperitoneal injection with 55 mg/kg freshly prepared streptozotocin (STZ) dissolved in 0.1 mol/l citrate buffer (pH 4.4). At the end of the first week after STZ injection, blood sugar measurements were taken for diagnosing diabetes. Rats that became neuropathic after 3 weeks of successful diabetes induction were pretreated with SNE (100, 300, 1000 mg/kg, *p.o*), pregabalin (PGB) (10, 30,100 mg/kg, *p.o*) and vehicle (0.5ml normal saline) respectively. Pain assessment tests; hot plate, cold water and the paw pressure analgesimeter were used to measure the pain threshold on alternate days. Blood serum was collected for biochemical analysis and assayed for malondialdehyde concentration and superoxide dismutase activity. Selected organs of interest were isolated and examined for any pathological alterations using the haematoxylin and eosin method

Results: *Synedrella nodiflora* (SNE) increased reaction latency to thermal and mechanical hyperalgesia dose- dependently and significantly ($p<0.01$ and $p<0.001$) in 300 and 1000 mg/kg doses. Tail withdrawal latency in cold water also increased ($p<0.01$) with 1000 mg/kg dose. Histopathological examination of isolated organs showed slight variation between treatment and non-treatment groups. There was no increase in superoxide dismutase activity and no significant decrease in malondialdehyde concentration ($p>0.05$).

Conclusion: *Synedrella nodiflora* has analgesic activity against hyperalgesia (thermal and mechanical) and cold allodynia in diabetic neuropathy in rats.

CHAPTER ONE

INTRODUCTION

1.0 Background

The neuropathic pain developing in patients with diabetes are known to be heterogonous by their symptoms, pattern of neurologic involvement, course, risk covariates, pathologic alterations, and underlying mechanisms (Tesfaye *et al.* 2010). The pain is caused by a damage in the peripheral nerves and can also occur in the central nervous system (Iannitti *et al.* 2014). It is secondary to diabetes mellitus (Daousi *et al.* 2004), shingles (Jung *et al.* 2004) multiple sclerosis (Mori *et al.* 2010) spinal cord injury (Woller *et al.* 2014), stroke (Alstadhaug and Prytz 2012), HIV infection (Liu *et al.* 2016), and cancer (Falk *et al.* 2014). Neuropathic pain (NP) in diabetes is pain arising as a direct consequence of abnormalities mostly in the peripheral somatosensory system (Treede *et al.* 2008). The prevalence of NP in the diabetic population is difficult to estimate as definitions have varied enormously among studies. However, it is crudely estimated that about 25% of patients might experience NP (Boulton *et al.* 2004). The symptoms are distal, symmetrical, often associated with nocturnal exacerbations, and commonly described as prickling, deep aching, sharp pain like electric shock, and a burning sensation with hyperalgesia and frequently allodynia upon examination (Boulton *et al.* 2004). The symptoms are usually associated with the clinical signs of peripheral neuropathy;hyperalgesia and allodynia. Major treatment options include the reduction of hyperglycemia, which causes pain to persist (Lee *et al.* 1990). Pain relief of only 30% to 50% can be achieved in most patients taking higher doses of medication (Wong *et al.* 2007). Uncontrolled chronic hyperglycemia resulting from absolute insulin deficiency (type 1 diabetes) or insulin resistance with or without insulin deficiency (type 2 diabetes) is one of the primary causes of diabetic complications in a number of organs (Chawla *et al.* 2016). Type 1

diabetes mellitus is caused by cell-specific autoimmune destruction of the insulin producing beta cells in the pancreas (Yoon and Jun 2005). Type 2 diabetes occurs as a result of the failure of beta cells to compensate for insulin resistance (Li *et al.* 2012) or selective loss of pancreatic beta cells due to viral infections or toxic damage. Hyperglycemia-induced oxidative and nitrosative stress has been singled out as one of the major links between diabetes and diabetic complications (Oyenihi *et al.* 2015). It leads to generation of free radicals due to autoxidation of glucose and glycosylation of proteins (Oyenihi *et al.* 2015), Even though medications for neuropathic pain are effective, they present with severe side effects and therefore agents of natural sources that can be well tolerated are needed as alternatives to these chemical agents (Taesotikul *et al.* 1998, Woode *et al.* 2009). Traditionally, most plant materials are used as medicines for curing and prevention of most diseases in Ghana. One of such plants mostly exploited for its medicinal properties is *Synedrella nodiflora*. It is an animal herb which is a native tropical American weed but now dispersed pan-tropically and occurring through-out the West African sub-region (Mshana *et al.* 2000). Presently the aqueous extract of the whole plant is drunk for the treatment of epilepsy and pain (Forestieri *et al.* 1996, Mshana *et al.* 2000). A considerable amount of investigation into its medicinal properties has been done and is found to demonstrate the following activities: anti-inflammatory activity (Abad *et al.* 1996), analgesic activity (Woode *et al.* 2009), antioxidant activity (Amoateng *et al.* 2011), anticonvulsant activity (Amoateng *et al.* 2012), , and ameliorating vincristine and paclitaxel induced-neuropathic pain (Amoateng *et al.* 2015, Amoateng *et al.* 2017). To further establish and confirm the plants traditional use in alleviating and managing pain, the present study was conducted to determine the analgesic effects of the ethanolic extract of *Synedrella nodiflora* in streptozotocin-induced diabetic neuropathy in rats.

1.1 Problem statement

The prevalence of neuropathy in diabetic patients is about 30%, whereas up to about 50% of these patients ultimately develop neuropathy (Han *et al.* 2014). It has been projected that worldwide prevalence of diabetes will increase to about 472 million by 2030. Diabetic neuropathy can be categorized into general polyneuropathies thus focal or multifocal (Fila *et al.* 2008). The polyneuropathies can be further categorized into typical and atypical in relation to development, course, associations, clinical presentation and pathophysiology. Diabetic neuropathic pain is a chronic, symmetrical distal sensorimotor polyneuropathy. It is the most reported presentation of the peripheral micro vascular damage in diabetes (Deshpande *et al.* 2008). Diabetes has gradually attained an epidemic status universallywith China alone having 90 million people of the entire population diagnosed with diabetes (Yang *et al.* 2010), representing a greater number of the universal diabetic population. To worsen the status of the global epidemic, the financial and economic cost of diabetes is increasing appreciably. A report by the American Diabetes Association stated that the total 2012 estimated medical expenditure of diabetes in the United States had reached a total of about $176 billion, with workplace absenteeism and reduced workplace productivity reaching $69 billion (Eizirik *et al.* 2013). It was reported that 21.5 million people are affected with diabetes resulting in about half a million diabetes-related deaths in sub-Sahara Africa in the year 2013 (Gatimu *et al.* 2016). There is however a wide variation in the prevalence of diabetes in various age groups with adults being at a greater risk compared to the younger groups (Albert and Duffy 2012). For instance, the prevalence of diabetes has been estimated to be between 7.7 to 20% and 5 to 8.8% for adults aged 45 years and more in Kenya and South Africa respectively (Gatimu *et al.* 2016). This suggests that old age is found to be associated with increased risk of acquiring diabetes and its complications, this association could

be explained by the cumulative effect of early life exposure to biological, social and behavioral determinants of diabetes (Gary-Webb *et al.* 2013).The micro and macro-vascular complications of diabetes are potentially devastating and affect multiple organs and tissues, including the eyes, kidney, heart, and nerves. Approximately 60% of all diabetic patients develop diabetic peripheral neuropathy (DPN), the most common micro-vascular complication (O'Brien *et al.* 2014). DPN is characterized by a progressive distal-to-proximal degeneration of peripheral nerves, which results in sensory symptoms, including pain, weakness, and/or loss of sensation. DPN is associated with significant morbidity and mortality and aside tight glycemic control there is no ideal agent which is safer for effective management. DPN pathophysiology is not completely understood; determining the underlying mechanisms that cause nerve injury and prevent nerve regeneration is of paramount importance for the development of successful therapeutic interventions. Meanwhile, current goal of treatment of DN is to diminish pain, increase the functionality and improve the quality of life of every patient (Hosseini and Abdollahi 2013). Despite efforts to ensure a tight glycemic control to stop the progression of DN, the condition still develops in most diabetic patients. The current medications available for DPN provide pain relief to an extent but not without untoward side effects which causes patients to discontinue medications.

1.2 Justification

Even though medications for neuropathic pain are effective, they present with severe side effects and therefore agents of natural source that can be well tolerated are needed as alternatives to these chemical agents (Taesotikul *et al.* 1998). Traditionally, most plant materials are used as medicines for curing and prevention of most diseases in Ghana. One of such plants mostly exploited for its medicinal properties is *Synedrella nodiflora*. It is an animal herb which is a

native tropical American weed but now dispersed pan-tropically and occurring through-out the West African sub-region (Mshana *et al.* 2000). Traditionally the aqueous extract of the whole plant is drunk for the treatment of epilepsy and pain (Forestieri *et al.* 1996). A considerable amount of investigation into its medicinal properties has been done and is found to demonstrate the following activities: anti-inflammatory activity (Abad *et al.* 1996), analgesic activity (Woode *et al.* 2009), antioxidant activity (Amoateng *et al.* 2011), anticonvulsant activity (Amoateng *et al.* 2012), , and ameliorating vincristine and paclitaxel induced-neuropathic pain (Amoateng *et al.* 2015, Amoateng *et al.* 2017). These remarkable investigations of the plant extract have made it an ideal plant material to exploit, due to its established hypoglyceamic and analgesic activities in-vivo. Further investigations into the medicinal properties of the plant may provide a new and a promising alternative in the management of diabetes and diabetic neuropathy. Currently pharmacological management remains the most important therapeutic option for chronic neuropathic Pain. However draw backs in the use of these major therapeutic interventions and, the high cost of newer and more effective antiepileptic drugs have led to a greater proportion of patients in Ghana and possibly other third world countries, resorting to the use of traditional medicine. There is therefore a universal and local need for continued research into the development of newer and cost effective agents for the management of this disorder. Plant sources of drugs dominate therapy in the developing countries and have often served as an effective means of getting lead compounds, from which newer and effective drugs can be developed

1.3 Research Question

1. Will the effect of *Synedrella nodiflora*, as used in the treatment of vincristine-induced neuropathy, be beneficial in diabetic neuropathy?

2. What will be the mechanism of the analgesic effect of the plant extract?

1.4 Aim

To investigate the analgesic effects of the ethanolic extract of *Synedrella nodiflora* in streptozotocin-induced diabetic neuropathy in rats.

1.5 Objectives

- To obtain an ethanolic extract of the whole plant of *Synedrella nodiflora*.
- To investigate the analgesic effect of the ethanolic extract in diabetic neuropathic rats.
- To conduct behavioural, physiological and functional assessment of diabetic neuropathic rats.
- To investigate the antioxidant activity of the *Synedrella nodiflora* extract.

CHAPTER TWO

LITERATURE REVIEW

2.0 Diabetes and Diabetic neuropathy

Diabetes can impact the peripheral nervous system in a multitude of ways, it is responsible for such a greater proportion of all peripheral nerve presentations, such that the terms diabetic DSP, and diabetic neuropathy are sometimes used interchangeably. Neuropathic patients usually experience numbness, tingling, and painful sensations that progress in the feet upwards and generates proximally in a length-dependent manner (stocking and glove distribution) (Albers and Pop-Busui 2014). The manifestation of the symptoms is symmetric, with more pronounced sensory symptoms with little or no motor participation. Many patients experience an apparent anomaly of insensibility and excruciating sensitivity at the same time. Amongst the two, the one that prevails varies dramatically from patient to patient (Callaghan *et al.* 2012a). Diabetic neuropathic pain associated with numbness often causes balance problems which can lead to falls (D'Silva *et al.* 2016). Comparatively patients with diabetic neuropathy are much more susceptible to falls than those with diabetes and without neuropathy (Peltier *et al.* 2014). The condition leads to an increased risk of acquiring foot ulcers and lower extremity amputations during the course of their disease (Dabkana *et al.* 2018). Chronic and long standing hyperglycemia is the principal cause of lower extremity amputations, which has a higher probability of occurring in those that develop neuropathic pain (Al-Rubeaan *et al.* 2015). A greater number of lower extremity amputations are performed within patients with diabetic foot ulcers each year (Ferreira *et al.* 2018). The overall impact of diabetes on the patient standard of health is undesirable, especially in those who have developed the complication of neuropathic pain (Alleman *et al.* 2015). DN is one of the leading health problems that incapacitate patients

suffering from diabetes, currently there are no therapeutic agents available for its treatment this leads to a remarkable patient distress (Goldman *et al.* 1999) . An overall estimation of the diabetic neuropathy stands at 10% to 20% of the diabetic population, and 40% to 60% with documented neuropathy (Callaghan *et al.* 2012a) these reported figures are underrated, as 12% of patients with DNP at no time in past or future discussed this condition with their doctors (Callaghan *et al.* 2012a). Symptoms of DN are very much consistent with the symptoms of other forms of neuropathic pain distinguished by electric-stabbing sensations with or without insensibilities. Clinical presentation of DN on examination is expressed as painful sensations to innocuous stimuli and exaggerated response to pain, clinically termed as allodynia and hyperalgesia respectively.

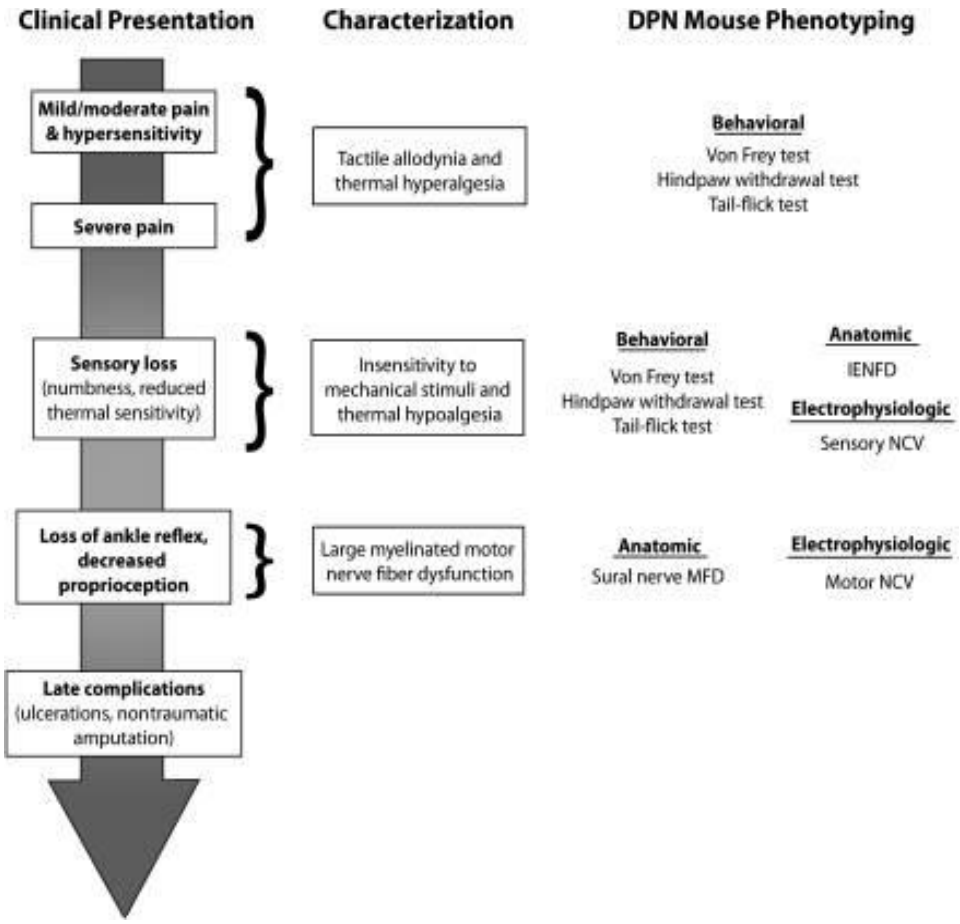

Figure 1: Typical presentation of common diabetic peripheral neuropathy (DPN) features.

(O'Brien *et al.* 2014).

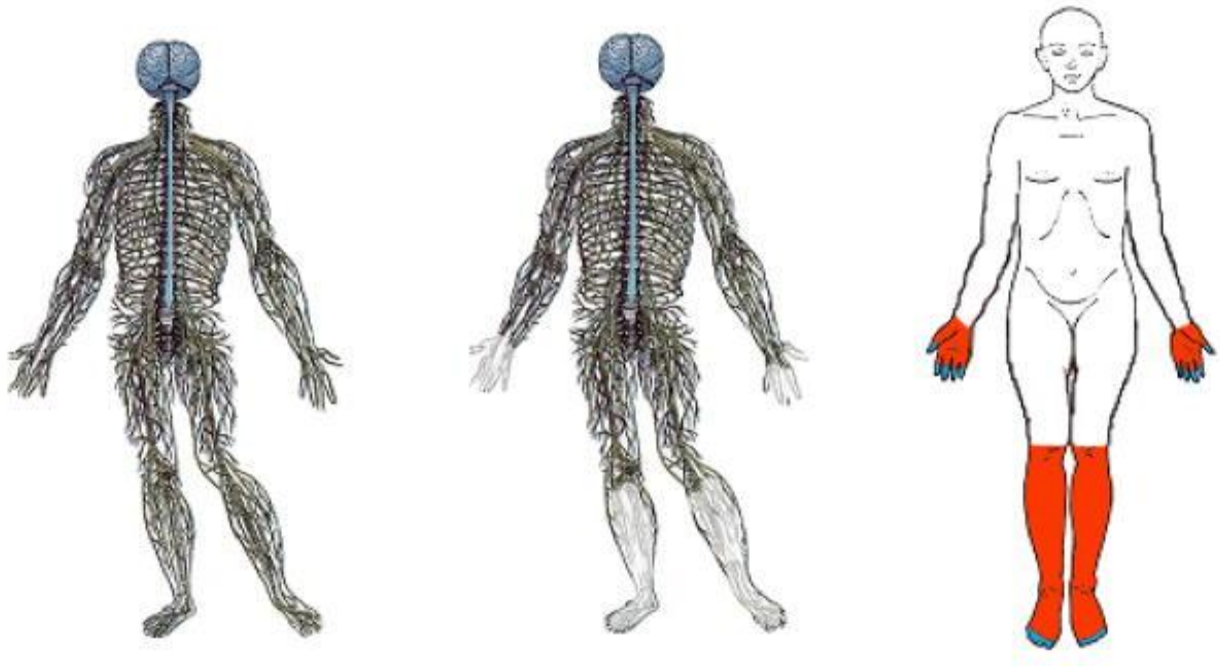

Figure 2: Stocking Glove Configuration of DPN

Diabetic neuropathy is dependent on axon length, initiating in the toes and progressing upward until reaching the calf. Neuropathy presents at the fingertips at this point.(Edwards *et al.* 2008)

2.1 Oxidative stress and diabetic neuropathy

Reactive oxygen species activated stress has been identified as one of the common pathways in the development of diabetic neuropathy. Major studies over several years has documented major routes involved in micro vascular complications of the nerves (Edwards *et al.* 2008). Oxidative stress rooting from long term hyperglycemia has been established as a link that provides a unified mechanism of tissue damage (Negi *et al.* 2011). Significant changes in oxidative stress biological markers in DN and the over expression of superoxide in sciatic nerve resulting in altered vaso-relaxation is accountable for nerve malfunctions (Pacher *et al.* 2005). Superoxide elevation reduces vascular activity which further leads to a block in nutritive supply to the sciatic

nerve. Decreased glutathione and antioxidant enzymes are also implicated in diabetic neuropathy (Figueroa-Romero *et al.* 2008). There was an observed DNA fragmentation in peripheral nerve sections of diabetic neuropathic animal models (Sullivan *et al.* 2007), natural cell death in the dorsal root ganglion (DRG) and vagus ganglion in streptozotocin- induced diabetic models (Guo *et al.* 2004). However, in other research papers it was established that increased ROS as a result of chronic hyperglyceamia did not lead to natural cell death in the peripheral neurons (Zherebitskaya *et al.* 2012). Zherebitskaya et al. concluded that increased blood glucose levels leads to the reduced expression of antioxidant enzymes and results in ROS mediated distortions in DRG, however it did not bring about the elevation in apoptotic reactions. Natural cell death in peripheral nerves and diabetes presentation still remains unclear; many suggest that it should be overlooked when diabetic nerve degeneration is reviewed. There is however enough evidence linking chronic increase in blood glucose levels to reactive ROS accumulation as hyperglycemia induced oxidative stress is usually associated with an increase in markers like superoxide and peroxynitrite ions, it is therefore critical to look at early expression of oxidative biological markers in the diabetes. This will provide a quick diagnosis of the condition and offer an appropriate response in halting further progression of diabetic neuropathy (Yagihashi *et al.* 2011)

2.2 Anatomy of diabetic neuropathic pain.

Pain is the body's response to actual or potential damage to the nerve or tissue by noxious stimuli. The sensory afferent nerves carry sensations from the skin, joints, and viscera via large and small fibres. Large fibres, such as A-alpha, are responsible for limb proprioception and A-beta fibres carry sensations of limb proprioception, pressure, and vibration. Large A-delta myelinated fibres and small C unmyelinated fibres are mainly responsible for carrying nociceptive sensations. Superficial pain is often a sharp or pricking sensation and is transmitted

by A-delta fibres. A deep-rooted burning, itching, aching type of pain is mostly associated with hyperalgesia and allodynia and is carried by slow, unmyelinated C fibres. Tissue distraction results in the production of inflammatory mediators (prostaglandins, bradykinins, and histamines) at the site of inflammation. This initiates the depolarization of pain receptors, leading to the generation of action potentials (Willis and Westlund 1997). The action potential carries the pain sensation, through the dorsal root ganglion (DRG), to the dorsal horn of the spinal cord. The production of glutamate, substance P results in the relay of pain sensations to the spino-thalamic tract, thalamus, and, subsequently, the cortex, where pain is interpreted and perceived (Willis and Westlund 1997). Nociceptive pain is the normal response to noxious insult or injury of tissues such as skin, muscles, visceral organs, and joints. The pain usually subsides upon the healing of the tissue injury, neuropathic pain however arises as a direct consequence of a lesion or disease affecting the somatosensory system without any noxious stimuli. This type of pain is caused by damage or pathological change and is characterized by the activation of abnormal pathways of pain at the peripheral nerves, posterior roots (peripheral neuropathic pain), spinal cord and brain (central pain) (Aslam *et al.* 2014).

2.3 Pathophysiology of Diabetic Neuropathy

Although the precise etiology of Diabetic peripheral Neuropathy (DPN) remains largely unclear, the growing consensus is that DPN results from the impact of metabolic and physiologic imbalances within the nerve environment. Chronic hyperglycemia and elevated levels of plasma glucose produce metabolic abnormalities and contribute to nerve damage by triggering dysfunctional biochemical mechanisms (Vincent *et al.* 2011). This includes the generation of advanced glycation end products (Jack and Wright 2012), increased activity of the polyol pathway (Oates 2002), protein kinase C (Geraldes and King 2010), inflammation (Pucić *et al.*

2011), impaired insulin signaling (Kim and Feldman 2012), mitochondrial reactive oxygen species production (Sena and Chandel 2012), endoplasmic reticulum stress (Lupachyk *et al.* 2013) and dyslipidemia (Hinder *et al.* 2013).Vascular disease is also present in diabetes with micro-vascular pathophysiology contributing to neuropathy in both humans and experimental models of diabetes (Tesfaye *et al.* 1994). As DPN progresses neuronal dysfunction closely coincides with the development of endoneurial microangiopathy (Britland *et al.* 1990, Malik *et al.* 1989), capillary basement membrane thickening (Ashton 1974) and endothelial hyperplasia (Timperley *et al.* 1985). These vascular abnormalities promote diminished oxygen tension and hypoxia resulting in neuronal ischemia. Further confounding our understanding of DPN pathologic mechanisms is the recent suggestion that DPN in T1DM and T2DM may be inherently separate disorders (O'Brien *et al.* 2014). This premise is based on results from a number of clinical trials examining the efficacy of strict glucose control on diabetic peripheral neuropathy prevalence. Whereas maintaining glucose control attenuated DPN onset and progression in T1DM populations, no similar beneficial correlations have been observed in T2DM populations, suggesting that there are fundamental differences in DPN etiology in T1DM and T2DM (Callaghan *et al.* 2012b)

2.4 Pathways typically attributed to the development of diabetic peripheral neuropathy

2.4.1 Polyol Pathway

The Polyol focuses on the enzyme aldose reductase. This enzyme normally serves the purpose of reducing toxic aldehydes to the less toxic inactive alcohols but when glucose concentration in the cell becomes too high aldose reductase converts glucose into sorbitol and later oxidized into fructose. In this process, the aldose reductase consumes the cofactor NADPH which is essential in generating glutathione which is critical in reducing intracellular oxidative stress (Brownlee

2005). In a study performed by Engerman et al, diabetic dogs were treated for 5 years with an aldose reductase inhibitor and when the treatment was stopped, the diabetes-induced defect in nerve conduction velocity was prevented (Engerman *et al.* 1994).

2.4.2 Advanced glycated end products.

Intracellular Production of Advanced glycated end products (AGE) precursor's damage to cells is in three separate mechanisms. The first mechanism involves the endothelial cell. It modifies intracellular proteins including proteins involved in gene transcription. The second mechanism is that the AGE will diffuse out of the cell and modify extracellular matrix molecules nearby and this changes the signaling between the matrix and the cells causing cellular dysfunction. This pathway can be seen in the cross linking of collagen, tendon and ligament pathology. The final mechanism is that AGE products diffuse out of the cell and modify circulating proteins in the blood such as albumin. The proteins will then activate AGE's causing the production and release of inflammatory cytokines and growth factors which in turn lead to vascular pathology (Winocour *et al.* 1987).

2.4.3 Protein Kinase C Pathway

The Protein Kinase C activation is precipitated when hyperglycemia increases the synthesis of diacylglycerol and activates the cofactors for protein kinase C. PKC affects gene expression such as downregulation of endothelial nitric oxide (NO) and upregulation of vasoconstrictor endothelin-1. This will result in changes to Schwann cell metabolism and ultimately axonal flow (Greene *et al.* 1985)

2.4.4 Hexosamine Pathway

The final pathway precipitated by hyperglycemia is the hexosamine pathway. When intracellular glucose is high it is metabolized through glycolysis. Some of the fructose 6-phosphate gets diverted into a signaling pathway in which an enzyme called GFAT converts it into UDP (uridine phosphate) N-acetyl glucosamine. At an end stage, it binds to serine and threonine which leads to changes in gene expression. (Nawroth *et al.* 2017)

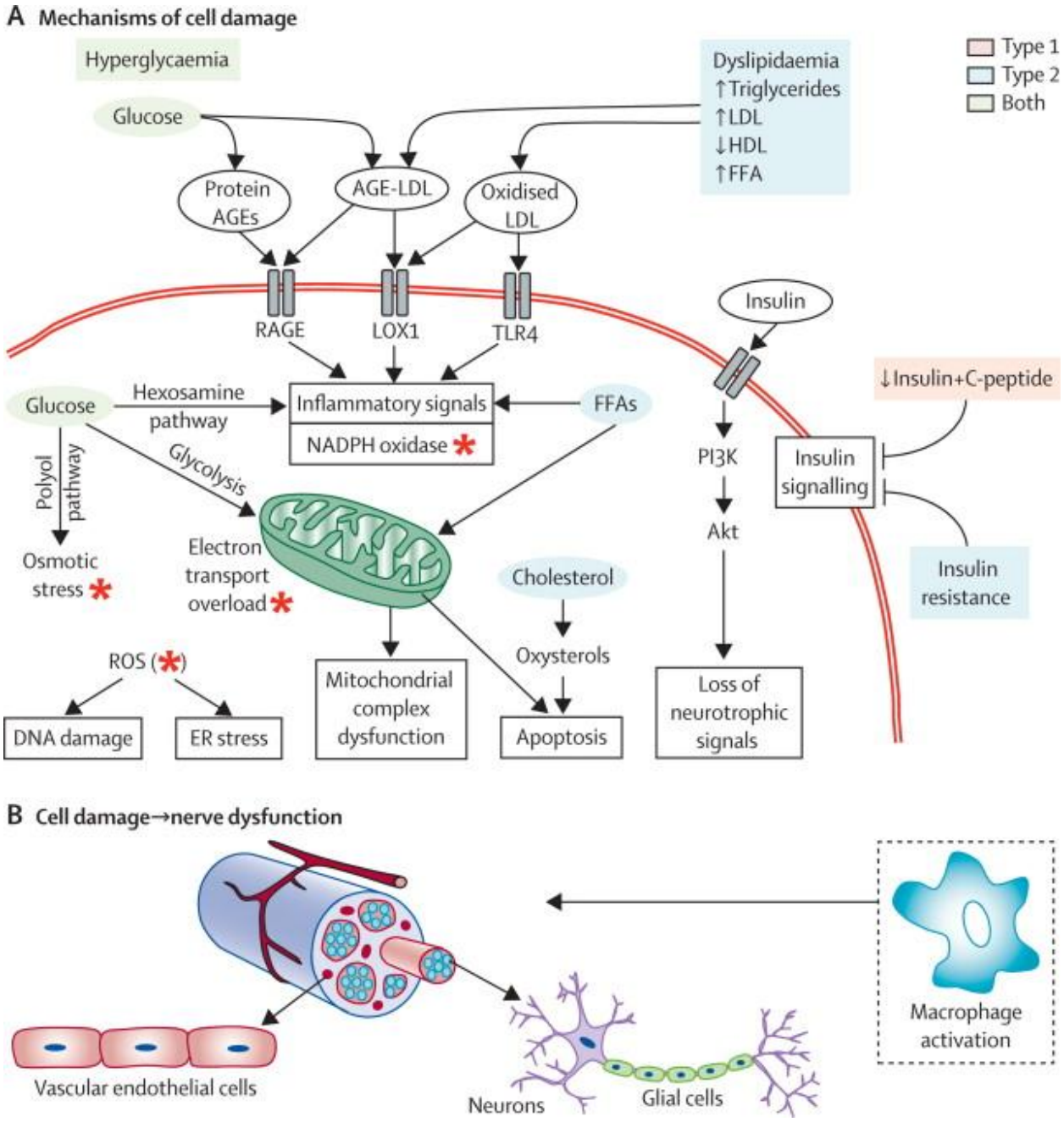

Figure 3: Mechanisms of diabetic neuropathy

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2.5 Pharmacotherapy of Diabetic neuropathic pain

2.5.1 Antidepressants and diabetic neuropathy

The pain-relievingg properties of antidepressants is non-dependent on their anti-depressant activity. Their analgesic property may be as a result of their effect on descending modulatory inhibitory controls, there are other pathways which involve the antagonism of sodium channels and glutamate receptors, and its action on β2 adrenergic receptors have been discussed (Khan *et al.* 1999). Several antidepressants are employed in the treatment of neuropathic pain (NP): tricyclic antidepressants (TCAs), amitriptyline being often used (TCAs usually have same effects comparatively with each other). Serotonin–norepinephrine reuptake inhibitors (SNRIs) in current publications and test trials have confirmed duloxetine to be efficacious in painful diabetic neuropathy and its effectiveness extends to some other NP associated conditions (Attal and Bouhassira 2015). Side effects of the antidepressants include somnolence and constipation which have been established in clinical trials, Dry mouth is mostly with TCA whereas duloxetine administration is much more with nausea but tertiary amine TCA have extremely poor side effects comparatively with the other class of antidepressants, these effects include ; postural hypotension, sedation (Attal and Bouhassira 2015) .

2.5.2 Cannabinoids and diabetic neuropathy

Oro-mucosal cannabinoids (2.7 mg delta-9-tetrahydrocannabinol and 2.5 mg cannabidiol) have been found to be effective in 2 trials in multiple sclerosis–associated pain and for refractory peripheral NP associated with allodynia, (Attal *et al.* 2010, Rog *et al.* 2005) but several published and unpublished trials in the same NP conditions were negative in the primary outcome (Attall *et al.* 2011). Common side effects include dizziness, fatigue, somnolence, and

nausea. Cannabis may potentially exacerbate psychiatric conditions, and therefore cannabinoids are not recommended for patients with psychiatric disorders (Fischer *et al.* 2011)

2.5.3 Opioids and diabetic neuropathy

Opioid agonists (particularly oxycodone and morphine) have been reported to be moderately effective in peripheral Neuropathic pain (Eisenberg *et al.* 2005).Most common adverse effects are constipation, nausea, vomiting, tiredness, somnolence, dizziness, dry mouth, and itch. After several years, opioid use may be associated with risk of abuse, particularly with high doses in young patients as well as potential cognitive impairment, endocrine and immunologic changes (Provenzano and Viscusi 2014, Pujol *et al.* 2018). There are concerns about an increase in prescription for opioid and its associated overdose. These concerns accounts for the increase in mortality rate, diversion, misuse, and other opioid related morbidities (Fischer *et al.* 2014). It is therefore necessary to track the daily dose in morphine and monitor more closely when patients require higher daily doses.

Tramadol is also of the opioid group of analgesics which exerts its activity via the blockade of serotonin and norepinephrine reuptake, another type of opioid norepinephrine reuptake blocker is tapentadol They are however abused, misused and with possible dependency than the other opioids. Tramadol is moderately effective in the treatment of Neuropathic Pain and should be used with caution in the elderly (risk of confusion) and when in combination with antidepressants (risk of serotonin syndrome) in the treatment of pain associated with other conditions (Vinik *et al.* 2014)

2.5.4 Anti-epileptic drugs and diabetic neuropathy

In preclinical studies the analgesic effects of pregabalin and gabapentin are mainly related to a decrease in central sensitization and nociceptive transmission through the action on the alpha-2-delta subunit of calcium channels. (Kehlet *et al.* 2006, Luo *et al.* 2002) Their efficacy is established in peripheral or central NP, but the number of weak or negative trials have increased over the last 5 years (Simpson *et al.* 2010, Yowtak *et al.* 2011). Extended-release formulations of gabapentin (gabapentin extended release) have similar efficacy as gabapentin in clinical trials and can be used twice daily ((Finnerup *et al.* 2015, Rauck *et al.* 2013) Similar efficacy as compared to TCA has however been reported (Finnerup *et al.* 2010). Common side effects include somnolence, dizziness, and weight gain. Anti-epileptics other than pregabalin and gabapentin (eg, topiramate, oxcarbazepine, carbamazepine, valproate, zonisamide, lacosamide) have weak or inconsistent results in Neuropathic pain with the notable exception of carbamazepine in trigeminal neuralgia (Attal and Bouhassira 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.0 Plant collection

Fresh whole plant of *Synedrella nodiflora* was collected from the University of Ghana botanical garden (GH-GA548-8183) in June 2016 and was identified by a botanist at the Department of Plant and Environmental Science (UG) and a voucher specimen (PA01/UGSOP/GH17) was kept in the university's Herbarium

3.1 Plant extraction

The plant was air-dried indoors for about three weeks and then milled into fine powder using a blender. A total of about 3kg of the powdered sample was extracted using three and half liters of 70% v/v ethanol to cold macerate. The mixture was allowed to stand for 48 hours. The mixture was filtered and the filtrate collected in a round bottom flask. It was then concentrated under reduced pressure using the rotary evaporator until the extract evaporated into brown syrup mass. A 10.5% w/w yield was obtained and labeled as SNE. The concentrate was kept in a refrigerator till required .

3.2 Experimental animals

Female Sprague-Dawley rats (150-200 g) was obtained from the Centre for Plant Medicine Research, Mampong, Ghana, and housed in the Department of Animal Experimentation, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon. The animals were housed in groups of eight in stainless steel cages (34 cm ×47 cm ×18 cm) with soft wood shavings as bedding. They were fed with normal commercial pellet diet (GAFCO, Tema) and given water ad libitum. The experimental animals were maintained under laboratory

conditions (temperature 24-28 C, relative humidity 60-70%, 12 hour light-dark cycle). All procedures and techniques used in these studies were in accordance with the Noguchi Institute Animal Care and Use Committee (NIACUC) with protocol number NIACUC-2012-01-1E.

3.3 Preliminary Phytochemical Analysis

The presence of saponins, tannins, alkaloids, triterpenes, flavonoids, glycosides, and reducing sugars was tested by simple qualitative test as described by Trease and Evans (1989) and Sofowora (1993).

3.4 Drugs and chemicals

Pregabalin (Lyrica) was purchased from Pfizer Pharmaceuticals, SOD kits (sigma Aldrich) thiobabituric acid (sigma Aldrich), streptozotocin (Sigma-Aldrich), Blood glucose kits (one touch select plus®, China).

3.5 Induction of diabetic neuropathy

Diabetes was induced by intraperitoneal injection of 55 mg/kg freshly prepared streptozotocin (STZ) dissolved in 0.1mol/L Citrate buffer (pH 4.4). At the end of the first week after STZ injection, blood sugar measurements were taken for diagnosing diabetes using tails vein blood (OneTouch® Select Plus glucometer, China). Rats with fasting blood glucose of 11mmol/L and over were selected. After the induction of diabetes, reaction latencies to paw withdrawal and tail flicks were recorded on alternate days over a period of 3 weeks. A reduction in pain threshold over this period to the hot plate and cold water was characteristic of thermal hyperalgesia and cold allodynia. This significant increase in pain to these innocuous stimuli confirmed the successful induction of diabetic neuropathy with Streptozotocin (STZ).

3.6 Extract/drug treatment of streptozotocin-induced neuropathic pain

After a successful induction of diabetic neuropathy with Streptozotocin (STZ), observed as an exaggerated response (pain) to the cold water (cold allodynia) and hot plate (thermal hyperalgesia), the rats were then treated daily with SNE (100, 300 and 1000 mg/kg, p.o), PGB (10, 30 and 100 mg/kg, p.o) and vehicle (normal saline) for 7 days.

3.7 Physiological biomarkers of peripheral neuropathy in STZ-induced diabetic rats

After the one week treatment, the physiological effects of SNE and pregabalin were assessed using the conventional open field. The open field consisted of a transparent plexi glass box (45 × 45 cm, height: 50 cm). For the assessment, the rats were placed individually in the center of the test box and the following behavioral and physiological parameters: total frequency of mobility and frequency of rearing (as a measure of vertical activity) and grooming (rubbing the body with paws or mouth and rubbing the head with paws) in the open field were analyzed for 5 min using a behavioral tracker (Crawley 1985, Shabani *et al.* 2011).

3.8 Behavioural assessment of neuropathic Pain

Three animal models of pain assessment were employed: cold allodynia using cold water at 4 ^{0}C; mechanical hyperalgesia using the Randall-Sellito test and thermal hyperalgesia using the hotplate test as previously described. Prior to each determination, baseline measurements were determined.

3.9 Cold allodynia

The effect of the extract (SNE), pregabalin (PGB) and normal saline as the vehicle in cold allodynia was determined by submerging the rat's tail into cold water (4 °C) as previously described (Rautenberg *et al.* 1978). The reaction time for tail removal was measured with a timer with a cut-off latency period of 20 s.

3.10 Mechanical hyperalgesia

The effect of the extract (SNE) (100, 300,and 1000 mg/kg), pregabalin (PGB) (10,30 and100 mg/kg) and the vehicle on mechanical hyperalgesia was calculated with the Randall-Selitto paw pressure analgesimeter (IITC Life Science Model 2888 Woodland Hills, CA, USA) as formerly described (Ameyaw *et al.* 2014, Görlitz and Frey 1972). Briefly, the hindpaw of the rat was placed into a pressure applicator and an increasing pressure (cut-off of 200 g) was applied to the dorsal surface of the paw until withdrawal or vocalization. For each animal, two recordings were made for each hind paw and the average (force) recorded as the pain threshold.

3.11 Thermal hyperalgesia

This was determined in the diabetic rats by using the hotplate analgesiometer as previously described (Muthuraman *et al.* 2011, Thiagarajan *et al.* 2013). Briefly, the animals were gently dropped onto a pre-heated hot plate at 55°C with a reaction stoppage time of 20 s. Time for the removal of paws, shaking, or licking of either hind paw was noted as the pain threshold. This response was measured for each experimental animal prior to PGB or SNE administration and taken at times (0), 1 hour post drug and extract administration.

3,12 Preparation of specimen

On the 8th week, all animals were weighed. They were then chloroformed and sacrificed. Blood samples were collected from the jugular vein to into serum separating gel tubes (BD Vacutainer® blood collection Tube Product,USA) and EDTA tubes (Mediplus vacutainer K3, Sunphoria Co. Ltd., Taiwan) for serum preparation and haematological analysis respectively. Rat organs (liver, kidney, lungs, brain and heart) were harvested, cleaned and dried with tissue. Organs were then weighed. All organs were examined by trained veterinary doctors for any anomalies and placed in labeled containers containing 10% neutral buffered formalin. The organs were then transported to a pathology laboratory for processing and examination

Three (3) to four (4) millilitre quantities of blood allowed clotting at room temperature (27 °C) in the gel tubes after they were collected. The tubes were then centrifuged (Heraeus Labofuge 300, UK) at 3000 rpm for 20 min. A clear supernatant obtained was collected by pipetting into plain tubes. These tubes were labeled accordingly and taken to the lab for intended biochemical analysis.

3.13 Evaluation of the antioxidant activity as a mechanism of action of *Synedrella Nodiflora*

3.13.1 Determination of SOD Activity

A commercially available kit (Sigma-Aldrich, 3050 Spruce Street St.Louis MO 63103 USA) was used for the SOD assay. WST (water soluble tetrazolium salt) working solution was prepared by diluting 1 ml of WST Solution with 19 ml of Buffer Solution. The enzyme working solution was centrifuged for 5 seconds and mixed by pipetting. It was then mixed with 2.5ml of the dilution buffer. For each sample (blood serum) four wells were selected and labeled sample, blank 1 blank 2 and blank 3 respectively. 20 µl of sample solution was added to each sample (serum) and blank 2 well. 20 µl of ddH_2O (double distilled water) was also added to each blank 1 and blank 3 well. A 200 µl of WST Working Solution was subsequently added to each well and mixed. Then 20 µl of Dilution Buffer was added to each blank 2 and blank 3 well. Finally 20 µl of enzyme working solution was added to each sample (serum) and blank 1 well and then mixed thoroughly. These wells were incubated at 37 °C for 20 min. The absorbance at 450 nm was read using a microplate reader. SOD activity (inhibition rate %) was calculated using the following equation:

$$\text{SOD activity (inhibition rate \%)} = \frac{(blank1 - bank3) - (sample - blank\ 2)}{(blank\ 1 - blank3)}$$

3.13.2 Determination of the presence malondialdehyde (TBARS)

A 0.5ml serum of SNE treated rat was mixed with 0.5ml of ice-cold 10% trichloacetic acid and incubated for 30 minutes, 1.0ml of 0.67% thiobarbituric acid was subsequently added to the mixture, mixed properly and heated at 100 ºC for 30 minutes. The mixture was then allowed to cool. A 4.0ml of n-butanol was added to the mixture and vortexed for 30 s. It was then centrifuged at 500 g for 10 minutes. The supernatant obtained was drawn into 96- well plates

using a micropipette. Each sample was duplicated and the absorbance was then measured at 535 nm using the spectrophotometer (GloMax Explore GM 3500, Promega Corporation, Madison, Wisconsin, USA). The results expressed in umol/l using the extinction coefficient of 1.56 X105 M-1 cm-1 (Mohan Murali Achary *et al.* 2008)

3.14 Histopathology of isolated organs

All animals were sacrificed and their organs (liver, kidney, lungs, spleen, heart and brain) were harvested and placed in labeled containers containing 10% neutral buffered formalin. The organs were transported to a pathology laboratory for processing and examination.

Portions of the organs were selected into labeled tissue processing cassettes and processed into paraffin blocks. Each was passed through ascending grades of alcohol (70%, 80%, 90% and absolute) and further two changes of absolute alcohol for dehydration, cleared in three changes of xylene and finally infiltrated and embedded in paraffin wax. Five micron sections were cut from each block and mounted on microscope slides and stained by the haematoxylin and eosin method.

Haematoxylin and Eosin Method.

STEP	ACTIVITY
1	Sections on slides were put in two changes of xylene for 15 minute each
2	Section through three changes of absolute ethanol for 5 minutes each
3	Sections to 70% ethanol for 15 minutes
4	Sections to 50% ethanol for 15 minutes
5	Sections to tap water for 30 minutes
6	Sections to distilled water for 2 minutes
7	Sections to Mayer's haematoxylin stain for 15 minutes
8	Sections to tap water for 30 minutes
9	Sections to working eosin solution for 5 minutes
10	Sections to 80% ethanol for 5 minutes
11	Sections to two changes of absolute ethanol for 5 minutes
12	Sections to three changes for 5 minutes each
13	Sections mounted in DPX
RESULTS	Nuclei----blue black Background and cytoplasm,---varying shades of pink

3.14,1 Examination of slides

The sections of the liver, heart, spleen, lung and kidney samples of the sacrificed animals were examined by a qualified histologist who was blind to the experimental profiles under a light microscope after been stained with eosin and heamatoxylin.

3.15 Analysis of results

GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determination. $P < 0.05$ was considered statistically significant in all analysis. The graphs were plotted using Sigma Plot for Windows Version 11.0 (Systat Software Inc., Germany).

CHAPTER FOUR

RESULTS

4.0 Phytochemical analysis

Phytochemical analysis carried out on the extract tested positive for alkaloids, saponins, tannins, terpenoids, phenols, flavonoids and glycosides.

Table 1; Results of preliminary phytochemical analysis of SNE

CONSTITUENT	RESULT
Alkaloids	+
Saponins	+
Tanins	+
Steroids	+
Phenols	+
Volatile oils	-
Terpinoids	+
Flavonoids	+
Cardiac glycosides	+

-: Absent +: present

4.1 STZ-induced diabetic neuropathy (cold allodynia Test)

There was a significant difference between tail withdrawal at day1 and day 7. A two-way ANOVA followed by a Bonferroni ‘s post-hoc test showed a significant difference (Group 1 p= 0.0058 , Fig 4.a), (Group 2, p= 0.0006 , Fig 4.b) (Group 3, p = 0.0016, Fig 4.c) and (Group4, p= 0.0004 ,Fig 4.1) an overall significant reduction of tail removal latencies between day 1 and day 7 is an indication of the induction of diabetic neuropathy (Figure 4.).

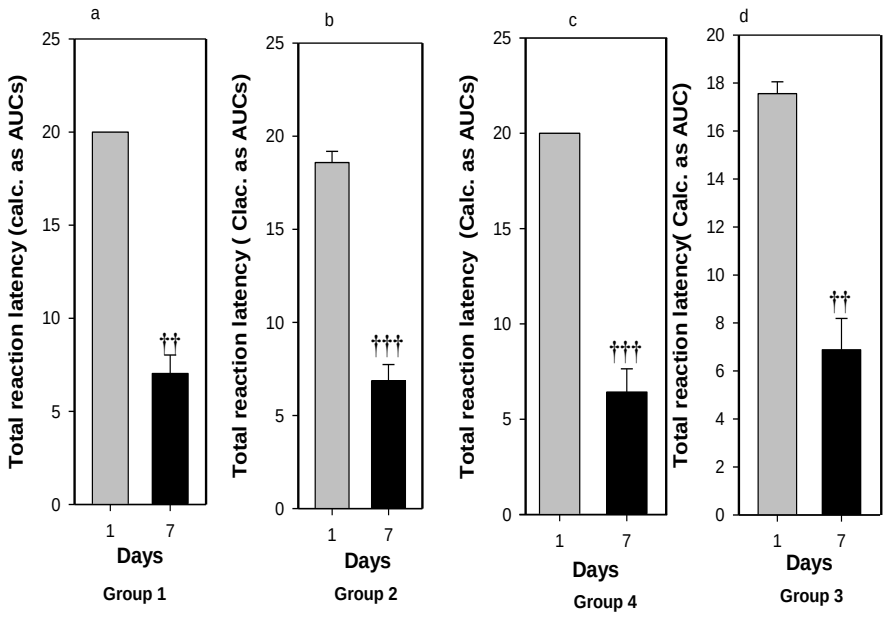

Figure 4: A comparison of tail withdrawal (as a measure of cold allodynia) on day 1 and day 7 after STZ-induced diabetes.A comparison of tail withdrawal (as a measure of cold allodynia) on day 1 and day 7 after STZ-induced diabetes. Data are mean ± SEM (n = 5) † P < 0.05, †† P< 0.01, ††† P < 0.001 compared between groups (two-way ANOVA followed by a Bonferroni’s post-hoc test)

4.2 STZ-induced diabetic neuropathy (Thermal hyperalgesia)

A determination of thermal pain during the induction of diabetic neuropathy produced a slow reduction of paw removal from the hot plate observed from day 1 through to day7. A two-way ANOVA followed by a Bonferroni's post-hoc test exhibited significant increase in pain intensity from days 1 and 7 (Group 1, p= 0.0501), (Group 2, p= 0.0095) (Group 3, p = 0.0028) and (Group4, p= 0.0434) (Figures 5.a, 5.b, 5.c, 5.d) an overall significant reduction of the paw withdrawals between day 1 and day 7 in all groups suggests an induction of peripheral neuropathy in the diabetic rats. (Figure 5)

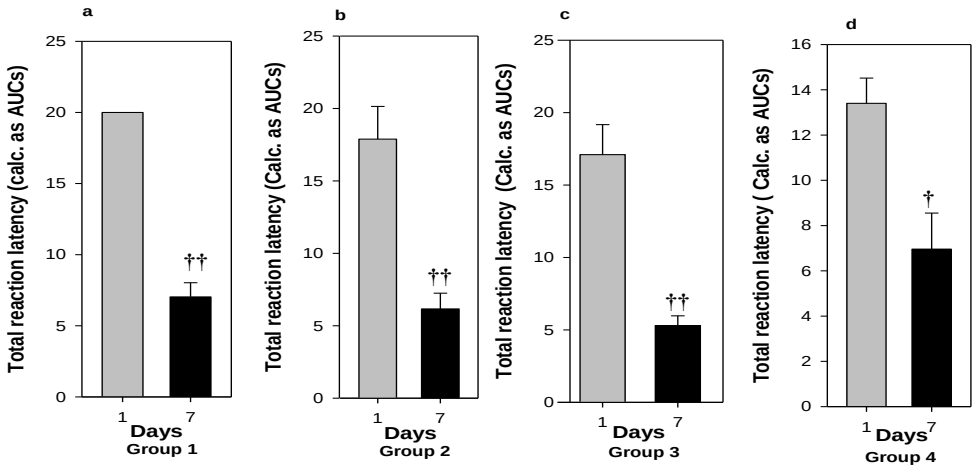

Figure 5: A comparison of paw withdrawals (as a measure of the onset of thermal hyperalgesia) on day 1 and day 7 after STZ administration. Data are mean ± SEM (n = 5). † P< 0.05, †† P< 0.01, †††P < 0.001 compared between groups (two-way ANOVA followed by a Bonferroni's post-hoc test).

4.3 Effects of SNE on Cold allodynia

The extract (SNE) increased the tail removal latency in the cold water but with no significance. The overall anti-allodynia effects of all the extract doses were not significant ($p = 0.2268$, $F_{3,14}= 1.633$ Figure 6.a, 6.b). However, PGB (30 and 100 mg/kg) doses dependently and significantly increased tail withdrawal in the neuropathic rats (Figure 6.d). The overall anti-allodynic activity of PGB was significant ($p = < 0.0001$, $F_{3,14} = 56.05$) at doses 30 and 100 mg/kg (Figure 6.d).

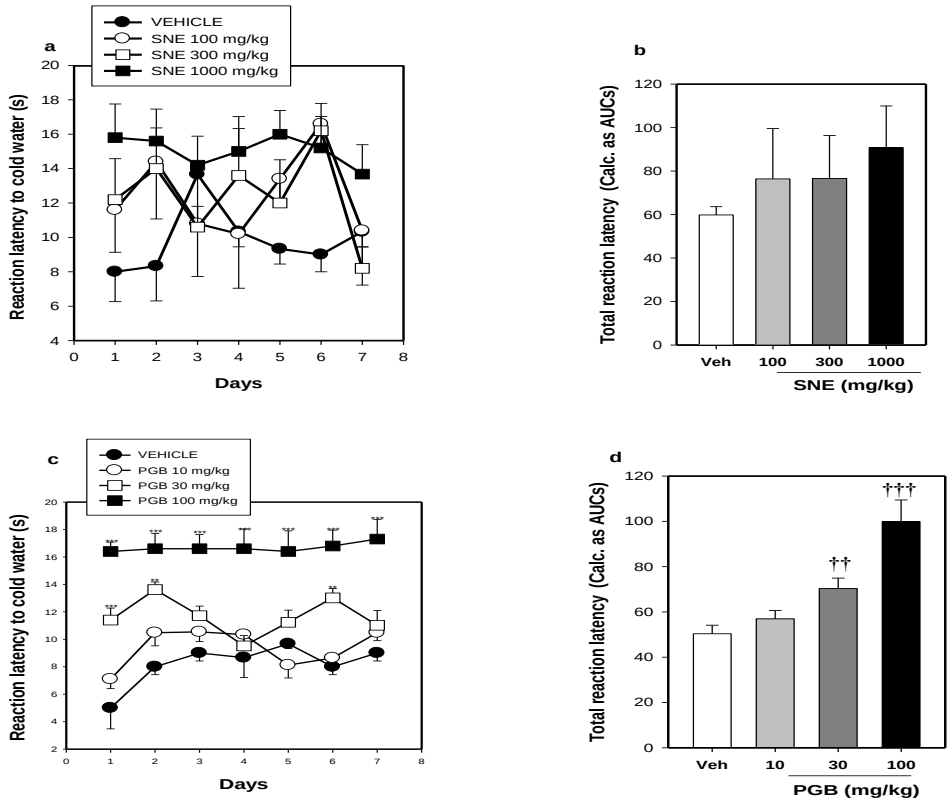

Figure 6: The results of SNE (100, 300, 1000 mg/kg, p.o) and PGB (10, 30, 100 mg/kg, p.o) on cold allodynia in daibetic neuropathic rats. The left figures (a and c) shows a time-course results of SNE (a) and PGB (c) in the treatment of diabetic neuropathic pain. The right figures (b and d) shows the total anti-cold allodynic results (calculated from the AUCs) of SNE (b) and PGB (d). Data are mean ± SEM (n = 5). *p ≤ 0.05, ** p ≤ 0.01 and ***p ≤ 0.001 compared to vehicle group (two-way ANOVA followed by a Bonferroni's post-hoc test †† P<0.01 and ††† P<0.001) (one-way ANOVA followed by a Dunnett's multiple comparison test).

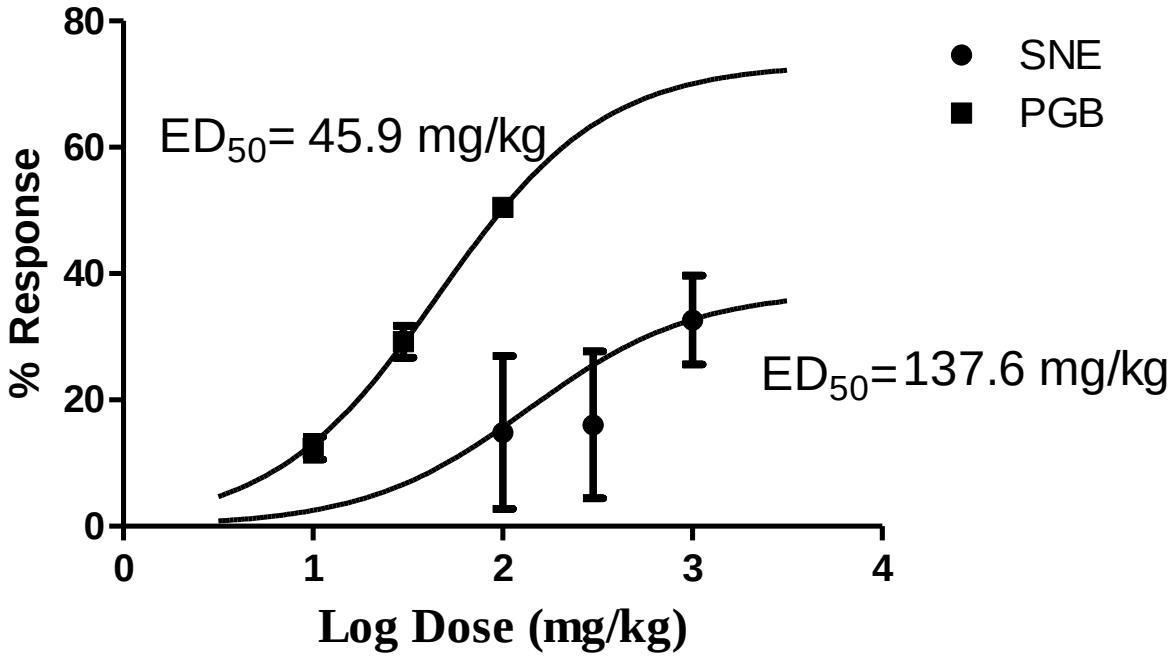

Figure 6.1: A log dose response curve of SNE and PGB comparing the ED_{50} as a measure of potency for SNE and PGB respectively in a reduction of cold allodynia.

4.4 Effects of SNE on Thermal hyperalgesia

There was a significant activity in the paw withdrawal latency of SNE 1000 mg/kg ($p = 0.0035$, $F_{3,14}= 7.297$, Fig 7.a) compared with the vehicle treated group. The overall SNE 1000 mg/kg activity on thermal pain was significant on the days 3 and 4 of treatment ($p < 0.05$ and $P < 0.01$ Fig 7.b), but there was no significant activity with SNE 100 and 300mg/k. PGB 30 mg/kg exhibited significant anti-hyperalgesic effects on days 2 and 4 during drug administration, PGB 100 mg/kg significantly produced anti-hyperalgesic activity on all days of drug administration and the overall anti-hyperalgesic effects was significant ($p < 0.0001$ $F_{3,14}= 52.51$, Fig 7.d)

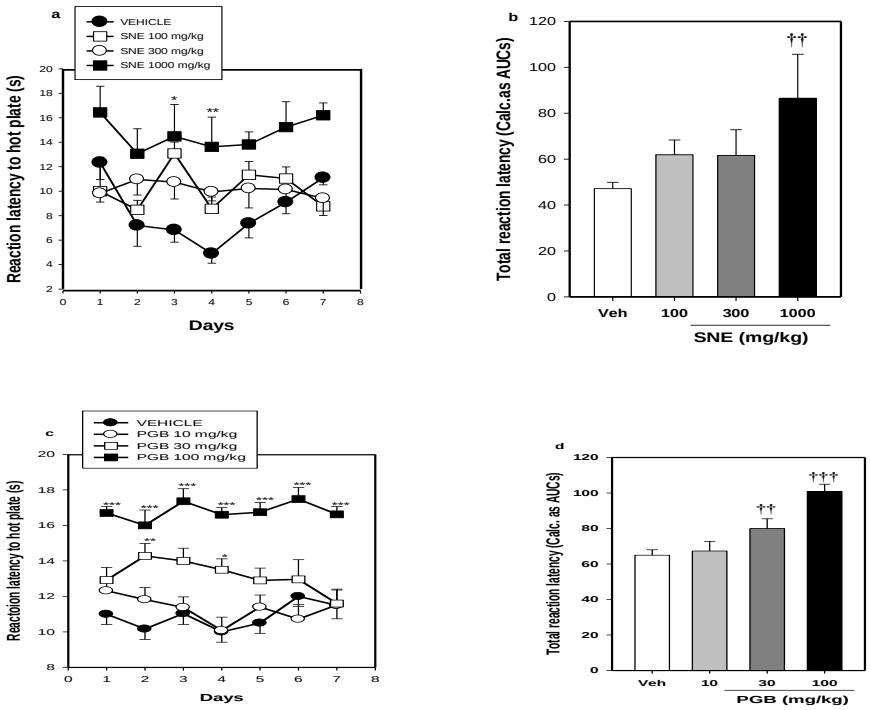

Figure 7: The effect of SNE (100, 300, 1000 mg/kg, p.o) and PGB (10, 30, 100 mg/kg, p.o) on thermal hyperalgesia in diabetic neuropathic rats. The left figures (a and c) are time-course events of SNE (a) and PGB (c) post extract and drug administration for the treatment of neuropathic pain. The right figures (b and d) also represent the total analgesic effects of SNE (b) and PGB (d). Data are mean ± SEM (n = 5). *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 compared to vehicle group (two-way ANOVA followed by a Bonferroni's post-hoc test). †† P<0.01 and ††† P< 0.001 compared to vehicle group (one-way ANOVA followed by a Dunnett;'s multiple comparison test).

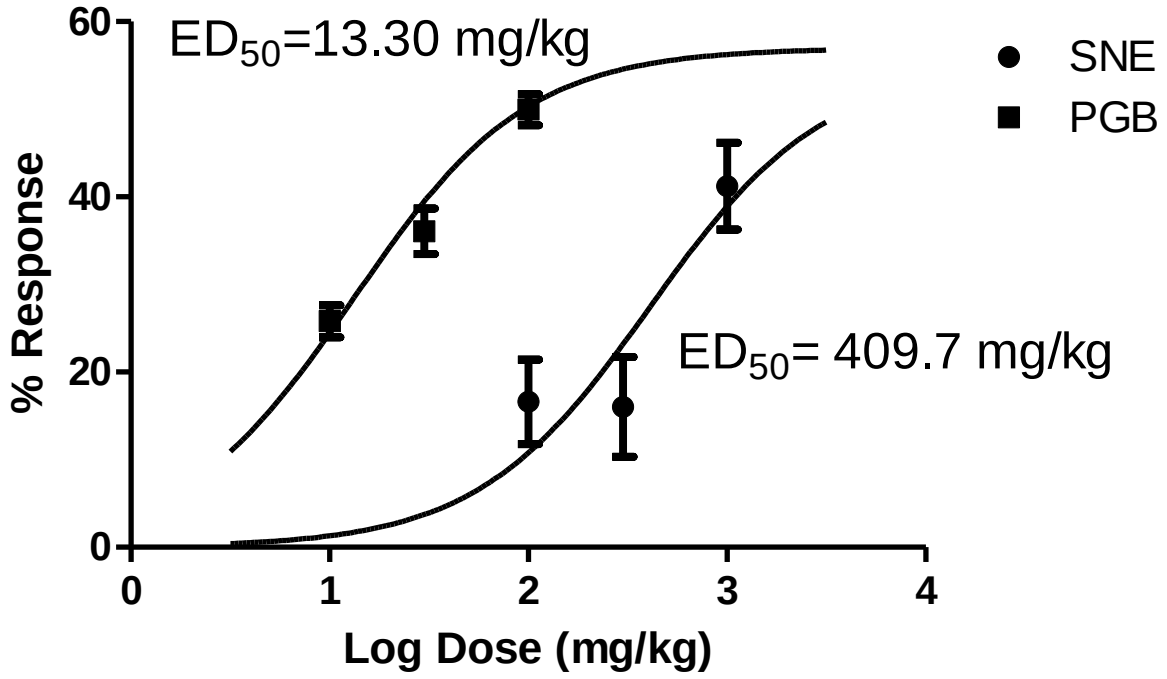

Figure 7.1: A log dose response curve of SNE and PGB comparing the ED_{50} as a measure of potency for SNE and PGB respectively in a reduction of thermal hyperalgesia.

4.5 Effects of SNE on Mechanical hyperalgesia

SNE 100 and 300 mg/kg produced an increase in the time for paw removal to an applied force but however only SNE 1000 mg/kg significantly increased paw removal latency ($p=0.0076$, $F_{3,14}=5.990$, Fig 8.b) in contrast with vehicle-designated group. PGB significantly and dose-dependently increased the paw withdrawals ($p= 0.001$, $F_{3,24} = 16.51$, Figure 8.c and 8.d),the analgesic effect was significant for all doses of PGB 10, 30 and 100 mg/kg (Figure 8.d) .The overall anti-hyperalgesic effects were significant ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$) for 10 mg/kg, 30 mg/kg, 100 mg/kg respectively.

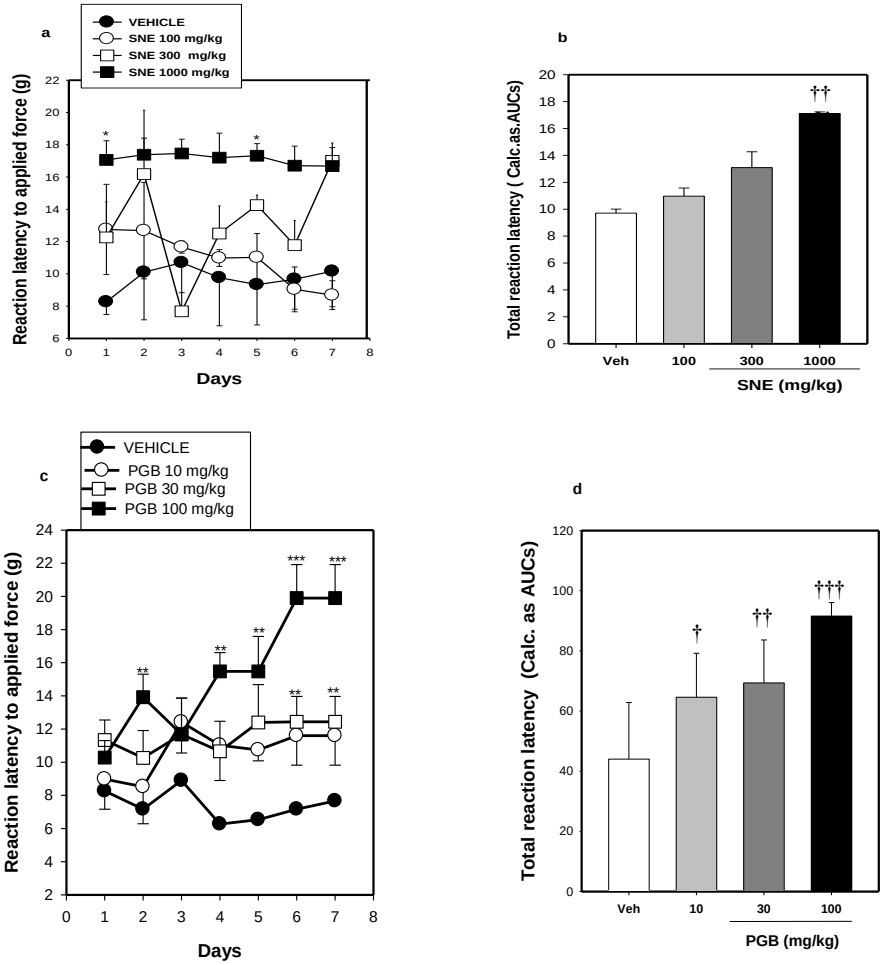

Figure 8: The effect of SNE (100 – 1000 mg/kg, p.o) and PGB (10 – 100 mg/kg, p.o) on mechanical hyperalgesia in diabetic neuropathic rats. The left figures (a and c) shows time-course events of SNE (a) and PGB (c) after the treatment of neuropathic pain. The right figures (b and d) also show the total analgesic effects (calculated as AUCs). Data are mean ± SEM (n = 5). * p ≤ 0.05 , ** p ≤ 0.01 and ***p ≤0.001 in contrast with vehicle treated group (two-way ANOVA followed by a Bonferronis post-hoc test † P<0.05, †† P<0.01, ††† P<0.001 compared to vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison test).

4.6 Effects SNE on Locomotor Activity

The locomotor activity of rats were assessed by measuring the horizontal, vertical activities of the rats treated with SNE (100, 300, 1000 mg/kg), PGB (10, 30,100 mg/kg) and vehicle. On

mobility SNE increased mobility among the treated animals with respect to the vehicle treated groups dose dependently but not significantly ($p = 0.0870$, $F_{3,12}= 2.776$, Fig 9.a) for all SNE doses, PGB dose dependently and significantly increased mobility among treated rats ($p= 0.0015$, $F\ 3_{3,14} = 8.962$. Fig 9.b).The effect of SNE on rearing (recorded as a measure of vertical activity), SNE increased the frequency of rearing with no significance in comparison to vehicle treated groups at all dose levels ($p=0.3771$, $F_{3,14}= 1.127$, Fig 9.c). PGB at all dose levels increased the frequency of rearing in comparison to vehicle treated groups but did so significantly and dose independently ($p<0.05$, $p<0.05$ and $p< 0.01$,Fig. 9.d). Also SNE increased grooming at 300 mg/kg and 1000 mg/kg doses respectively, but non significantly and dose independently (Fig 9.e), PGB 10 mg/kg and 100 mg/kg doses increased grooming in comparison to the vehicle treated groups ($p=0.1915$, $F_{3,14}=1.810$, Fig 9.f) non significantly and dose independently.

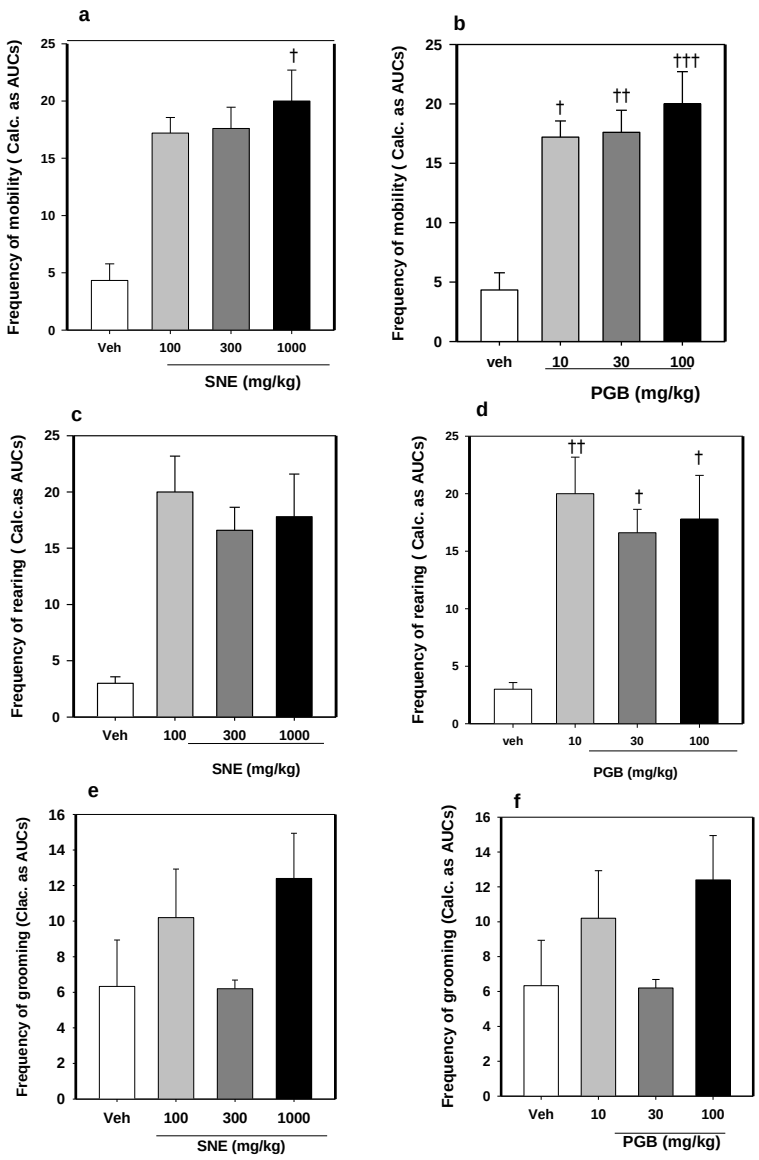

Figure 9: The effects of SNE (100, 300, 1000 mg/kg) and PGB (10, 30,100 mg/kg) on the frequency of mobility (a and b), frequency of rearing (c and d) and frequency of grooming (e and f) calculated as AUCs. Data presented as mean ± S.E.M. n=5). † P<0.05, †† P<0.01 and ††† P< 0.001 compared to vehicle-treated group (One-way ANOVA followed by Dunnett's Multiple Comparison Test)

4.7 SOD and MDA assay

For SOD activity, there was a decrease in MDA concentration for SNE 1000 mg/kg (Fig 10.a) in comparison to the vehicle treated group but 100 and 300 mg/kg (Fig 10.b) increased in respect to the vehicle group. SOD also showed a dose dependent decrease in activity the highest decrease was in the SNE 1000 mg/kg which was the only significant decrease SOD activity ($p< 0.0339$, $F_{3,12=}.029$ Fig10.b). Decrease in the other extract groups were not significant ($p>0.05$,Fig10.b).

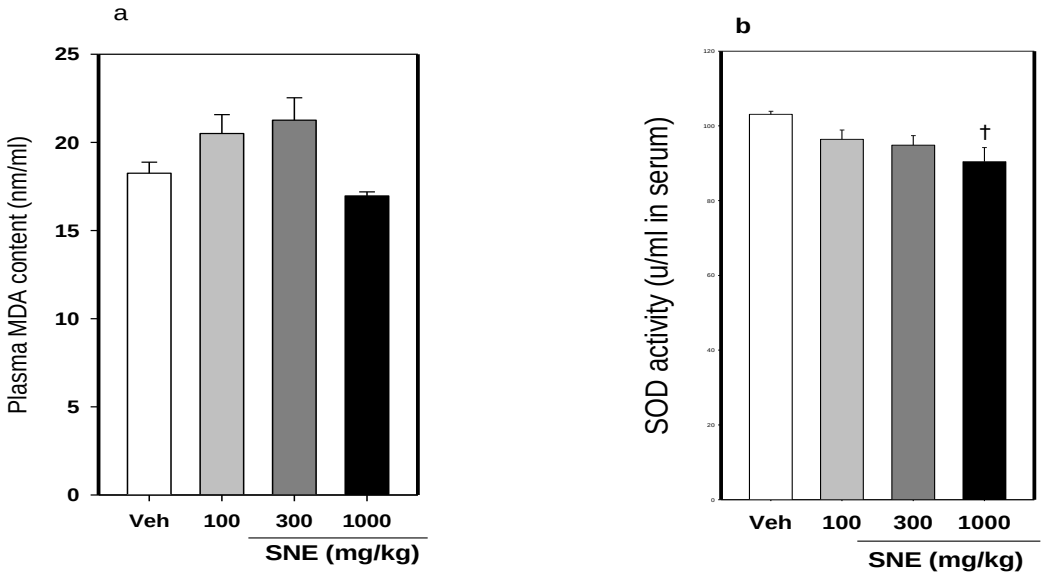

Figure 10: The effect of SNE (100,300 and 1000 mg/kg,) on MDA concentration (a) and SOD activity (b) in STZ -induced diabetic rats treated for 7 days. Data are mean± SEM (n=5). Data are mean ± SEM (n = 5). †P<0.05, ††P<0.01, †††P<0.001 compared to vehicle group (one –way ANOVA followed by a Bonferroni's posthoc test)

Table 2: Organ weight of Vehicle and SNE treated groups of STZ-induced diabetic neuropathic rats after 7-days administration.

WEIGHTS	VEHICLE	CONTROL	SNE 100MG	SNE 300MG	SNE 1000MG	P VALUE
HEART	0.6567±0.01667	0.7480±0.1484	0.7325±0.04423	0.7075±0.08816	0.6040±0.02064	0.7623
LIVER	8.967±0.9207	9.234±0.6584	8.213±0.4377	7.675±1.136	6.732±0.4528	0.1268
SPLEEN	0.3567±0.07172	0.4600±0.06237	0.5125±0.1163	0.4600±0.1526	0.3540±0.08606	0.7639
KIDNEY 1	0.8900±0.04359	0.7980±0.05643	0.9450±0.08026	0.9375±0.1148	0.8380±0.03216	0.5010
KIDNEY 2	0.8867±0.003333	0.8040±0.06392	0.9375±0.06524	0.8850±0.1248	0.8140±0.03750	0.6473
BRAIN	1.620±0.03055	1.644±0.08322	1.643±0.06196	1.453±0.06676	1.576±0.04490	0.2558
LUNGS	2.173±0.5042	2.014±0.1191	1.830±0.3918	3.8201.439	1.602±0.1690	0.1815

Data expressed as means ±SEM (n = 5), No changes ($p > 0.05$) was recorded when vehicle group (0.5 % NaCl) was compared to the normal control group (Non-diabetic). No significant changes in organ weights seen when SNE treatment groups ($p > 0.05$) were compared to the Vehicle using (one-way ANOVA followed by Dunnett's Multiple Comparison Test)

Table 3: Biochemical analysis of SNE (100,300 and 1000 mg/kg) after a 7-day treatment period

Parameter	Normal control	Diabetic control (vehicle)	SNE 100 mg/kg	SNE 300mg/kg	SNE 1000 mg/kg	P value
RENAL FUNCTION (MMOL/L)						
Urea	14.62±2.178	9.066±0.541	13.65±1.22	9.85±0.484	9.794±1.112	0.0086*
Creatinine	38.40±2.219	67.08±9.805	57.20±5.766	36.03±9.559	28.44±2.547	0.0050
LIPID PROFILE (MMOL/L)						
Total cholesterol	2.10±0.211	2.100±0.282	2.793±0.2877	2.480±0.4022	2.338±0.2721	0.5423
Triglycerides	2.550±0.5976	2.452±0.4503	2.628±0.6241	1.930±0.3592	1.470±0.2673	0.3276
HDL	1.057±0.3089	0.8140±0.1262	1.100±0.1566	1.018±0.2091	1.120±0.0762	0.6429

LDL	0.483±0.0433	0.6840±0.1327	0.5575±0.077	0.5950±0.071	0.5680±0.042	0.6443
LIVER FUNCTION						
Total protein	76.60±4.163	85.58±2.521	87.35±5.229	80.25±7.485	71.103±2.90	0.1149
Albumin(g/l)	29.07±1.506	39.56±1.073	35.88±2.363	30.43±3.187	30.42±3.172	0.0429*
Globulin (g/l)	47.53±4.328	46.04±2.100	51.48±3.389	49.83±4.428	40.68±1.864	0.149
ALT (u/l)	1567±959.6	719.1±434.0	1121±964.8	247.5±56.82	461.1±150.7	
AST (u/l)	1336±395.4	1277±652.6	210.6±7.712	427.1±87.71	827.6±283	0.3943
ALP (u/l)	1292±149.0	404.4±176.6	1111±192.1	1017±316.9	1313±304.6	0.1386

Data presented as means ± SEM (n=5). *P≤0.05: significant difference in urea and creatinine between SNE 100 mg/kg treated group compared to non-diabetic control group *P≤0.05: significant difference observed in Albumin levels between diabetic non- treated groups and SNE treated groups (one-way ANOVA, then Dunnett's multiple comparisons test).

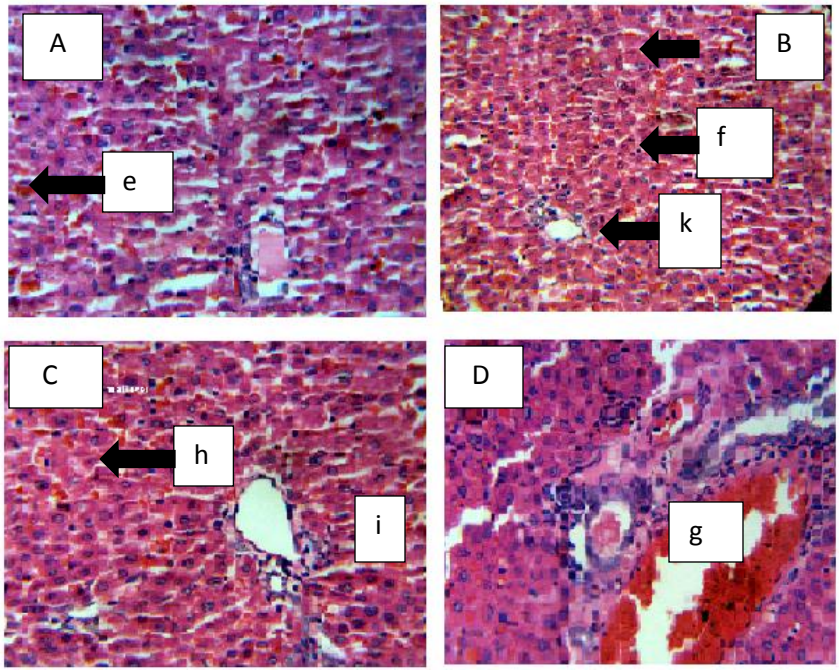

Figure 11: Photomicrographs of livers isolated from rats after after 7-day continuous treatment with (A) SNE 100 mg/kg, (B) SNE 300 mg/kg, (C) Vehicle, (D) 1000 mg/kg (H&E Staining 40χ). The arrow (g) shows central vein inclusion with (j) perivascular lymphoid aggregation in the vehicle group comparatively with (f), (h) and (e) which shows normal hepatocytes with (i) and (k) normal central veins. (A) And (B) also show normal hepatocytes with slight lymphoid infiltration.

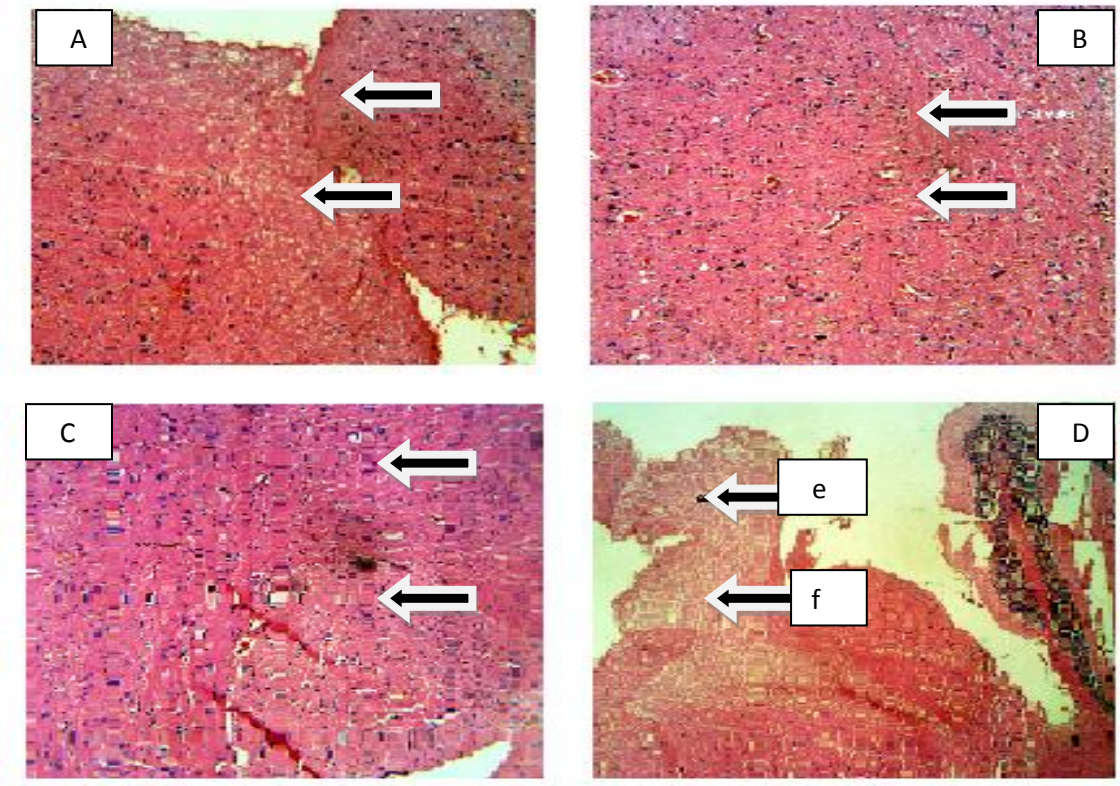

Figure 12: Photomicrographs of the cereberal cortex of the brain isolated from rats after 7-day continuous treatment with (A) SNE 100mg/kg, (B) SNE 300 mg/kg, (C) SNE 1000 mg/kg, SNE administration group showed normal myelinated regions and the Vehicle treated group (D) showed a high region of demyelinated neurons indicated by arrows(e) and (f)

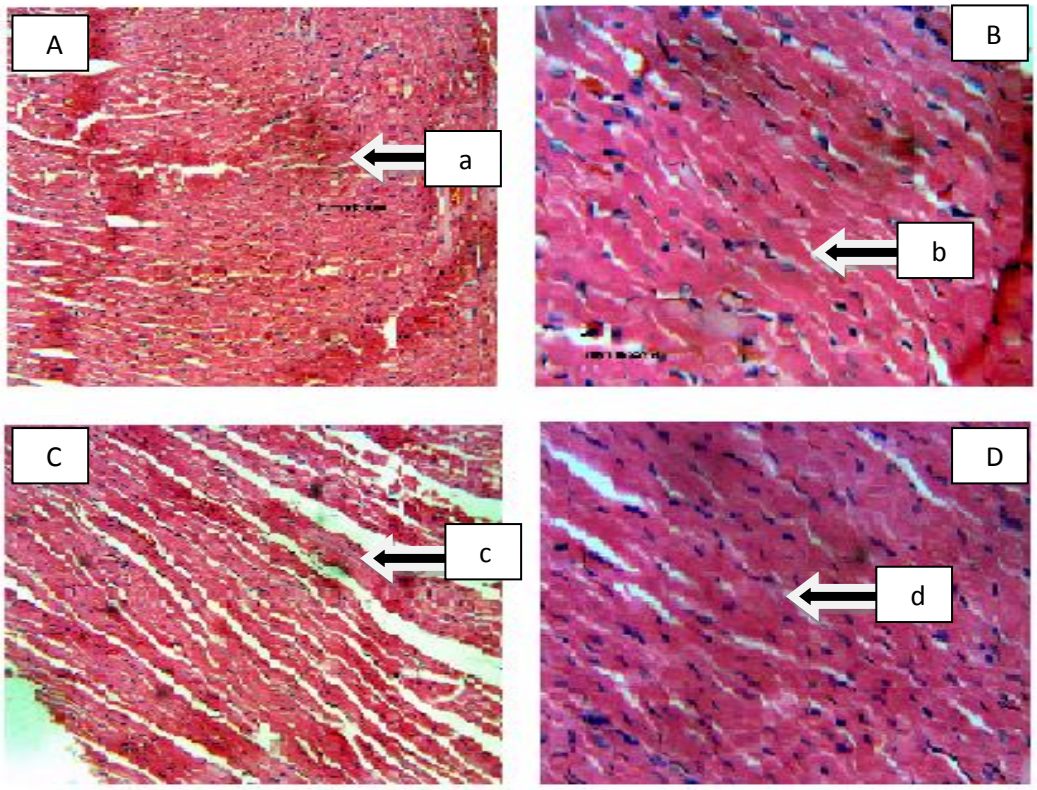

Figure 13: Photomicrographs of the tissues of the heart isolated from rats after 7-day continuous treatment with (A) SNE 100 mg/kg, (B) SNE 300 mg/kg, (C) SNE 1000 mg/kg, and the Vehicle-treated group (D), there was no pathological differences observed in the heart structure between extract and vehicle treatment groups.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.0 Discussion

The present study was conducted to determine the possible analgesic effect of a hydro-ethanolic extract of *Synedrella nodiflora* whole plant in relieving and attenuating hyperalgesia and allodynia in streptozotocin-induced diabetic neuropathy. Intraperitoneal injection of streptozotocin (STZ) in a single dose administration to the rats resulted in the induction of type 1 diabetes, 2 weeks after induction there was a development of diabetic complications (diabetic neuropathy). Pain assessment test revealed a significant decline in the reaction latencies to thermal, cold and mechanical application indicating a successful induction of neuropathic pain. Pancreatic β cells exposure to streptozotocin results in cell damage due to its cytotoxic effects in animals, damage to Pancreatic β cells is expected to take effect within 48-72 hours after administration but the level and onset of the damage is dependent on the dose administered, thus resulting in chronic hyperglyceamia (Junod *et al.* 1967).

STZ mediated diabetic neuropathy results in structural changes in A-delta fibres which produces hyperalgesia and allodynia, nerve or tissue damage caused by this structural changes results in the release of inflammatory mediators, (bradykinins, histamines, cytokines) around the area of inflammation; this causes the depolarization of pain receptors, activating action potentials. The action potentials leads to carrying of pain sensations, by the dorsal root ganglion (DRG), to the dorsal horn of the spinal cord this releases glutamate and substance P which relays pain sensations subsequently to the cortex, where pain is interpreted and perceived (Westlund. 1997). SNE significantly attenuated hyperalgesia in the SNE treatment groups, observed as a decline in paw withdrawal latency in both thermal and mechanical hyperalgesia, it also possesses anti-

allodynic effects in the STZ-induced diabetic neuropathic pain consistent with the findings of Amoateng et al., in the vincristine-induced and paclitaxel-induced neuropathic pain. The analgesic effects of SNE is liable for its favourable effects against the STZ-induced neuropathic pain (Woode *et al.* 2009). Phytochemical screening indicated the presence of glycosides, tannins, terpenoids, alkaloids, and saponins. These phytochemical constituents present are responsible for the extracts pharmacological activity observed in this study.

The effects of pregabalin in attenuating hyperalgesia and allodynia shows the clinical effectiveness of PGB in managing neuropathic pain (Blommel and Blommel 2007, Graversen *et al.* 2012). It binds to the voltage- gated calcium-gated channels, inhibiting the production of glutamate, norepinephrine and substance P and preventing the binding of the neurotransmitter to their receptor, thus reducing the neuronal hyperexcitability (Micheva *et al.* 2006, Taylor *et al.* 2007). These effects of PGB and subsequent activation of other events may result in the reduction in pain sensitivity and perception. It is however likely that the extract, among other pathways, may have antagonized pain sensations in this model by blocking calcium channels similar to pregabalin.

The open field is a useful tool for assessing locomotive impairment in animal models of neuromuscular disease (Tatem *et al.* 2014) and the efficacy of therapeutic drugs that may improve locomotion and/or motor function (Castellan Baldan *et al.* 2014). The open field activity assessment provides a different measure of locomotor activity and animal exploratory behavior. The perception of pain is not simply a sensory, nociceptive experience, but one that often disrupts a patient's quality of life (O'Connor 2009). Unquestionably, mechanical and thermal hypersensitivity are often associated with human chronic pain disorders (Baron *et al.* 2010, Maier *et al.* 2010). If the experimental animal is indeed experiencing spontaneous pain similar to

humans, there should be an impact on its daily life activity and affective state and behaviour (Urban *et al.* 2011). Locomotion (mobility), rearing and grooming represent different forms of behaviour and motor activity in rats (Kozler *et al.* 2017) in this current study changes in these activities were analysed in relation to impaired function or lesion in the micro-vasculature of the peripheral nerve of the rat as a result of chronic hyperglyceamia associated with diabetic neuropathy. Mobility (as a measure of frequency of movement) within the SNE treatment groups improved compared to the vehicle treated groups this pain reduction and improvement in movement amongst SNE treated animals is a confirmation of the extracts inherent analgesic properties. Frequency of rearing was enhanced among the SNE treatment groups this was manifested as long and frequent vertical movement on the two hind paws of the SNE treated rats, this increase in the ability of the experimental animals to rear is indicative of the reduction or absence of neuropathic pain. PGB significantly increased the frequency of vertical activity in the treatment of neuropathic pain this pattern was similar to that observed in the SNE treatment groups. This is suggestive of the extracts analgesic effects in comparison to PGB and both may have similar mechanisms of alleviating pain. Animal grooming serves a variety of adaptive function; on the other hand, it is the principal means of caring for the external body surface, ridding it of surface debris and parasites (Deseure and Adriaensen 2002) Furthermore, grooming has also been documented to be involved in counter-irritation, and, thermoregulation (Egerton *et al.* 1969), spreading of pheromones for social signaling(Vos *et al.* 1998) and de-arousal and stress reduction (Delius 1970, Kavushansky *et al.* 2006). In this present study however the SNE did significantly reduce grooming within the experimental treated animals though there were differences in the frequency of grooming between SNE treated and vehicle treated groups similar pattern was observed in the PGB treated groups. The increase in the frequency of grooming was

not consistent with pain models that suggest that an increase in grooming activities is somehow related to pain sensitization as observed in the increased licking of paws and rubbing of painful sites of the body in the formalin induced nociception. Increased grooming amongst treated animals is an adaptive function being carried out in the experimental animals which includes stress reduction (Delius 1970, Kavushansky *et al.* 2006) and care for external body surface (Spruijt and Gispen 1983) but not necessarily a response to pain.

Lipid peroxidation and oxidative stress mediated distraction of DNA measured by malondialdehyde has been found to be associated with diabetes complications and hyperglycemia has been identified as elevating oxidative stress resulting in microvascular complications (Hirsch and Brownlee 2005). Based on this premise an assessment of the level of malondialdehyde and SOD activity was evaluated. However SOD activity was found be lowered in the SNE treatment. MDA (malondialdehyde) content was also measured to determine the ability of the extracts antioxidant effects as a possible mechanism of action in attenuating diabetic neuropathic pain. However, SNE did not show significant reduction in MDA levels in the treatment groups of SNE this observed pattern is not uncommon since it is possible for an extract to show in vitro antioxidant effect and not show that in vivo in a diseased model.

There were no changes in the organ weights and biochemical parameters of SNE treated groups, compared with diabetic non-treatment and normal non diabetic animals confirming that the SNE extract does not impact any physical or chemical alterations in body organs except for SNE 100 mg/kg dose group which showed significant differences in both creatinine and urea levels. This can be attributed to inherent genetic intra specie variation amongst experimental animals (Hosten 1990). This suggest that both acute and chronic administration of the extract are devoid of causing any changes in body organs (Amoateng *et al.* 2016).

Histopathalogical assessment of the liver did not showed any severe organ abnormalities for SNE 100, 300 and 1000 mg/kg but showed normal liver cells a characteristic hexagonal arrangement of hepatocytes in lobules surrounding a central vein but vehicle treated group revealed infiltration of inflammatory cells with central vein dilatation surrounded with petechial hemorrhages and perivascular lymphoid aggregation. The presence of this liver inflammation in the liver of diabetic neuropathic animals is chronic hyperglyceamia increase in reactive oxygen species (Mohanty *et al.* 2000) and its impact on liver cells . This normal hepatocytes observed in the SNE treated groups is due to the immunoprotective and hepatoprotective properties of SNE (Gnanaraj *et al.* 2016).

Brain cells of the extract group were normal with no pathological changes in the cerebral cortex of brain tissues but however brain tissues of the diabetic non-treated groups had multifocal areas of gliotic degeneration with various grade of gliosis and demyelination. The gliotic degeneration and demyelination in brain neurons is indicative of a neuro-degeneration in the central nervous system (Carletti *et al.* 2011). This observed pattern seen in the non- treatment group is due to the progression of diabetic neuropathy resulting from the persistent hyperglycemia (Boulton and Malik 1998, Yagihashi *et al.* 1990). SNE is however not known to offer such protective mechanism against nerve degeneration but this mechanism can be further studied to provide a better understanding of this observation. There was also no pathological alterations observed in the heart tissues for all experimental animals there was no significant differences between the SNE treated groups and the vehicle group. This is suggestive of the extracts safety on the cardio vascular system.

5.1 Conclusion

Findings of this present study indicates that whole plant extract of *Synedrella nodiflora* possess analgesic effects in thermal, mechanical hyperalgesia and cold allodynia in a streptozotocin–induced diabetic neuropathy in rats.

5.2 Recommendations

An attempt should be made to isolate and characterize the active phytoconstituent(s) responsible for these anti-allodynia and anti-hyperalgesic activities.

Further research demonstrating clearly the possible neuroprotective actions of SNE in the rats is warranted.

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APPENDIX A

Phytochemical screening

Alkaloids (Dragendorff's Test)

A 0.5 g quantity of SNE was extracted with 20 ml of ammoniacal alcohol and filtered. The residue obtained after evaporating the filtrate was shaken with 1 % H2SO4 and filtered. The filtrate was made alkaline with dilute ammonia solution and shaken with chloroform; the chloroform layer was separated and evaporated to dryness. The residue was dissolved in 1 % H2SO4 and one drop of Dragendorff's reagent (Sigma Aldrich Co. Ltd. Irvine, UK) was added. An orange-red precipitate shows alkaloids are present.

Saponins (Frothing test)

A 1 g quantity of SNE was dissolved in distilled water and filtered. The filtrate was given a vigorous shake and left to stand for 5 minutes. The presence of persistent froth on standing indicated the presence of saponins in this extract.

Phytosterols (Lieberman-Burchard's test)

Using 5 ml chloroform, 0.5 g of SNE was extracted with and filtered into a test tube. Several drops of acetic anhydride (Sigma Aldrich Co. Ltd. Irvine, UK) were added and mixed carefully. Two (2) drops of concentrated H2SO4 (BDH Laboratories, England) was added to test tube gently through the side. Formation of violet to blue coloured ring at the junction of two liquids shows the presence of steroid moiety.

Terpenoids (Salkowski test)

5 ml chloroform,was added to 0.5 g of SNE, and filtered into a test tube. Careful addition of concentrated H2SO4 (BDH Laboratories, England) to test tube wall produced a violet colouration showing the presence of terpenoids.

Glycosides (General test)

A 0.5 g quantity SNE was extracted by warming with 5 ml of dilute H2SO4 on a water bath for 2 minutes and filtered. The filtrate was made alkaline by adding 2-5 drops of 20 % NaOH (BDH Laboratories, England). 1 ml of Fehling's solution A and B (Sigma Aldrich Co. Ltd. Irvine, UK) was added to the filtrate and dried on a water bath for 2 minutes. The presence of a brick-red precipitate indicates the presence of glycosides.

Tannins (Ferric Chloride test)

A 0.5 g quantity of SNE was boiled with distilled water for 5 minutes. The boiled extract was cooled, filtered and made up to 25 ml. To 1 ml of the extract, 10 ml of distilled water was added followed by 2-10 drops of 1 % Ferric chloride solution (Sigma Aldrich Co. Ltd. Irvine, UK). A blue-black or blue-green colouration showed a positive test for tannins.

Flavonoids (Kumar test)

A 0.5 g quantity of SNE was dissolved in water and filtered; to this 2 ml of 10 % NaOH (BDH Laboratories, England) solution was added to produce a yellow colouration. A change in colour from yellow to colourless on addition of dilute HCl (BDH Laboratories, England) indicates the presence of flavonoids.

APPENDIX B

Preparation citrate buffer

1.76 grams of sodium citrate (Mwt- 294, Zigma-Aldrich, St Loius ,USA) was dissolved in 60 of distilled water to obtain a 0.1M. 1.05grams of citric acid monohydrate powder (Mwt-210, Zigma-Aldrich, St Loius ,USA) was also dissolve in 50 mls of water to obtain 0.1M. 44.5mls of 0.1M citric acid was added to 55.5mls sodium citrate to obtain 100mls of citrate buffer with a pH of 4.5. The amount of streptozotocin needed was weighed and dissolved in the citrate buffer.

APPENDIX C

Histopathology

Preparation of specimen

All animals were sacrificed and their organs (liver, kidney, lungs, spleen, heart and brain) were harvested and placed in labeled containers containing 10% neutral buffered formalin. The organs were transported to a pathology laboratory for processing and examination.

Processing

Portions of the organs were selected into labelled tissue processing cassettes and processed into paraffin blocks. Each was passed through ascending grades of alcohol (70%, 80%, 90% and absolute) and further two changes of absolute alcohol for dehydration, cleared in three changes of xylene and finally infiltrated and embedded in paraffin wax. Five micron sections were cut from each block, mounted on microscope sides and stained by the haematoxylin and eosin method

Preparation of serum

Three (3) to four (4) millilitre quantities of blood allowed to clot at room temperature (27 °C) in the gel tubes after they were collected. The tubes were then centrifuged (Heraeus Labofuge 300, UK) at 3000 rpm for 20 minutes. A clear supernatant obtained was collected by pipetting into plain tubes. These tubes were labeled accordingly and taken to the lab for intended biochemical analysis.

STEP	ACTIVITY
1	Sections on slides were put in two changes of xylene for 15 minute each
2	Section through three changes of absolute ethanol for 5 minutes each
3	Sections to 70% ethanol for 15 minutes
4	Sections to 50% ethanol for 15 minutes
5	Sections to tap water for 30 minutes
6	Sections to distilled water for 2 minutes
7	Sections to Mayer's haematoxylin stain for 15 minutes
8	Sections to tap water for 30 minutes
9	Sections to working eosin solution for 5 minutes
10	Sections to 80% ethanol for 5 minutes
11	Sections to two changes of absolute ethanol for 5 minutes
12	Sections to three changes for 5 minutes each
13	Sections mounted in DPX
RESULTS	Nuclei----blue black Background and cytoplasm,---varying shades of pink