EVALUATION OF ETHANOLIC EXTRACT OF *OTTELIA ALISMOIDES (L.)* PERS ON THE PAIN THRESHOLD RESPONSE IN STZ INDUCED DIABETIC NEUROPATHIC PAIN MODEL IN RATS

Dissertation Submitted to

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In partial fulfilment for the requirements for the award of the degree of

MASTER OF PHARMACY

IN

BRANCH – IV- PHARMACOLOGY

Submitted by

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October - 2017

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"EVALUATION OF ETHANOLIC EXTRACT OF *OTTELIA ALISMOIDES (L.)* PERS ON THE PAIN THRESHOLD RESPONSE IN STZ INDUCED DIABETIC NEUROPATHIC PAIN MODEL IN RATS"** submitted by **Register No: 261525503** to The Tamil Nadu Dr. M.G.R Medical University, Chennai, in partial fulfilment for the degree of **Master of Pharmacy** in **Pharmacology** is the bonafide work carried out under guidance and direct supervision of **Mrs.G.SUMITHIRA, M. Pharm., Assistant Professor** at the Department of Pharmacology, **The Erode College of Pharmacy and Research Institute**, **Erode-638112** and was evaluated by us during the academic year 2016-2017.

1. INTERNAL EXAMINER **2.EXTERNAL EXAMINER**

3. CONVENER OF EXAMINATION

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This is to certify that the dissertation work entitled **"Evaluation of Neuroprotective Effect *Plecospermum Spinosum* in Experimentally induced Diabetic neuropathic pain in rats"** submitted by **Register No: 261525502** to The Tamil Nadu Dr. M.G.R Medical University, Chennai, in partial fulfilment for the degree of **Master of Pharmacy in Pharmacology** is the bonafide work carried out under my guidance and direct supervision at the Department of Pharmacology, The Erode College of Pharmacy and Research Institute, Erode-638112, during the academic year 2016-2017.

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DECLARATION

I do hereby declare that the dissertation work entitled **"EVALUATION OF ETHANOLIC EXTRACT OF *OTTELIA ALISMOIDES (L.)* PERS ON THE PAIN THRESHOLD RESPONSE IN STZ INDUCED DIABETIC NEUROPATHIC PAIN MODEL IN RATS"** submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai, in the partial fulfilment for the Degree of **Master of Pharmacy in Pharmacology**, was carried out by myself under the guidance and direct supervision of **Mrs. G.SUMITHIRA, M. Pharm., Assistant Professor,** at the Department of Pharmacology, The Erode College of Pharmacy and Research Institute, Erode-638112, during the academic year 2016-2017.

This work is original and has not been submitted in part or full for the award of any other Degree or Diploma of this or any other University.

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Reg.No:261525503

LIST OF ABBREVATIONS

ADA	:	American Diabetes Association
AEGs	:	Advanced Glycosylation products
AI	:	Atherogenic index
ANOVA	:	Analysis of variance
ATP	:	Adenosine Triphosphate
AC	:	Action Potential
CVD	:	Cardiovascular Disease
CNS	:	Central nervous system
CTS	:	Carpel tunnel syndrome
DM	:	Diabetes Mellitus
DNA	:	Deoxyribonucleic Acid
DN	:	Diabetic Neuropathy
EEOA	:	Ethanolic extract of Ottelia alismoides
$EAAT_S$	:	Excitatory amino acid transporters
FBG	:	Fasting Blood Glucose
GAD	:	Glutamic acid Decarboxylase
GLP	:	Glucogon like peptide
GTP	:	Guanosine Triphosphate
GLUT	:	Glucose transporter
GDM	:	Gestational diabetes mellitus
HNF	:	Hepatic Nuclear Factor
HLA	:	Human Leukocyte Antigen
IDDM	:	Insulin Dependent Diabetes Mellitus
IGT	:	Impaired Glucose Tolerance

IL	:	Interleukin
IFN	:	Interferon
IAPP	:	Islet Amyloid polypeptide
ICA	:	Islet Cell Antibodies
IGF	:	Insulin like Growth Factor
IFC	:	Interferential current
LADA	:	Latent Autoimmune Diabetes in Adults
LD50	:	Median Lethal Dose
MODY	:	Maturity onset of Diabetes in young
MAPK	:	Mitogen Activated Protein Kinase
MHC	:	Major Histocompatibility Complex
MIRE	:	Monochromatic infrared photo energy (MIRE)
NIDDM	:	Non Insulin Dependent Diabetes mellitus
NADH	:	Nicotinamide Adenine Di nucleotide
NADPH	:	Nicotinamide Adenine Di nucleotide Phosphate
NMDA	:	N- methyl Daspartate
NGA	:	Nerve growth factor
OGTT	:	Oral Glucose Tolerance Test
OECD	:	Organisation of Economic Co-operation and Development
PAG	:	Periaqueductal gray (PAG)
SEM	:	standard error mean
SGOT	:	Serum Glutamate Oxaloacetate Transaminase
SGPT	:	Serum Glutamate Pyruvate Transaminase
STT	:	Spinothalamic tract
SRT	:	Spinoreticular tract
SMT	:	Spinomesencephalic tract

SHT	:	Spinohypothalamic tract
TG	:	Triglycerides
TNF	:	Tumour Necrosis Factor
TENS	:	Transcutaneous electrical nerve stimulation
WHO	:	World Health Organisation
Fig	:	Figure
Cm	:	Centimetre
dL	:	Decilitre
i.p.	:	intra peritoneal
Kg	:	Kilogram
Min	:	Minute
Mg	:	Milligram
Ml	:	Millilitre
mmol/L	:	millimoles per litre
Nm	:	nano meter
p.o.	:	per oral
b.w.	:	body weight
qs	:	quantity sufficient
Sec	:	Seconds
◦C	:	degree Celsius
µL	:	micro litre
%PT	:	Percentage protection
GSH	:	Reduced Glutathione
LPO	:	Lipid Peroxidation
MDA	:	Malondialdehyde

SOD	:	superoxide dismutase
CAT	:	Catalase
GPx	:	Glutathione peroxidase

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1.INTRODUCTION

Diabetes is a serious, chronic disease that occurs either when the pancreas does not produce enough insulin (a hormone that regulates blood glucose), or when the body cannot effectively use the insulin it produces. Raised blood glucose, a common effect of uncontrolled diabetes, may, over time, lead to serious damage to the heart, blood vessels, eyes, kidneys and nerves. More than 400 million people live with diabetes. There are four types of diabetes mellitus, theyareType-1diabetes (previously known as insulin-dependent, juvenile or childhood-onset diabetes), Type2 diabetes (formerly called non-insulin-dependent or adultonset diabetes), Gestational diabetes (GDM) is a temporary condition that occurs in pregnancy and carries longterm risk of type 2 diabetes and MODY (Maturity onset Diabetes of the young)[1].

Globally, an estimated 422 million adults are living with diabetes mellitus, according to the latest 2016 data from the World Health Organization (WHO)[2]. Diabetes prevalence is increasing rapidly; previous 2013 estimates from the International Diabetes Federation put the number at 381 million people having diabetes[3]. The number is projected to almost double by 2030.Type 2 diabetes makes up about 85-90% of all cases. Increases in the overall diabetes prevalence rates largely reflect an increase in risk factors for type 2, notably greater longevity and being overweight or obese[2].

Long standing diabetes mellitus leads to multiple organ damage and it is associated with an increased prevalence of microvascular disease (Nephropathy, Neuropathy& Retinopathy) and macro vascular diseases

(Peripheral vascular disease, Ischemic heart disease& Stroke). Poor glycemic control, a factor that has been observed in the Indian population with diabetes put them at risk of complication including neuropathy-24.6%, cardiovascular disease-23.6%, kidney problem-21.1%, retinopathy- 16.6% and foot ulcer-5.5%[4].

Globally diabetic neuropathy affects approximately 132 million people as of 2010 (1.9% of the population).Diabetes is the leading known cause of neuropathy in developed countries, and neuropathy is the most common complication and greatest source of morbidity and mortality in diabetes. It is estimated that neuropathy affects 25% of people with diabetes. Diabetic neuropathy is implicated in 50–75% of nontraumatic amputations.

The main risk factor for diabetic neuropathy is hyperglycemia. In the DCCT (Diabetes Control and Complications Trial, 1995) study, the annual incidence of neuropathy was 2% per year but dropped to 0.56% with intensive treatment of Type 1 diabetics. The progression of neuropathy is dependent on the degree of glycemic control in both Type1 and Type2 diabetes. Duration of diabetes, age, cigarette smoking, hypertension, height, and hyperlipidemia are also risk factors for diabetic neuropathy[5]. Diabetic neuropathy is a complication of diabetes in which nerves are damaged due to long-term high levels of blood sugar or hyperglycemia. Diabetic neuropathy can affect many parts of the body including the legs, feet, bladder, heart, gastrointestinal system, and reproductive system. Diabetic neuropathy generally develops slowly over a period of months as ongoing high blood sugar levels damage the nerves of the body. Symptoms of diabetic neuropathy can include a

sensation of pain, numbness, tingling, or prickling that begins in the feet. In later stages of diabetic neuropathy, the hands can be affected as well. In some cases of diabetic neuropathy, the abnormal sensations can extend to the arm, legs and trunk[6].

Several medications are used to relieve nerve pain, but they don't work for everyone and most have side effects that must be weighed against the benefits they offer. There are also a number of alternative therapies, such as capsaicin cream (made from chili peppers), physical therapy or acupuncture, that may help with pain relief medications. Anti-seizure medications drugs such as gabapentin (Gralise, Neurontin), pregabalin (Lyrica) and carbamazepine (Carbatrol, Tegretol) Tricyclic antidepressant drugs such as amitriptyline, desipramine (Norpramin) and imipramine (Tofranil), serotonin and norepinephrine reuptake inhibitors (SNRIs), such as duloxetine (Cymbalta), can relieve pain with fewer side effects[7].

NATURALHERBES FOR THE TREATMENT OF DIABETIC NEUROPATHY

Various herbal remedies for diabetic neuropathy described in the ancient healthcare system of India. These herbs are effective in restoring the sensation in feet, healing the ulcers and keeping the sugar levels under control without causing any side effects. The herbs act together to keep nourishing the nerves damaged by diabetic neuropathy. These herbal remedies are combination of various herbal supplements which are otherwise useful in many other ailments like sexual weakness, erectile dysfunction, lack of stamina and strength, ageing related problems. The herbs can also be used by females to restore libido, fatigue, general weakness and pain in the

calf muscles due to diabetic neuropathy as well as high sugar levels[8]. The herbs are totally natural without any preservatives or chemicals. The herbs like *Plecospermum spinosum*[9] *Ashwagandha, Chandraprabha Vati, Shilajit, Indian gooseberry, Turmeric, Flaxseed oil, Ginger, Fennel seed, Castorl oil,Holy basil, Jamun and Fenugreek, Bitter melon, Ginseng, Cayenne Pepper, cinnamon and Bilberry leaves.* Above herbs for diabetic neuropathy may be alleviate or decrease the symptoms of neuropathy.

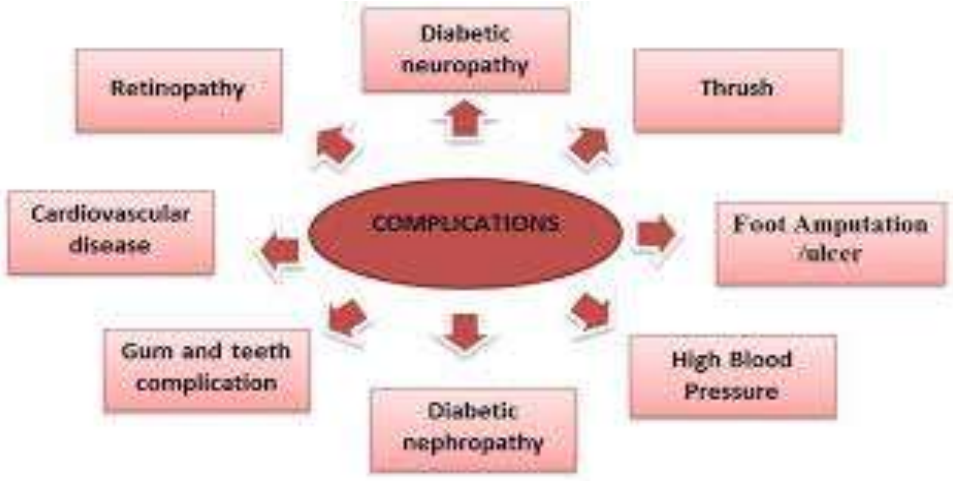
2. REVIEW OF LITERATURE

2.1 DIABETES:

Diabetes is a disease that affects body ability to produce or use insulin. Insulin is a hormone. When body turns the food into energy (also called sugar or glucose), insulin is released to help transport this energy to the cells. Insulin acts as a “key”. Its chemical message tells the cell to open and receive glucose. If body produce little or no insulin, or are insulin resistant, too much sugar remains in blood. Blood glucose levels are higher than normal for individuals with diabetes[10]. All types of diabetic patients insulin dependent diabetes mellitus (IDDM), non-insulin dependent diabetes mellitus (NIDDM) and secondary diabetes patients can develop neuropathy[11].

2.1.1 COMPLICATINS OF DIABETES:

Long term complication of diabetes develop gradually. The longer have diabetes and less controlled blood sugar the higher the risk of complications. Eventually diabetic complications may be disabeling or even life threatening.



Fifgure No: 1 : Complications of diabetes.

The most common long term complication diabetes divided into macrovascular and microvascular Macrovascular complications include damage to the large blood vessels of heart, brain and legs. Microvacular complications include damage to the small blood vessels causing problem in the eyes, kidneys, feet and nerves.

Other parts of body can also be affected by diabetes including the digestive system, the skin, sexual organs, teeth and gums and the immune system[12].

2.2 MACROVASCULAR DISEASE:

The common macrovascular disease are cardiovascular disease (in the heart). cerebrovascular disease (in the brain), and peripheral vascular disease (in the limbs)[13].

2.2.1 Cardiovascular disease:

Diabetes dramatically increases the risk of various cardiovascular problems including coronary artery with chest pain(angina), heart attack, stroke and narrowing of arteries(atherosclerosis), blood vessel diseases. The risk is greater for people with diabetes, who often have increased cholesterol, blood pressure levels. Smoking having a family history of cardiovascular disease being inactive also increase the risk[14].

2.2.2 Cerebrovascular disease:

Cerebrovascular disease is a vascular disease of the cerebral circulation. Arteries supplying oxygen to the brain are affected resulting in one of a number of cerebrovascular diseases[15]. It occurs when high cholesterol level, together with inflammation in the arteries of the brain. cause cholesterol buildup in the vessel are a thick , waxy plaque that can narrow and block blood flow in the arteries[16]. Cerebrovascular transient ischemia attacks(TIAS), which often herald a completed stroke, are also more common among patients with diabetes[17].

2.2.3 Peripheral vascular disease:

A condition in which the arteries in the legs, and sometimes the arms[18]. Peripheral vascular disease 20 times more common in people with diabetes than in the general population. Along with diabetes, other risk factor for peripheral vascular disease are smoking, inactivity and high blood lipid levels (cholesterol and triglyceroids). In people with diabetes chronic high blood glucose raises the risk of developing peripheral vascular disease.

2.3 MICROVASCULAR DISEASE:

Microvascular complications include damage to eyes (retinopathy) leading to blindness, to kidneys (nephropathy) leading to renal failure and to nerves (neuropathy) leading to impotence and diabetic foot disorders (which include severe infections leading to amputation)[19].

2.3.1 Diabetic retinopathy:

Diabetic retinopathy is a leading cause of blindness and visual disability. It is caused by small blood vessel damage to the back layer of the eye, the retina, leading to progressive loss of vision, even blindness.

2.3.2 Diabetic nephropathy:

Diabetic kidney disease is also caused by damage to small blood vessels in the kidneys. This can cause kidney failure, and eventually lead to death. In developed countries, this is a leading cause of dialysis and kidney transplant.

2.3.3 Diabetic neuropathy:

Diabetes causes nerve damage through different mechanisms, including direct damage by the hyperglycemia and decreased blood flow to nerves by damaging small blood vessels. This nerve damage can lead to sensory loss, damage to limbs, and impotence in diabetic men. It is the most common complication of diabetes.

2.4 NEUROPATHY:

Neuropathy the medical term for a condition in which there are problems with nerves in the body either they have been damaged or are affected by a disease. Usually, neuropathy affects the peripheral nervous system rather than the central nervous system (brain and spine)[20].

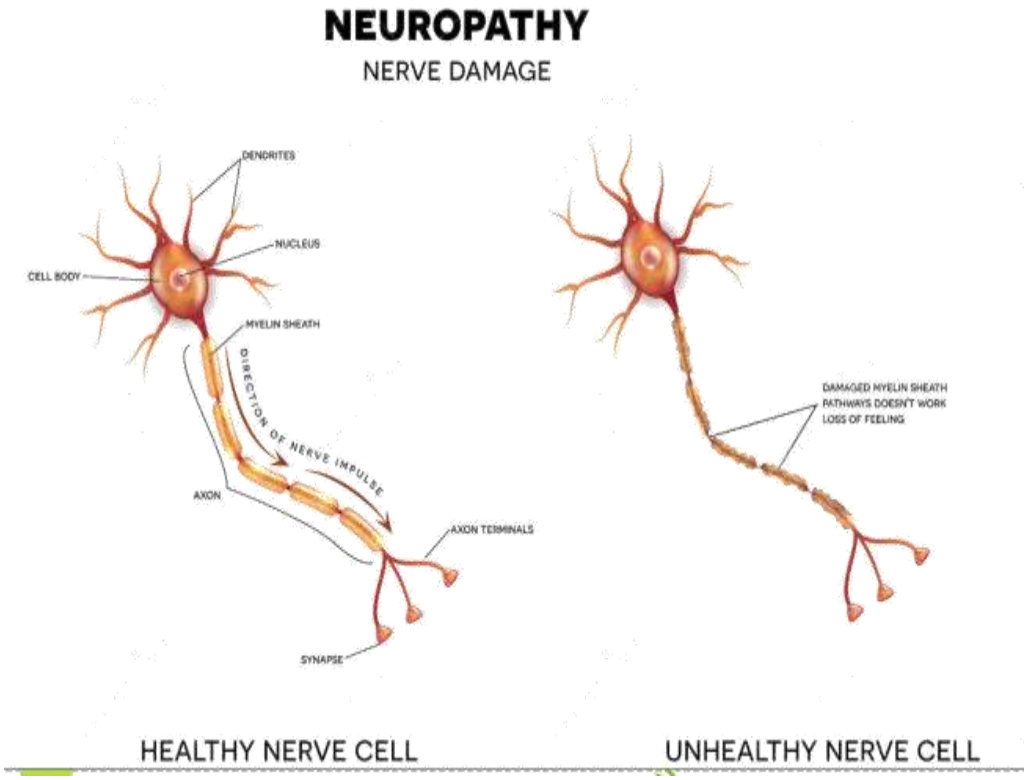


Fig.No.2 Difference between healthy nerve cell and damaged cell

In the peripheral nervous system, there are three primary types of nerves:

- **Sensory nerves** control the senses and the body's interpretation of different sensations.
- **Motor nerves** control muscle movement and power.
- **Autonomic nerves** control bodily systems like the gastrointestinal and urinary systems.

2.4.1 Complication of Diabetic Neuropathy:

Diabetic neuropathy can cause a number of serious complications, including:

- Loss of a limb.
- Charcot joint.
- Urinary tract infections and urinary incontinence.
- Hypoglycemia unawareness.
- Low blood pressure.
- Digestive problems.
- Sexual dysfunction.
- Increased or decreased sweating

2.4.2 Types of Neuropathy:

Diabetic neuropathy can be broken into several types. This is because we have different kinds of nerves in our bodies that serve different functions. The symptoms and treatments depend on which type of diabetic neuropathy[21].

There are four types of diabetic neuropathy:

- Peripheral neuropathy (also called diabetic nerve pain and distal polyneuropathy)
- Proximal neuropathy (also called diabetic amyotrophy)
- Autonomic neuropathy
- Focal neuropathy (also called mononeuropathy)

Peripheral Neuropathy:

Peripheral neuropathy refers to the conditions that result when nerves that carry messages to and from the brain and spinal cord from and to the rest of the body are damaged or diseased[22]. Peripheral diabetic neuropathy goes by various names peripheral diabetic nerve pain and distal polyneuropathy. Peripheral neuropathy can affect one nerve (mononeuropathy), two or more nerves in different areas (multiple mononeuropathy) or many nerves (polyneuropathy)[23]. It may be chronic (a long-term condition where symptoms begin subtly and progress slowly) or acute (sudden onset, rapid progress, and slow resolution)[24-26]. The peripheral nerves make up an intricate network that connects the brain and spinal cord to the muscles, skin, and internal organs. Peripheral nerves come out of the spinal cord and are arranged along lines in the body called dermatomes. Typically, damage to a nerve will affect one or more dermatomes, which can be tracked to specific areas of the body. Damage to these nerves interrupts communication between the brain and other parts of the body and can impair muscle movement, prevent normal sensation in the arms and legs, and cause pain[22].

Proximal Neuropathy:

Proximal neuropathy can also be called diabetic amyotrophy. That *myo* in the word means muscle, so this is a form of neuropathy that can cause muscle weakness. It specifically affects the muscles in the upper part of your leg(s), buttocks, and hips[27].

Sometimes, proximal neuropathy can also involve nerve pain, especially pain that shoots from the low back and down the leg[28]. The technical medical term for that is *radiculopathy*, although most people refer to it as sciatica. If there's also shooting nerve pain involved, this form of neuropathy can also be called polyradiculopathy-diabetic amyotrophy. Proximal neuropathy is the second most common type of diabetic neuropathy (second only to peripheral diabetic neuropathy). It usually affects elderly people with diabetes; as opposed to peripheral neuropathy, it usually resolves with time or treatment[21].

AUTONOMIC NEUROPATHY:

Autonomic neuropathy is a group of symptoms that occur when there is damage to the nerves that manage every day body functions such as blood pressure, heart rate, sweating, bowel and bladder emptying, and digestion[29]. Autonomic neuropathy may be seen with Alcohol abuse, diabetes (diabetic neuropathy), disorders involving scarring of tissues around the nerves, Parkinson disease, Spinal cord injury, Surgery or injury involving the nerves[30]. The first objective of management of a patient with autonomic neuropathy is to administer specific treatment for treatable conditions. For example, if an autoimmune neuropathy is present, attempted management with immunomodulatory therapies should be considered[31].

Focal Neruopathy:

Focal neuropathy by contrast, affects one specific nerve It's focused neuropathy and also called mononeuropathy. Focal neuropathy which comes

on suddenly, most often affects nerves in the head (especially ones that go to the eyes). It can also affect the torso and legs. The diabetic patients are also susceptible to a variety of asymmetric and focal neuropathies[32].

Types of focal neuropathy:

a. Cranial neuropathy

b. Truncal neuropathy

c. Entrapment neuropathy

a. Cranial Neuropathy :

When nervous in the brain (or) brain stem affected areas like face and eyes. It is called cranial neuropathy[33]. The cranial nerve control such a function as vision, hearing, facial movement and the actions of some of the organs in the head chest and abdomen[33]. Third, fourth, and sixth cranial nerves are commonly involved. Elderly patients are the most affected. Two specific types of cranial neuropathy are optic neuropathy and auditory neuropathy. Optic neuropathy refers to damage or disease of the optic nerve that transmits visual signals from the retina of the eye to the brain. Auditory neuropathy involves the nerve that carries signals from the inner ear to the brain and is responsible for hearing[34].

b. Truncal Neuropathy :

Symptomatic truncal polyneuropathy though less common, tends to occur in the setting of long standing diabetes with other microvascular

complications especially peripheral neuropathy[35]. Truncal neuropathy is an important cause of chest and abdominal pain[36]. It is also called as thoracoabdominal neuropathy, thoracia poly rediculopathy, truncal mononeuropathy. On examination, hypoaesthesia or hyperaesthesia may be present in the appropriate thoracic segment and abdominal muscle weakness leading to abdominal swelling[37].

C. Entrapment neuropathy :

Nerve compression syndrome or compression neuropathy, also known as entrapment neuropathy, is a medical condition caused by direct pressure on a nerve.[38] It is known colloquially as a *trapped nerve*, though this may also refer to nerve root compression (by a herniated disc, for example). Its symptoms include pain, tingling, numbness and muscle weakness. The symptoms affect just one particular part of the body, depending on which nerve is affected[39].

2.4.3 CAUSES AND PATHOGENESIS OF DIABETIC NEUROPATHY:

Causes of diabetic neuropathy:

- **Diabetes:** Diabetes is the condition most commonly associated with neuropathy. The characteristic symptoms of peripheral neuropathy often seen in people with diabetes are sometimes referred to as diabetic neuropathy. The risk of having diabetic neuropathy rises with age and duration of diabetes. Neuropathy is most common in people who have had diabetes for decades and is generally more severe in those who have had difficulty controlling their

diabetes, or those who are overweight or have elevated blood lipids and high blood pressure[40].

- **Vitamin deficiencies:** Deficiencies of the vitamins B12 and folate as well as other B vitamins can cause damage to the nerves.

- **Autoimmune neuropathy:** Autoimmune diseases such as rheumatoid arthritis, systemic lupus, and Guillain-Barre syndrome can cause neuropathies.

- **Infection:** Some infections, including HIV/AIDS, Lyme disease, leprosy, and syphilis, can damage nerves.

- **Post-herpetic neuralgia:** Post-herpetic neuralgia, a complication of shingles (varicella-zoster virus infection) is a form of neuropathy.

- **Alcoholic neuropathy:** Alcoholism is often associated with peripheral neuropathy. Although the exact reasons for the nerve damage are unclear, it probably arises from a combination of damage to the nerves by alcohol itself along with the poor nutrition and associated vitamin deficiencies that are common in alcoholics.

- **Genetic or inherited disorders:** Genetic or inherited disorders can affect the nerves and are responsible for some cases of neuropathy. Examples include Friedreich's ataxia and Charcot-Marie-Tooth disease.

- **Amyloidosis:** Amyloidosis is a condition in which abnormal protein fibers are deposited in tissues and organs. These protein deposits can lead to varying degrees of organ damage and may be a cause of neuropathy.

- **Uremia:** Uremia (a high concentration of waste products in the blood due to kidney failure) can lead to neuropathy.

- **Toxins and poisons can damage nerves.** Examples include, gold compounds, lead, arsenic, mercury, some industrial solvents, nitrous oxide, and organophosphate pesticides.

- **Drugs or medication:** Certain drugs and medications can cause nerve damage. Examples include cancer therapy drugs such as vincristine (Oncovin, Vincasar), and antibiotics such as metronidazole (Flagyl), and isoniazid (Nydrazid, Laniazid).

- **Trauma/Injury:** Trauma or injury to nerves, including prolonged pressure on a nerve or group of nerves, is a common cause of neuropathy. Decreased blood flow (ischemia) to the nerves can also lead to long-term damage.

- **Tumors:** Benign or malignant tumors of the nerves or nearby structures may damage the nerves directly, by invading the nerves, or cause neuropathy due to pressure on the nerves.

- **Idiopathic:** Idiopathic neuropathy is neuropathy for which no cause has been established. The term idiopathic is used in medicine to denote the fact that no cause is known.

2.4.2 Pathogenesis of diabetic neuropathy:

The cause of diabetic Neuropathy remains unknown but ischemic and metabolic complication are implicated. The following mechanisms seem to be involved:

1. Increased flux through the polyol pathway, mediated by aldose reductase and sorbitol dehydrogenase, leading to accumulation of sorbitol and depletion of myo-inositol. The latter reduction is associated with reduced Na+-K+-ATPase activity[41].

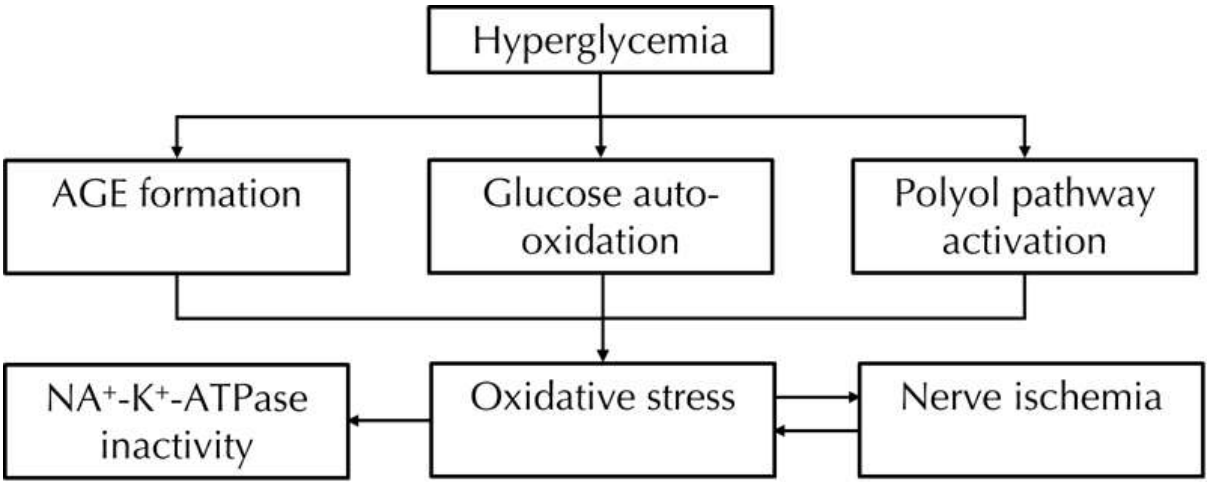


Fig. No.3 Pathogenesis of diabetic neuropathy

a. Endoneurial microvascular damage and hypoxia due to nitric oxide inactivation[42].

b. Accumulation of advanced glycation end products (AGEs) that exert their damaging effects by binding to specific receptors on the surface of neurons. Binding of AGEs to their receptors causes oxidative stress and activates nuclear factor-κB (NF-κB). There is increasing evidence

that the diverse agents able to activate NF-κB elevate levels of reactive oxygen species (ROS). Also, chemically distinct antioxidants and over expression of antioxidant enzymes can inhibit NF-κB activation[41-44].

c. Increased nerve lipid peroxidation in vivo. The most reliable index of increased oxidative stress is reduction in GSH[42].

d. Activation of protein kinase C (PKC) by increased release of intracellular diacylglycerol (DAG) due to glycolysis. Hyperglycemia activates PKC, especially its βII isoform through increased de novo synthesis of DAG. The increased activity of PKCβ may impair endoneurial blood flow. Recently, hyperglycemia has been associated with activation of PKC and increase in Na1.7 tetrodotoxin-sensitive voltage-gated sodium channel isoform; both of which play a critical role in the perception of pain[45-48].

e. Alterations in mitogen-activated protein kinases (MAPKs) result in a signaling cascade involved in the pathogenesis of peripheral diabetic neuropathy[49].

f. Abnormal Ca2+ homeostasis and signaling[50].

2.4.5 Diagnosis of diabetic neuropathy:

Diagnose of neuropathy on the basis of symptoms and a physical exam[51]. The diagnosis of DN in time is very important because effective intervention will be possible only during the subclinical or early phase of dysfunction[52]. During the exam, check blood pressure, heart rate, muscle

strength, reflexes, and sensitivity to position changes, vibration, temperature, or light touch.

Morphological testing:

Measures such as sural nerve biopsies provide an opportunity to study the biochemical and morphometric parameters of myelinated and unmyelinated fiber populations using the light microscope. Analysis includes examination of the myelinated nerve fibre size and distribution, myelinated nerve fiber density, index of circularity, and a measure of focal fiber loss[53]

Superficial Pain Testing:

Pain sensation can be tested with a sterile safety pin. The site of testing varies with the specific algorithm but may include the dorsum of the great toe or the plantar aspect of the distal first, third and fifth toe of each foot. Most commonly, the stimulus is applied once per site. Results are scored accordingly[54-55].

Vibration perception thresholds:

Vibration thresholds is performed using a handheld device (Bio-Thesiometer). This instrument quantitatively tests vibratory sensation with a specialized probr set at 100-1Hz and hasan adjustable amplitude ranging from 0-50 volts[56]. As an easy and traditional way to test vibratory sensation, the128Hz standard (non-graduated) tuning forkis a tool of screening for diabetic neuropathy. The risk of foot ulceration is increased 3-4 fold if the vibration perception threshold exceeds 25 volts. Vibrameter is also based on

the principle of biosthesiometer but results are given directly in mm of probe displacement[52].

Light touch sensation:

Light touch perception can be evaluated by using a number of methods from a finger, to cotton, to specifically calibrated devices. The best known of the calibrated devices is the Semmes- Weinstein 10-g monofilament, a nylon filament embedded in a plastic handle[57]. A series of increasingly thick filaments are tested, and the threshold at which the first one can be felt when buckling is noted. The inability to feel the 10 gm filament indicate that patient is prone to foot ulceration[52].

Thermal thresholds:

The Thermal threshold parameters studied were: (1) sensory thresholds to cold and heat and (2) pain thresholds induced by cold and heat on both palms and on the dorsum and soles of both feet[50]. The equipment used for thermal threshold assessment are expensive and mostly used for research purposes. Pain threshold can be determined either by application of high or low temperature or by using the “Pinchometer” or a series of weighted needles[52].

Electrophysiology:

All electrophysiological studies were performed on a multiple channel EMG (Macro electromyography) apparatus[56]. Often performed along with

nerve conduction studies, electromyography measures the electrical discharges produced in the muscles.

Nerve conduction studies. These measure the ability of peripheral nerves to conduct electrical impulses and are abnormal when pathological changes are present in myelinated nodes of Ranvier and axons[51].

Autonomic testing

Symptoms of autonomic neuropathy, may request special tests to look the blood pressure in different positions and assess the ability to sweat[51].

A check of heart rate variability

It shows how the heart responds to deep breathing and to changes in blood pressure and posture.

Ultrasound:

It uses sound waves to produce an image of internal organs. An ultrasound of the bladder and other parts of the urinary tract, for example, can be used to assess the structure of these organs and show whether the bladder empties completely after urination.

Foot Exams:

Experts recommend that people with diabetes have a comprehensive foot exam each year to check for peripheral neuropathy. People diagnosed with peripheral neuropathy need more frequent foot exams[51].

2.5 PAIN:

Pain has been defined as “an unpleasant sensory or emotional experience associated with actual or potential tissue damage”[60].

Classification of pain[61]:

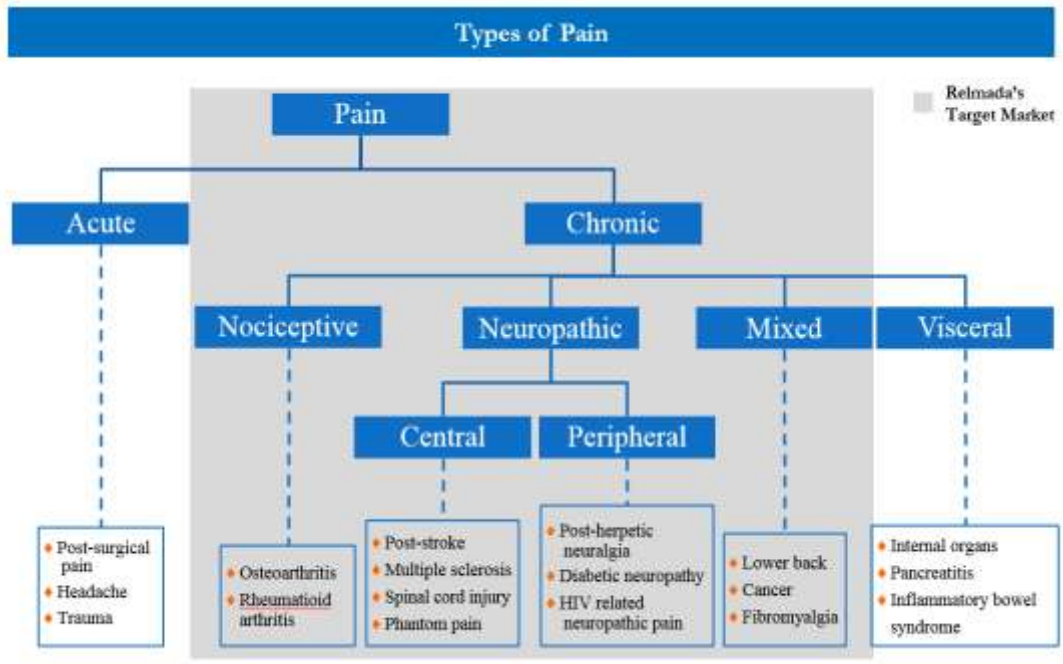


Fig. No. 4 Classification of pain

2.5.1 NOCICEPTOR:

A nociceptor is a type of receptor at the end of a sensory neuron's axon that responds to damaging or potentially damaging stimuli by sending pain signals to the spinal cord and the brain. This process is called nociception[62].

The peripheral terminal of the mature nociceptor is where the noxious stimuli are detected and transduced into electrical energy. When the electrical energy reaches a threshold value, an action potential is induced and driven towards the central nervous system (CNS). The sensory specificity of

nociceptors is established by the high threshold only to particular features of stimuli. Only when the high threshold has been reached by either chemical, thermal, or mechanical environments are the nociceptors triggered. The majority of nociceptors are classified by which of the environmental modalities they respond to. Some nociceptors respond to more than one of these modalities and are consequently designated polymodal. Other nociceptors respond to none of these modalities (although they may respond to stimulation under conditions of inflammation) and are referred to as sleeping or silent[63].

Nociceptors have two different types of axons. The first are the Aδ fiber axons. They are myelinated and can allow an action potential to travel at a rate of about 20 meters/second towards the CNS. The other type is the more slowly conducting C fiber axons. These only conduct at speeds of around 2 meters/second. This is due to the light or non-myelination of the axon. As a result, pain comes in two phases. The first phase is mediated by the fast-conducting Aδ fibers and the second part due to (Polymodal) C fibers. The pain associated with the Aδ fibers can be associated to an initial extremely sharp pain. The second phase is a more prolonged and slightly less intense feeling of pain as a result of the acute damage. If there is massive or prolonged input to a C fiber, there is a progressive build up in the spinal cord dorsal horn; this phenomenon is similar to tetanus in muscles but is called wind-up. If wind-up occurs there is a probability of increased sensitivity to pain[64-65].

Inflammatory mediators (e g bradykinin, serotonin, prostaglandins, cytokines, and H+) are released from damaged tissue and can stimulate nociceptors directly. They can also act to reduce the activation threshold of nociceptors so that the stimulation required to cause activation is less. This process is called primary sensitisation[66].

2.6 NEUROPATHIC PAIN:

Neuropathic pain is pain caused by damage or disease affecting the somatosensory nervous system. Neuropathic pain may be associated with abnormal sensations called dysesthesia or pain from normally non-painful stimuli (allodynia). It may have continuous and/or episodic (paroxysmal) components. The latter resemble stabbings or electric shocks. Common qualities include burning or coldness, "pins and needles" sensations, numbness and itching[67].

2.7 PAIN PROCESS:

There are four steps involved in how our bodies process pain[11].

- Transduction
- Transmission
- Perception
- Modulation

Transduction:

Transduction is the conversion of a noxious stimulus (mechanical, chemical or thermal) into electrical energy by a peripheral nociceptor (free afferent nerve ending)[11].

This process begins when peripheral nerve terminals of nociceptor C fibres and A-delta (Aδ) fibres are depolarised by noxious mechanical, thermal or chemical energy. The membranes of these terminal contains protein and voltage-gated ion channels that convert thermal, mechanical or chemical energy into action potencial (AP). Nociceptor terminals spread densely throughout the skin[68]. Nociceptors are exposed to noxious stimuli when tissue damage and inflammation occurs as a result of, for example trauma, surgery, inflammation, infection, and ischemia.

The nociceptors are distributed in the:

- Somatic structures (skin, muscle, connective tissue, bones, joints)
- Visceral structures (Visceral organs such as liver, gastro-intestinal tract)
- The C fibre and A-delta fibres are associated with different qualities of pain.

The chemical mediators like Prostaglandin, bradykinin, serotonin, suptance P, Potassium, Histamine are activate and / or sensities the nociceptor to the noxious stimuli. In order for a pain impulse to be generated an exchange of sodium and potassium ions (depolorisation and re-

polarisation) occurs at the cell membranes. This results in an action potential and generation of a pain impulse[68].

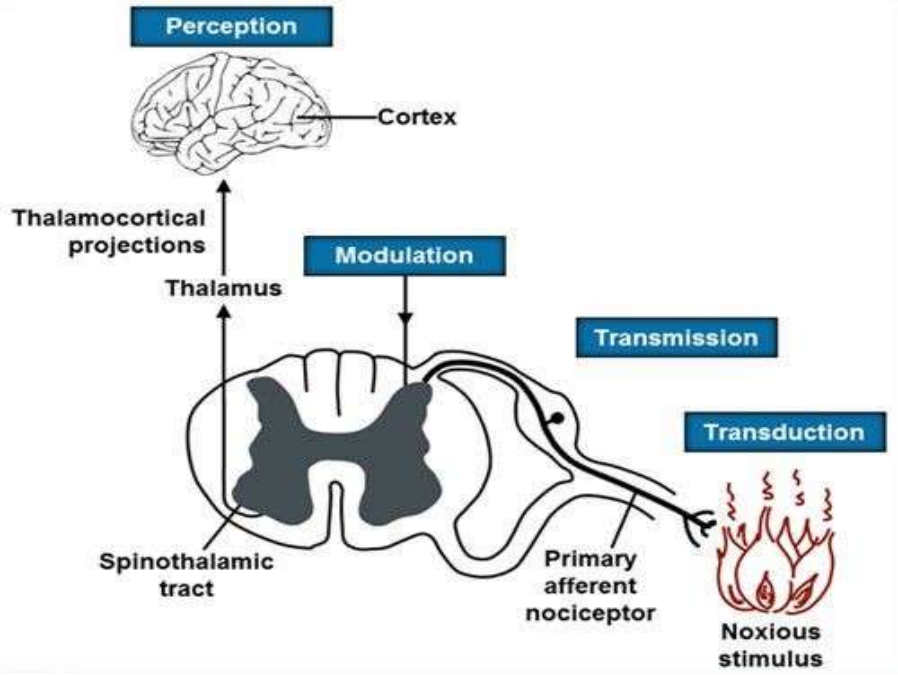


Fig. No. 5 Pain Process

2.7.2 TRANSMISSION:

The transmission process in three stage. The pain impulse is transmitted:

1. From the site of transduction along the nociceptor fibres to the dorsal horn in the spinal cord.
2. From the spinal cord to the brain stem.
3. Through connections between the thalamus, cortex and higher levels of the brain.

The C fibre A-delta fibres terminate in the dorsal horn of the spinal cord. There is a synaptic cleft between the terminal ends of the C fibre and A-delta fibres and the nociceptive dorsal horn neurons (NDHN). In order for the pain

impulses to be transmitted across the synaptic cleft to the NDHN. Excitatory neurotransmitters are released, which bind to specific receptors in the NDHN. These neurotransmitters are:

- Adenosine triphosphate
- Glutamate
- Calcitonin gene-related peptide
- Bradykinin
- Nitric oxide
- Substance P

The pain impulse is then transmitted from the spinal cord to the brain stem and thalamus via two main nociceptive ascending pathways. These are the spinothalamic pathway and the spinoparabrachial pathway.

The brain does not have a discrete pain centre, so when impulses arrive in the thalamus they are directed to multiple areas in the brain where they are prosessed[68].

2.7.3 MODULATION:

Modulation of nociceptive transmission is an adaptive process involving both excitory and inhibitory mechanisms. Peripheral modulation can be accomplished by[69]:

- Inhibiting the sensitization of nociceptor terminals
- Inhibiting depolarization and repolarization of the axonal membrane by preventing the generation or conduction of an action potential by

blocking the influx of sodium through voltagegated sodium channels located along first and second order afferents.

- Inhibiting the inflammatory responses to trauma with hydrocortisone.
- Stimulating the large fast Aβ fibers in the area of injury can induce interneurons in the dorsal horn to release GABA and glycine which inhibit the release of glutamate from the primary afferent terminal, there by preventing depolarization of the second order neuron. Mechanical stimulation and transcutaneous electrical nerve stimulation (TENS) are believed to reduce the perception of pain by activating fast Aβ fibers[R].

2.7.4 PERCEPTION:

Perception of nociceptive pain is dependant upon neural processing in the spinal cord and several brain regions. Pain becomes more than a pattern of nociceptive action potentials when they reach the brain. Action potentials ascending the spinothalamic tract are decoded by the thalamus, sensorimotor cortex, insular cortex and the anterior cingulate to be perceived as an unpleasant sensation that can be localized to a specific region of the body. Action potential ascending the spinobulbar tract are decoded by the amygdala and hypothalamus to generate a sense of urgency and intensity. It is the intergration of sensations, emotions and cognition that result in our perception of pain[69].

2.8 PAIN PATHWAY:

There are two pain pathways they are ascending pathway and descending pain pathway.

2.8.1 Ascending Pathway:

The central processes of the afferent neurons enter the brain or spinal cord and synapse upon interneurons there. The central processes diverge to terminate on several, or many, interneurons and converge so that the processes of many afferent neurons terminate upon a single interneuron. The interneurons upon which the afferent neurons synapse are termed second-order neurons, and these in turn synapse with third-order neurons, and so on, until the information (coded action potentials) reaches the cerebral cortex[70].

Various sensory signals take two different paths to reach the brain, both of which start in a given part of the body and end in the brain's somatosensory cortex[15]. Each of these paths consists of a chain of **three neurons** that pass the nerve impulses from one to the next. Where these two paths differ is in the location where they cross the midline in the spinal cord. The nerves responsible for sensory inputs, as well as those responsible for motor control, are crossed. In other words, the neural pathways from the left side of the body terminate in the right hemisphere of the brain, and vice versa. Hence, at some point in the body, these pathways must cross the body's midline (in scientific terminology, they must "decussate").

Any incoming sensory impulse-whether for touch, pain, heat, or proprioception-follows from the spinal cord to the brain. Regardless of the sensory modality, the three neurons in the form of a chain running from one side of the spinal cord to the other, and the cell body of the **first** neuron in this chain is always located in a spinal (dorsal root) ganglion. This neuron is said

to be T-shaped, because its axon emerges as a short extension from its cell body and then soon divides into two branches going in opposite directions: one goes to the part of the body that is innervated by this spinal nerve, while the other immediately enters the dorsal root of the spinal cord (an essentially sensory part of the spinal cord, as opposed to the ventral root, which is a motor area). It is from this point on that the two pathways differ.

The pathway responsible for touch and proprioception is called the Lemniscal pathway. The first axon in this pathway runs along the dorsal root of the spinal nerve and up the dorsal column of the spinal cord. (Along the way, this axon also sends out collaterals: branches in the dorsal root that play a valuable role in the local inhibition of pain, among other functions.)

The primary axon, however, remains on the same side of the spinal cord as the side of the body that it innervates (the “ipsilateral” side) until it connects with the **second** neuron in the chain, which in the case of the lemniscal pathway is located in the medulla. The axon of this second neuron crosses the midline immediately. It then travels up through the medial lemniscus to the ventral poster lateral (VPL) nucleus of the thalamus, where it connects with the **third** neuron in the chain.

The pathway that carries information about pain and non-painful temperatures is called the neo spinothalamic pathway (or often simply the spinothalamic pathway). The **first** neuron in this pathway connects to the **second** neuron not in the medulla, but in the dorsal horn of the spinal cord, on the same side that the nerve impulse comes from. This second neuron has a

single axon, which immediately crosses the midline to the other (contralateral) side of the spinal cord and goes up to the brain along with the other axons forming the lateral spinothalamic tract. This part of the pathway is described as contralateral, meaning that it runs along the side of the body opposite to the area that its axons innervate.

The axon of the second neuron connects to the **third** and final neuron of this ascending pathway in the ventral posterolateral (VPL) nucleus of the thalamus. In both of these pathways, the third neuron sends its axon to the somatosensory cortex, the part of the brain that determines exactly where the original stimulus occurred in the body[71].

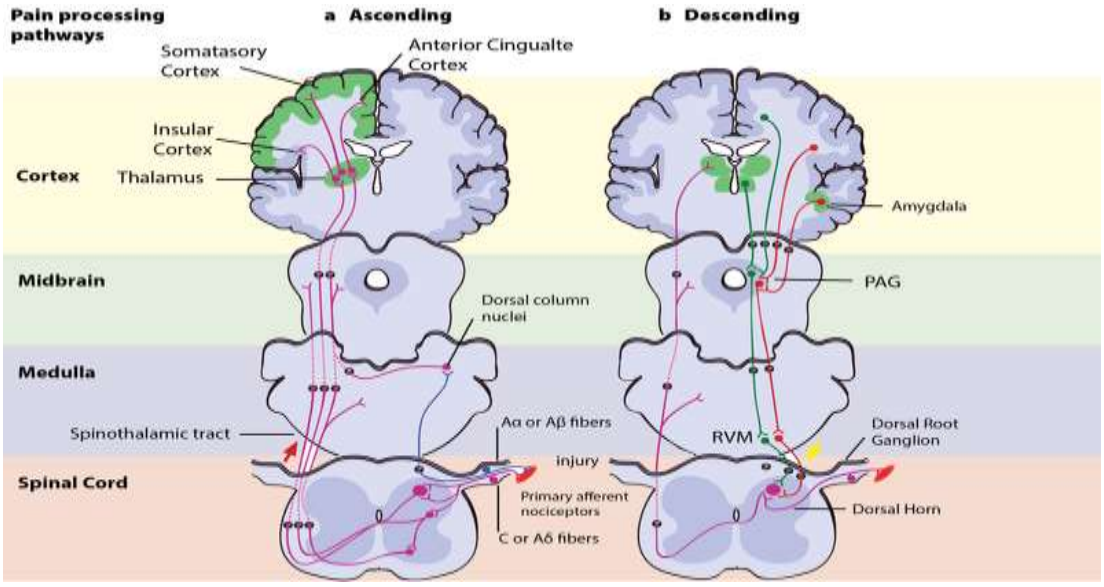


Fig. No. 6 Pain Processing Pathways

2.8.2 DESCENDING PATHWAYS:

A nerve pathway that go down the spinal cord and allow the brain to control movement of the body below the head. Descending pathways begin in the periaqueductal gray (PAG).Stimulation of the PAG has been shown to

produce analgesia, but no change in the ability to detect temperature, pressure, or touch. The neurons beginning in the PAG end on cells in the medulla, including the serotonergic cell bodies of the raphe nuclei. The serotonergic neurons then descend into the spinal cord to inhibit cell firing. Other cells in the PAG terminate close to the locuscoeruleus in the brainstem. Thus, there are at least two major pathways that descend to the spinal cord to inhibit the projection of pain[72].

2.9 TREATMENT OF DIABETIC NEUROPATHY:

The treatment of diabetic neuropathy can be broadly divided into two major groups[51,73-75]

1. Symptomatic treatment.
2. Treatment for nerve regeneration.

Symptomatic Treatment:

Pain is the most common symptoms which could be superficial, deep or aching. Simple reassurance that the pain is not permanent, does produce great relief from pain, pain related anxiety or depression. However, following measures can be take in order of preference for pain relief:

1. Tricyclic anti-depressants:

- Imipramine
- Amitriptyline
- Desipramine
- Nortriptyline
- Clomipramine

2.Anti-convulsants:

- Carbamazepine
- Clonazepam
- Phenytoin
- Gabapentin
- Pregabalin

3.Serotonin- Norepinephrine reuptake inhibitors:

- Duloxetine
- Venlafaxine

4.Selective serotonin reuptake inhibitors:

- Fluoxetine
- Paroxetine
- Sertraline
- Citalopram

5.Antioxidants:

- Alpha-lipoic acid

6.Opioids:

a. Typical opioid:

- Oxycodone

b.Atypical opioids:

- Tramadol
- Tapentadol

Topical agents:

Treatments that are applied to the skin typically to the feet. sprays or patches for the feet may relieve pain. Oil are suggest they may help relieve symptoms and improve nerve function in some patients.

- Capsaicin
- Clonidine
- Lidocaine patch
- Nitrate sprays or patches
- Primrose oil

Miscellaneous:

- Bed cradle can keep sheets and blankets from touching sensitive feet and legs.
- Acupuncture
- Biofeedback or physical therapy()
- Electrical nerve stimulation
- Magnetic therapy
- Laser or light therapy
- Hot wax
- Monochromatic infrared photo energy(MIRE)
- Interferential current(IFC)
- Therapeutic ultrasound

Treatment for nerve regeneration:

The pathologic basis for the treatment of diabetic neuropathy, it is important to enhance nerve regeneration as well as prevent nerve degeneration[76]. Nerve regeneration or sprouting in diabetes may occur not only in the nerve trunk but also in the dermis and around dorsal root ganglion neurons, thereby being implicated in the generation of pain sensation. Nerve regenerative capacity has been shown to be decreased in diabetic patients as well as in diabetic animals. Rationally accepted replacement therapy with neurotrophic factors has not provided any success in treating diabetic neuropathy. Aside from adverse effects of those factors, more rigorous consideration for their delivery system may be needed for any possible success. Although conventional therapeutic drugs like aldose reductase (AR) inhibitors and vasodilators have been shown to enhance nerve regeneration, their efficacy should be strictly evaluated with respect to nerve regenerative capacity.

2.10 TREATMENT DESIGNED TO MODIFY THE COURSE OF DIABETES:

The treatment of diabetic neuropathy is aimed at preventing the progression of neuropathy and providing symptomatic relief.

Glycaemia control:

Regulation and maintenance blood glucose levels within normal ranges; aim of the treatment of diabetes mellitus (by diet, oral hypoglycaemic agents or parenteral insulin), longterm glycaemic control reduces later

incidence of secondary diabetic complications[77]. Tight glycemic control is the only strategy convincingly shown to prevent or delay the development of neuropathy in patients with type 1 diabetes and to slow the progression of neuropathy in some patients with type 2 diabetes[78]. Studies have shown that good control can prevent or delay the onset of diabetic peripheral neuropathy. The effectiveness of normoglycaemia in improving damaged nerves has been documented in some patients who have undergone combined pancreatic and renal transplantation[79].

Aldose reductase inhibitors:

Aldose reductase is an enzyme that is normally present in many other parts of the body, and catalyzes one of the steps in the sorbitol(polyol) pathway that is responsible for fructose formation from glucose. Aldose reductase activity increases as the glucose concentration rises in diabetes in those tissues that are not insulin sensitive, which include the lenses, peripheral nerves and glomerulus. Sorbitol does not diffuse through cell membranes easily and therefore accumulates, causing osmotic damage which leads to retinopathy and neuropathy. The aldose reductase inhibitors prevent conversion of glucose to sorbitol in presence of hyperglycaemia, Therefore, it prevents the polyol pathway cascade. ARIs are alreastat, tolerestat, epalrestat, sorbinil, and zopolrestat. There is great controversy about the mechanisms of action of the ARIs, and suggestions range from altered phosphoinositide metabolism and Na+ - K+ adenosine triphosphate activity, through reduced glutathione levels, to vasodilation and improved blood flow to nerve[80-81].

Alpha-Lipoic acid:

The antioxidant alpha-lipoic acid (or ALA), taken in pill form, lessened pain in people with diabetic neuropathy or nerve damage resulting from diabetes. ALA is a sulfur-containing compound that is made in small amounts in the body but is not found in food[82-83]. It's potent antioxidant, prevents or improves nerve conduction attributes, endoneurial blood flow, and nerve Na^+ K^+ ATPase activity in experimental diabetes and in humans and may improve positive neuropathic sensory symptoms.

Carnitine:

Acetyl-L-carnitine (ALC) is deficient in diabetes[83]. Acetyl-L-carnitine (ALC), a constructive molecule in fatty acid metabolism, is an agent potentially effective for treating neuropathic pain (NP). It is also called as nerve productor or nervepredicting agent Acetyl L-Carnitine which involved in the energy production of human body. Carnitine made in liver and kidneys from the aminoacids. Acetyl L-Carnitine improves the cells ability to produce energy, this allows the cells to do their jobs. But more energy creates more free-radicals. Adding Alpha Lipoic Acid to the Aceytl L-Carnitine handles these free radicals. The cell producing better, can now use cholesterol and B vitamins to repair the nerve cells.

2.11 DIABETES INDUCING AGENTS:

At present time best and quickest way to induce diabetes is with use of chemicals (alloxan, streptozotocin, dithizone, monosodium glutamates etc.),

viruses and genetically diabetic rats. In recent years, scientists and technologists have worked toward refining techniques that have led to the discovery of chemical agents that physiologically alter the function of the pancreas. The main advantage of using such chemicals is that body changes during and after the induction of diabetes can be observed. The five major diabetogenic agents are chemicals, biological agents, peptides, potentiators, and steroids but most commonly used chemical agents are alloxon and streptozotozine[84]

Alloxan:

Alloxan (2, 4, 5, 6-tetra oxy pyrimidine; 2, 4, 5, 6- pyrimidinetetrone) is an oxygenated pyrimidine derivative which is present as alloxan hydrate in aqueous solution. It is most prominent chemical compound used in diabetogenic research. In research it is used for induction of Type 1 diabetes. Alloxan is a urea derivative which causes selective necrosis of the β- cells of pancreatic islets It has been widely used to induce experimental diabetes in animals such as rabbits, rats, mice and dogs with different grates severity by varying the dose of alloxan used[85-86].

Mechanism action of Alloxan:

Alloxan are toxic glucose analogues that preferentially accumulate in pancreatic beta cells via the GLUT2 glucose transporter. In the presence of intracellular thiols, especially glutathione, alloxan generates reactive oxygen species (ROS) in a cyclic redox reaction with its reduction product, dialuric acid. Autoxidation of dialuric acid generates superoxide radicals, hydrogen

peroxide and, in a final iron-catalysed reaction step, hydroxyl radicals. These hydroxyl radicals are ultimately responsible for the death of the beta cells, which have a particularly low antioxidative defence capacity, and the ensuing state of insulin-dependent 'alloxan diabetes'. As a thiol reagent, alloxan also selectively inhibits glucose-induced insulin secretion through its ability to inhibit the beta cell glucose sensor glucokinase.

Streptozotocin:

Streptozotocin is naturally occurring chemical; used to produce Type- 1 diabetes in animal model and Type- 2 diabetes with multiple low doses. It is also used in medicine for treating metastatic cancer of islets of Langerhans[87].

Streptozotocin was originally identified in the late 1950s as an antibiotic. The drug was discovered in a strain of the soil microbe *Streptomyces achromogenes*. The soil sample in which the microbe turned up had been taken from Blue Rapids, Kansas, which can therefore be considered the birthplace of streptozotocin.

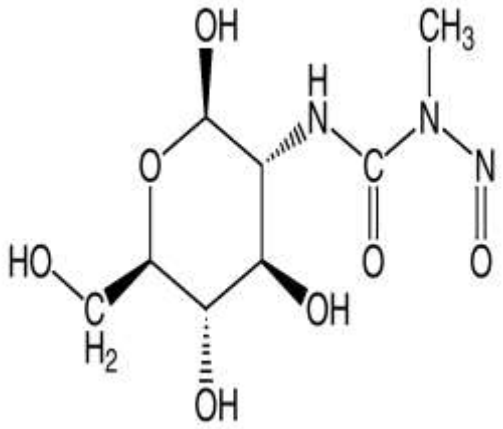


Fig. No.8 2-deoxy-2-{[methyl (nitroso) amino] carbonyl} amino)--D-glucopyranose

MECHANISAM OF STREPTOZOTOCIN:

STZ is a broad-spectrum antibiotic that is toxic to the insulin producing *β* cells of pancreatic islets. It is currently used clinically for the treatment of metastatic islet cell carcinoma of the pancreas and has been used investigationally in a wide variety of large and small animal species. The method of STZ action in *β* cell depletion has been studied extensively over the years. Streptozotocin prevents DNA (Deoxyribonucleic acid) synthesis in mammalian and bacterial cells, in the bacterial cells; it renders special reaction with cytosine groups, resulting in degeneration and destruction of DNA. The streptozotocin enters the pancreatic cell via a glucose transporter-GLUT2 (Glucose transporter 2) and causes alkylation of DNA. Further STZ induces activation of poly adenosine diphoshate ribosylation and nitric oxide release, as a result of STZ action, pancreatic -cells are destroyed by necrosis and finally induced insulin dependent diabetes[88-89].

Dithizone:

Dithizone induced the symptoms of diabetes in cats, rabbits, golden hamsters and in mice. In dithizonised diabetic animals, the levels of serum zinc, iron, and potassium were found to be higher than normal but copper and magnesium levels were unchanged. After treatment with insulin, most of these serum levels were normal, except for serum potassium and magnesium[90].

Mehanism of Dithizone:

Zinc-chelating agent such as dithizone is causes diabetes in laboratory animals. Dithizone has abilities to permeate membranes and to complex zinc inside liposomes with the release of protons, that can enhance diabetogenicity. When such complexing agents are added to lipid vesicles at pH 6 containing entrapped zinc ions, they acidify the contents of these vesicles. Such proton release occurs within the zinc-containing insulin storage granules of pancreatic beta-cells; solubilisation of insulin would be induced which leads to osmotic stress and eventually the granule rupture and finally diabetes is induced[91].

Gold thioglucose:

Gold thioglucose is diabetogenic compound, which is induced hyperphagia and severe obesity induced Type -2 diabetes.

Mechanism of Action Gold thioglucose:

Developed obesity induces diabetes in genetically normal mouse strains. Gold thioglucose treated DBA/2 (Dilute Brown Non- Agouti), C57BLKs, and BDF1 mice gained weight rapidly and significantly increase non fasting plasma glucose level within 812 weeks. These mice showed impaired insulin secretion, mainly in early phase after glucose load and reduced insulin content in pancreatic islets[92].

Monosodium glutamate:

Monosodium glutamate induces Type -2 diabetes without polyphagia.

Mechanism of Action:

Monosodium glutamate causes a very large insulin response after ingestion. It is developed glycosuria in both male and female mice but not induced polyphagia. Within 29 weeks level of glucose concentration in blood, total cholesterol and triglyceride were higher[93].

Other diabetogenic agents:

1. Dehydroascorbic acid 650/kg for three days in rat
2. Dehydroascorbic acid 1.5 mg/kg in rat
3. Dehydroglucoascarbic acid 3.5-3.9 gm/kg in rat
4. Methyl alloxan 53mg/kg in rat
5. Ethyl alloxan 53-130 mg/kg in rat
6. Oxime and dithizone 53 mg/kg in rabbit
7. Sodium diethyldithiocarbonate 0.5-1 gm/kg in rabbit
8. Pottasium xanthate 200-350 mg/kg in rabbit
9. Uric acid 1 gm/kg in rabbit

2.12 PREGABALINE:

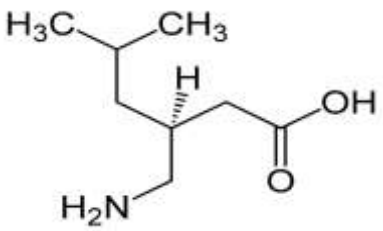


Fig. No. 9 Pregabalin,(S)-3-(aminomethyl)-5-methylhexanoic acid,

is a pharmacologically active S-enantiomer of a racemic 3- isobutyl gamma amino butyric acid analogue. It is a well-established anticonvulsant and analgesic agent. In fact Pregabalin is the first drug to receive an approved labelling from Food and Drug Association (FDA) for the treatment of diabetic neuropathy and post herpatic neuralgeia. The major advantage of pregabalin is its relative reliability, easy use and high tolerance in patients with neuropathic pain. Pregabalin is structurally related to the antiepileptic drug gabapentin and the site of action of both drugs is similar, the alpha2-delta (alpha2-delta) protein, an auxiliary subunit of voltage gated calcium channels.

Pregabalin, marketed under the brand name Lyrica among others, is a medication used to treat epilepsy, neuropathic pain, fibromyalgia, and generalized anxiety disorder. Its use for epilepsy is as an add-on therapy for partial seizures with or without secondary generalization in adults. Some off Label uses of pregabalin include restless leg syndrome, prevention of migraines, social anxiety disorder, and alcohol withdrawal. When used before surgery it does not appear to affect pain after surgery but may decrease the use of opioids.

MECHANISM OF PREGABALIN:

Pregabalin blocks the VGCC and hence decrease glutamate and sensory neuropeptides (substance P and CGRP) release at synapse by decreasing Ca2+ influx. EAATs (excitatory amino acid transporters) activity is increased by pregabalin which caused more decrease in synaptic availability of glutamate. Decreased glutamate levels further inhibited the activation of NMDA and decreased the neuronal firing. Additionally pregabalin also activates the KATP channels, which also contributes to inhibition of neuronal excitation. Pregabalin through all these pathways ultimately provides significant pain relief in various neuropathic pain states[94].

LITERATURE REVIEW OF DIABETIC NEUROPATHY:

95. Faisal Mohd et al., reported "The Pharmacological Evaluation of *Epigallocatechin-3-Gallate* (EGCG) Against Diabetic Neuropathy in Wistar rats". The observation of this indicates that hyperglycemia and oxidative stress is responsible for the development of diabetic neuropathy. Protective effect of EGCG against diabetic neuropathy could be due to controlling hyperglycemia and reducing oxidative stress. This could provide a rational for the use of Epigallocatechin-3-gallate in fever folk medicine.

96. "Evaluation of Neuroprotective Effect of *Ficus benghalensis* against alloxan induced diabetic neuropathy in rats". This study has revealed that oral administration of Methanolic extract of *Ficusbenghalensis* leaves not only attenuated the alloxan induced diabetic condition but also reversed the neuropathic pain. The probable mechanism could be via enhancing insulin

production and decreasing the glucagon production. The onset of neuropathic complications could be prevented by early gly cemiccontrol and it was reported by Stalin et al.,

97. Alsharari et al., revealed the “Ameliorative potential of *morin* in streptozotocin induced neuropathic pain in rats”. The present study is about*morin* ameliorative hyperglycemia induced mechanical or thermal hyperalgesia and lowers neuropathic pain by reducing oxidative stress in the nerve of diabetic rats by virtue of its antioxidative and antiinflammatory properties. Morphological assessments show that the sciatic nerve is also markedly reduced by the administration of *morin*. These findings suggest that *morin*traetment might be beneficial in chronic diabetics exhibiting neuropathy.

98. “Effects of Ethyl Acetate Fraction of *Ziziphus mauritiana*Lam. Leaves on STZ induced diabetes and diabetic neuropathy in mice”. The study indicates that STZ induced diabetic mice shows elevated blood glucose level and reduced pain threshold in thermal hyperalgesia and cold allodynia, and also reduced motorinco-cordination. Ethyl Aceteate fraction of Methanolic extract of *Ziziphusmaurititana* Lam. leaves has Antidiabetic activity and also protective against diabetic neuropathy in STZ induced diabetic mice was studied by Mohammed Mubashir et al.,

99. Amit Kumar Gangwar et al., reported “Investigation of Neuroprotective Effect of *Rasagiline* in diabetic neuropathy in Streptozotocin induced Type-2 diabetic rats”.This study indicates *Rasagline* combine with

glimiperide significant reduce in symptoms of diabetic neuropathy in a dose dependent amnner.

100. Parkar N &Addepalli V et al., studiedNobiletin on Diabetic Neuropathy in Experimental rats. This study concluded t hat treatment with nobiletin ameliorated diabetic neuropathy in STZ rats which was evident by improved nociceptive latency and nerve conduction velocity. Thus the results of the study lead to suggest the protective role of nobiletin in STZ induced diabetic neuropathy.

101. Neuroprotective effects of *Gymnema sylvestreon*streptozotocin-induced diabetic neuropathy in rats. The present study demonstrate the ameliorative properties of *GS*extract against chemically-induced DN in rats via its antihyperglycemic, antioxidative and anti-inflammatory properties. Furthermore, morphological examinations indicate that the damage caused to the sciatic nerve by STZ was markedly reduced following the administration of *GS*. Therefore, *GS* extract application may be useful for the treatment of neuropathy in patients with chronic diabetes was revealed by Amal Jamil Fatani et al.,

102. “Effect Of *CocciniaIndica* Leaf Extract On Diabetic Neuropathy Pain In Rats”. This study indicates that freshly prepared *Coccinia indica* attenuates hyperglycemia and diabetic neuropathy in STZ-induced diabetic rat, due to antihyperglycemic and analgesic activity. We also showed that higher dose of *Coccinia indica* plant extracts (500 mg/kg/day) have more

blood glucose lowering effects when treatment started one week after the STZ injection, It was proved by Ravikant et al.,

103. Sandeeep Kumar K et al., Experimentally studied the "Neuroprotective effect of ethanolic root extract of *Boerhaavia diffusa* (Linn.) against Streptozotocin induced Diabetic neuropathy in animal model". The present study has shown that, the ethanol extract of *B. diffusa* root have attenuated the STZ induced diabetic neuropathy in rats. These effects may be attributed to its potential antidiabetic, anti-oxidant and calcium channel blocking properties.

104. Pratibha D Nadig et al., reported "Effect of *Tinospora cordifolia* on experimental diabetic neuropathy". The *invitro* effect of TC on aldose reductase inhibition was observed. It showed an inhibitory effect although the IC50 was higher as compared to the standard (quercetin). Thus, the beneficial effect of TC on diabetic neuropathy appears to be due to its analgesic effect and unrelated to its antihyperglycemic effect. Aldose reductase inhibition may contribute to this action. An antioxidant role of TC cannot be ruled out as oxidative damage contributes to the causation of diabetic neuropathy and TC is proven to be an antioxidant in experimental models.

105. Ravichandran Ranjithkumar et al., "Standardized Aqueous *Tribulus terristris (Nerunjil)* extract attenuates hyperalgesia in experimentally induced diabetic neuropathic pain model: Role of oxidative stress and inflammatory mediators" this study confessed that *nerunjil* attenuated the neuropathic pain through modulation of oxidative stress and inflammatory

cytokine release in diabetic states. These findings suggest that *neunjil* may potentially have clinical applications to treat neuropathic pain in diabetic animals.

106. Ramdas B. Pandharel, et al., revealed that “Attenuating effect of seeds of *Adenanthera pavonina* aqueous extract in neuropathic pain in streptozotocin-induced diabetic rats: an evidence of neuroprotective effects”. pregabalin is well documented to exert its beneficial effect in neuropathic pain via inhibition of voltage gated calcium [Cav 2.2 (a2-δ subunit)] channels and therefore, it is proposed that potential anti-oxidative and neuroprotective actions of *Adenanthera pavonina* may be an important factor in attenuating STZ-induced diabetic peripheral neuropathic pain.

107. Gowhar Ali et al., revealed that “A streptozotocin induced diabetic neuropathic pain model for static or dynamic mechanical allodynia and vulvodynia: validation using topical and systemic gabapentin”. This study confessed that outcomes of topical and systemic gabapentin in the diabetic neuropathy model of vulvodynia tend to validate it as a useful non-clinical paradigm. The complex nature of the diabetic neuropathic pain syndrome and the unpredictable rate of absorption of gabapentin from the gel formulation warrent further research to correlate any antivulvodynia activity of gabapentin gel with gabapentin penetration after topical appilcation.

108. “Neuroprotective effect of *Cerebrolysin* on Diabetic neuropathy: A study on male rats” by Naseer Zangiabadi et al., In the present study it was observed that intraperitonial injection of *cerebrolysin* is effective in the

treatment of diabetic neuropathy and can improve the function of peripheral nerves.

109. Jurairat Khongrum et al., reported "*Moringa Oleifera* leaves extract attenuates neuropathic pain induced by chronic constriction injury". The possible mechanism underlying these studies may be *M.oleifera* exert the antioxidant effect. However, these, the precise underlying mechanism still require further investigation.

110. Saeed Esmaeili-Mahani et al., "*Olive (Oleaeuropaea L.)*Leaf extract attenuates early diabetic neuropathic pain through prevention of high glucose-induced apoptosis: In vitro and in vivo studies". The study suggest that olive leaf extract rescues PC12 cells against high glucose-induced neural damage and attenuates thermal hyperalgesia in diabetic rats. The mechanisms of these effects may be due, at least in part, to reduce neuronal apoptosis. Therefore, the data suggest therapeutic potential of o live leaf extract in attenuation of diabetic neuropathic pain.

111. Roman adhikari, jyothi yet al., "Combined effect of aqueous extract of *curcuma longa* Linn With metformin in diabetes induced neuroptahic pain in rats "This study reveals Uncontrolled blood glucose level leading to hypoglycemia, oxidative stress, and end-organ complications can ultimately become fatal. A number of commercially available OHAs though are effective in controlling the blood glucose, long-term use of the higher doses reports severe side effects. Hence, herbal drugs possessing anti-diabetic and anti-oxidant properties are desired for long-term treatment in order to reduce the

dose of commonly used OHA and avoid their side effects. The basic pathophysiology of diabetes involves the destruction of β-cell or its improper functioning in secreting insulin due to unwanted metabolic changes, hence β cells become more compromised to the stimuli that demands excess insulin for the body. CUR has a property of protecting the islets β-cell, decrease the insulin resistance and decrease the oxidative stress. Hence, the use of CUR along with the OHA could be useful in preventing the complications associated with Diabetes when subjected to prolonged treatment.

112. N Premkumar et al., reported Antinociceptive property of *Emblica Officinalis* garten in high fat diet fed/low dose Streptozotocin induced diabetic neuropathy in rats. This study reveals that *E.officinalis* fruit extract induced reduction in oxidative stress secondary to decrease in blood glucose level may be responsible for ameliorting axonal degenration and neuropathy in sciatic nerve EOE has produced a pronounced antinociceptive activity from 3week onwards but not quercetin and thus it can prevent as well as cure diabetic neuropathy. The curative and preventive property of EOE in diabetic neuropathy may be due to its improvement in glucose in tolereance and antioxidant property.

113. Archana jorige et al., “Neuroprodective and antioxidant role of pregabalin in sterptozotocine induced neurotoxicity” the present study revealed that 15mg/kg pregabalin significantly prevented the amelioration of defensive antioxidant enzyme levels and reduced the lipid peroxidation in sciatic nerves of STZ induced diabetic rat model. It also prevented the abnormal structural changes of sciatic nerve in diabetic rats. There by the

present study uncovered the neuroprotective action of pregabalin in STZ induced neurotoxicity. The anti-neuropathic effect of pregabailn was not only mediated through its action on voltage gated Ca^{+2} but also through its antioxidant action by the restoration of endogenous antioxidant enzyme levels.

114. Sameer Nasikkar et al., “Protective effect of *Lagerstroemia speciosa* leaves extract against streptozotocin induced diabetic neuropathy in experimental rats”. The present data conclude that the repeated dose treatment of Lagerstroemia Speciosa leaves extract for four weeks in diabetic rats not only attenuate the hyperglycemic condition but also reversed DPN symptoms. It is a potent anti-diabetic and antioxidant, which has observed as its ameliorative effects.

115. Solanki nilay D et al., “Experimental study on *operculina turpethum* in STZ induced diabetic neuropathy, neurodegeneration and cardiovascular complications”. These studies have concluded that there is definite role of operculina turpethum in the reversal of major diabetic complications especially neuropathy and cardiovascular complication. In this study number of animals utilized was limited as per ethical apporuval, so further extrapolation of this study need to he done by using other species of animals.

116.Nasser Zangiabadi et al., “The Effect of *Angipars* on Diabetic Neuropathy in STZ Induced Diabetic Male Rats”. In this study on Behavioural, Electrophysiological, Sciatic Histological and Ultra structural Indices.

According to the obtained results in the present study and the results of previous studies injection of Anipars for two weeks, in spite of not being effective on some indices, has some positive effects on the treatment and decrease of physiologic symptoms of neuropathy in male rats. Moreover it should be mentioned that Angipars at dose of 10ml/kg has the most positive effects on some indices of diabetic neuropathy.

117. Seigo Usuki et al., “Effect of *pre-germinated brown rice* intake on diabetic neuropathy in Streptozotocin induced diabetic rats”. Pre-germinated Brown Rice or White rice treatment shows efficacy for protecting diabetic deterioration and for improving physiological parameters of diabetic neuropathy in rats, as compared with a brown rice or white rice diet. This effect may be induced by a mechanism whereby Pre-germinated brown rice intake mitigates diabetic neuropathy by one or more factors in the total lipid fraction. The active lipid fraction is able to protect t he Na^+/K^+/ATP ase of the sciatic-nerve membrane from the toxicity of homocysteine thiolactone modified low density lipoprotein and to directly activate the homocysteine ase of high density lipoprotein.

118. Thierry C et al., “Neuroprotective Effect of *Docosahexaenoic Acid–Enriched Phospholipids* in Experimental Diabetic Neuropathy”. Docosahexaenoic acid phospholipids totally prevented the decrease in nerve conduction velocity and nerve blood flow observed during diabetes when compared with the nonsupplemented diabetic group. Docosahexaenoic acid phospholipids also prevented the Na,K-ATPase activity decrease in red blood cell but not in sciatic nerve. Moreover, docosahexoaenoic level in sciatic

nerve membranes was correlated with nerve conduction velocity.These results demonstrate a protective effect of daily doses of docosahexaenoic acid on experimental diabetic neuropathy. Thus, treatment with docosahexaenoic acid phospholipids could be suitable for evaluation in clinical trials.

119. Abhishek Bhanot et al., “A comparative profile of methanol extracts of *Allium cepa Allium sativum* in diabetic neuropathy in mice”. The results of the present study show that the extract of the outer scale of onion and the edible portion of both onion and garlic provided significant production in diabetic neuropathy in both preventive and curative groups. The methanol extract of the outer scales of onion has shown a most significant effect which may be due to the presences of higher quantities of phenolic compounds.

120. Trupti C et al., “Protective effect of ethyl acetate soluble fraction of ethanolic extract of *Terminalia Chebula* Retz. fruits on diabetic neuropathy in mice”. In the present study, diabetic animals shows elevated blood glucose level, reduced pain threshold and motor incoordination. EATC treatment restored body weight, blood glucose, along with pain threshold and motor coordination in diabetic mice.

121. Huda I Abd Elhafiz et al., “The Protective Effect of *Calcitriol* on Diabetic Neuropathy in STZ-induced Diabetic Rats”. Our study shows that calcitriol improved physiologic symptoms of neuropathy in diabetic rats. Also, it should be mentioned that co administration of calcitriol with insulin has the most positive effects on some indices of diabetic neuropathy. Results of the

current study suggest that calcitriol could have a potential therapeutic role in diabetic neuropathy.

122. Naveen Kumar Gupta et al., “Neuroprotective potential of *Azadirachta indica* leaves in diabetic rats”. Neuropathic pain or hyperalgesia is a manifestation of diabetic neuropathy severs studies have reported oversentivity(feeling of excessive pain) in diabetic patients and animals. Presently diabetic animals exhibited decrease in threshold for pain perception and reported hyperalgesia. Hyperalgesia develops mainly due to local metabolic and microvascular changes in the nervous tissue.The involvement of superoxide ,peroxynitrite and hydroxyl radicals has already been well established in various animal model of hyperalgesia. Besides this the role of cytokines and inflammatory makers cannot be ignored in neuropathic pain and diabetic complications. The results of this study also revealed metabolic changes and variation in the antioxidant profile of diabetic animals. Thus alternations in the biochemical parameters seem to be akin with microangiopathic effects which suggested the development of hyperalgesia in diabetic animals. However the ALE treatment decreased reduction in neuronal inflammation, LPO, increased GSH content suggested reduction in neuronal stress levels hence attenuated the hyperalgesia. Thus in totally this study suggests the oral administration of ALE to STZ induced diabetic animals resulted in neuroproduction against degenerative oxidative stress associated with metabolic and histopathological changes.

123.G.Sumithira , A.Ashmaet al., Evaluation of Neuroprotective Effect of *PlecospermumSpinosum* Trec in Experimentally induced Diabetic

Neuropathic pain in Rats. In this study revealed that *Plecospermum Spinosum* extract was proposed that in addition to its antidiabetic, the antioxidant properties is the prominent features in attenuation of diabetes induced neuropathy and its generating pain. These findings suggest that *Plecospermum Spinosum* treatment must be beneficial to treat pain in diabetic animals.

3. PLANT DESCRIPTION

Name **:** *Ottelia alismoides (L.)* Pers.

Synonym **:** Stratiotes alismoides

Damasonium alismoides

Family **:** Hydrocharitaceae

Vernacular names[124-125]:

Common name	: Duck lettuce, Waterplantain.
Tamil name	: Nirkuliri
Malayalam	: Ottel ambel
Kannada	: Hasiru neeru paathre
Bengali	: Parmikalla
Marathi	: Olek-alsem
Telugu	: Edukula thaamara, Neeru veniki
Dutch	: Duikerbloem
Malaya	: Keladi Ayer
Spanish	: Espada,Tangila

TAXONOMY[126]:

Kingdom	: Plantae
Subkingdom	: Tracheobionta
Super division	: Spermatophyta
Division	: Magnoliophyta
Class	: Liliopsida
Subclass	: Alismatidae
Order	: Hydrocharitales
Family	: Hydrochritaceae
Genus	: Ottelia Pers
Species	:*Ottelia alismoides* (L.) Pers

CULTIVATION AND COLLECTIONS[127-132]:

It grows in shallow waters, ponds and in rice fields. It faces no major threats and is therefore listed as Least Concern**.** It does well under strong light and harder water with a rich substrate. It is an annual and grows from seed each year. CO_2 fertilisation and pH control make growing this plant in the aquarium less difficult. It grows very large in the aquarium and is best used as a feature plant. seeds may remain viable for up to four years noted that fish prefer to eat the seeds, but it is unknown how this affects germination Seeds will germinate in 25-30 °C, and germination may be influenced by light availability and burial depth, but substratum (mud or sand) and oxygen availability had no significant effect.

DESCRIPTION:

Fig No :10

Fig No :11

Fig No :12

Fig No :13

Fig No:14

Fig No:15

Habitat:

This species grows in streams, lakes, marshes, ponds, ditches, canals[14]. Depth and turbidity of water affecting the penetration of light a clayey and reducing substratum and little biotic disturbance.(Fig no : 10).

Leaves:

Leaves are extremely variable, with short or long petioles according to the depth of the water. Blades of the submerged leaves are often narrow; the floating ones, ovate or somewhat rounded, with a rounded or often heart shaped base, thin and translucent 5 to 20 centimeters long. (Fig no : 11).

Flowers:

Flowers are white about 2cm in length Inflorescences 1-flowered; spathes 3-10 winged. Flowers sepals 10-15, stamens 3-12; ovary 1, 3-9 carpellate. wrapped within spathes, cylindrical structures 2-4 cm long, composed of green bracts that are ornamented with 3 or more ruffled wings. Spathes born on long, angled stalks that become spiraled after flowering. Sepals and short-lived petals of male flowers exert from the tip of the spathe just above the water surface. Spathes containing female and/or bisexual flowers are self fertile and remain submersed. Petals white, pink, blue or purple, often tinged with yellow at the base. Flowering period autumn to spring. (Fig no : 12).

Fruit:

Fruit is oblong, 2.5 to 4 centimeters long, ovoid to cylindrical and Fleshy encapsulated fruits contain as many as 2000 seeds Fruit with numerous seeds(Fig no : 13).

Seeds:

Seeds densely covered with whitish, unicellular hairs. Seeds fusiform 1–2 mm long, 0.3–0.7 mm in diameter, with 2 faint slightly curved longitudinal ridges. Testa light brown, dull to semi glossy, faintly wrinkled, with a tuft of unicellular white hairs. Hilum inconspicuous. Embryo linear-spatulate endosperm absent.(Fig no : 14).

Stems & Roots:

Stem small and corm-like, occasionally forked, with fibrous roots .The roots in the sediment in water to 2 feet deep and has short. (Fig no : 15).

DISTRIBUTION[133-134]:

Ottelia alismoides (L.) pers. is Widely distributed in northeastern Africa, South Korea, eastern and southeast Asia and the tropical regions of Australia, Bangladesh. It is introduced in United states china (Anhui, Fujian, Guangdong, Guangxi, Guizhou, Hainan, Hebei, Heilongjiang, Henan, Jiangsu, Jiangi, Jilin, Liaoning, Sichuan, Yunnan, Zhejiang),Egypt, Hong Kong, India, Srilanka. It has been reported from many countries across tropical and subtropical Asia and Australia and covers a wide geographic range. It is found

throughout India in tanks, ponds, streams and ditches. It was identified in several places like the aquatic herb at the back waters of Madhuban Dam near Dudhani, Silvassa region and Kunnathur tank Madurai. The plant was identified in the location of Agaram village kudapakkam villianur Pondicherry.

PHYTOCHEMISTRY[135-139]:

Phytochemical present in this genus contain different natural compounds like glycosides, Alkaloids, Flavonoids, Terpenoids, Tannins, Phenolic compounds. Present knowledge about this endangered species of medicinal plant is still limited with respect to its phytochemistry and biological activity. Major chemical constituents of *Ottelia alismoides* Studies revealed that isolated two diastereomeric 4-methylene-2-cyclohexenones, otteliones A and B, ten new diarylheptanoids (2,3,4,5a-d,6,7&8) together with a hydroxylated analog of otteliones A and B and 3a-hydroxyottelione.

ETHANOBOTANICAL AND MEDICINAL USES[140]:

The plants are used to improve the water quality in fish ponds by capturing floting mud particles. The petioles and leaves are eaten as a vegetable with excellent flavour, the leaves are used in Thailand for seasoning rice. The fruit is also edible. The plant is used in the treatment of haemorrhoids and applied as the poultice against fever. It is also grown as an aquarium plant

Table:1 Summarises the traditional use of different parts of the plant for various ailments.

Plants part	Uses/ailments
Leaves	1. Applied on poultice arm & leg in fever, Topical for haemorrhoids. 2. Decoction of ground leaves mixed with shoots of ipomoea aquatic, alium sativum, lasia spinosa, ocimum sanctum and Typha angustata for the treatment of pneumonia. 3. It is used as a rubefacient. 4. It is used as the treatment of stomach disorder. 5. It is used as spice for the rice and vegetables. 6. Leaves used to check bleeding.
Flowers	1. Flower paste 10-12 gram is taken orally early in morning to curing piles. 2. Local people eat lower part of the flower as raw or as vegetable.
Fruit	1. Arresting cough and inducing diuresis. 2. Fruit eaten as raw.
Whole plant	1. It is a promising drug raw material having anti-tuberculosis activity. 2. Plant prepared as paste and applied to abscesses of the breasts cancer, ulcers and burns. 3. It is also used for treatment of Asthma and applied externally for skin diseases. 4. Clearing away from heat. 5. Eliminating phlegm 6. It is used for the treatment of diabetes.

Earlier work done on *Ottelia alismoides (L.)* Pers.,

1. Li H, Qu X, Zhao D et al., Preliminary study on the anti-tubercular effect of Ottelia alismoides (L.) Pers. In this study a clinical trials, extract of *Ottelia alismoides* cured two cases of bilateral tuberculosis of cervical lymph glands within 3 months. Results suggest *Ottelia alismoides* to be a promising medicinal herb with anti-tubercular effect.

2. Seif-Eldin, Ayyad N et al., *Ottelione*s A and B: potently Cytotoxic 4-Methylene-2-cyclohexenones from *Ottelia alismoides*. In this study Isolated Ottelione A the natural products showed remarkable *in vitro* cytotoxicity against various cancer cell lines

3. Tsai-Yuan Chang, Yun-Peng Tu et al., Synthesis and Antiproliferative Activities of Ottelione A Analogues. In this study Ottelione A, isolated from the fresh water plant Ottelia alismoides, is among the most potent natural product that possess in vitro antiproliferative activity, with an IC50 in the pM-nM range against 60 human cancer cell lines. Study established the relationship of antimitotic ottelione against tubulin and various cancer cell lines.

4. SCOPE OF THE PRESENT STUDY

In the present situation, diabetes is possibly the world's largest growing metabolic disorders and as the knowledge on the heritogeneity of this disorder is advanced, the need for more appropriate therapy increases. People with diabetes can over time develop nerve damage throughout the body. About 60-70 % of people with diabetes have neuropathy. A number of allopathic drugs are used for the diabetic neuropathy effect like Aceclofenac, Aspirin, Gabapentin, Paroxetine and Imipramine which have danger of drug interaction, adverse effects etc. Traditional plant medicines are used throughout the world for a range of diabetic neuropathy presentations. There are many medicinal plants known to be used in the treatment of diabetic neuropathy and a number of plants had been screened positively for their Diabetic neuropathy effect. Most of these plants were found to belong to the chemical group glycosides, alkaloids and Flavanoids and Tannins.

Ottelia alismoides(L.) PERS whole plant is having the Diabetic neuropathy activity. As per the literature review, still no Diabetic neuropathy activity has been reported on this plant. Hence, this study has been taken to explore the Diabetic neuropathy activity of *Ottelia alismoides.* In Streptozotocin induced diabetes in Wistar albino rats.

5. AIM AND OBJECTIVES

Aim

The present investigation was aimed to screen the Diabetic neuropathy activity of *Ottelia alismoides (L.)* PERS on Streptozotocin induced Diabetic Wistar albino rats.

Objectives

The objective of the present study to:

a. Find out the phytochemical constituents present in the ethanolic extract of the whole plant of *Ottelia alismoides (L.)* PERS

b. Acute toxicity studies

c. Evaluation of anti-diabetic activity

- Blood glucose level
- Body Weight
- Organ Weight (Liver and Kidney)

d. Estimation of Antioxidant parameters

- Superoxide dismutase level (SOD)
- Catalase level (CAT)
- Reduced glutathione (GSH)
- Glutathione peroxidase (GPx)

Other parameter:

- Na+ ,K+, ATPase acivity

e. Estimation of Oxidative stress parameters

- Lipid peroxidation (LPO)

f. Estimation of Behavioral assessment parameters

- Hot plate (Thermal Hyperalgesia)
- Cold plate (cold allodynia)
- Tail immersion (Thermal hyperalgesia)
- Tail clip (Mechanical hyperalgesia)
- Tail Flick (Thermal hyperalgesia)
- Formalin (Chemical hyperalgesia)

g. Histopathological studies of sciatic nerve

h. Statistical analysis

6. PLAN OF WORK

1. Collection of plant.
2. Authentication of plant, shade drying of the Whole plant.
3. Extraction of plant materials with solvents.
4. Preliminary phytochemical screening of Ethanolic extract of Whole plant of *Ottelia alismoides (L.)* PERS
5. Evaluation of acute toxicity studies to determine LD_{50} value
6. Evaluation of anti-diabetic activity.
7. Estimation of Nociceptive threshold parameters.
8. Histopathological study of sciatic nerve.
9. Statistical analysis

7. MATERIALS AND METHODS

7.1 PLANT MATERIALS:

7.1.1 Collection and authentication, shade drying and granulation of plant material

The whole plant *Ottelia alismoides (L)* PERS., were collected in the month of February from Agaram Village Kudapakkam Villianur Pondicherry. The plant material was taxonomically identified and authenticated by Dr.N.Ayyapan Researcher, French Institute of Pondicherry, Pondicherry.

7.2 PREPARATION OF *OTTELIA ALISMOIDES* ETHANOLIC EXTRACT

The whole plant of *Ottelia alismoides(L)* PERS. were dried under shade and then powdered with a mechanical grinder. The powder was passed through sieve No:40 and stored in an airtight container for further use.

7.2.1 Method of Extraction

Soxhlet extraction:

Soxhlet extraction is the process of continuous extraction in which the same solvent can be circulated through the extractor for several times. This process involves in the extraction followed by evaporation of the solvent. The vapours of the solvent are taken to a condenser and the condensed liquid is returned to the drug for continuous extraction. Soxhlet apparatus designed for such continuous extraction consists of a body of extractor attached with a side tube and siphon tube. The lower of the extractor is attached to distillation

flask and the mouth of extractor is fixed to a condenser by the standard joints. The powdered crude drug is packed in the soxhlet apparatus directly or in a thimble of filter paper or fine muslin cloth. The diameter of the thimble corresponds to the internal diameter of the soxhlet extractor.

7.2.2 Chemicals and preparation of drug solutions:

Streptozotocin was obtained from Sisco research laboratories Pvt. Ltd, Mumbai, India and Pregabalin was perchased from Swapnaroop drugs & pharmaceuticals, Aurangabad, Maharastra, India. All otherchemicals and reagents used were of analytical grade.

7.3 PRELIMINARY PHYTOCHEMICAL SCREENING

Qualitative Phytochemical Analysis[141]

The Ethanolic extract of *Ottelia alismoides* was analyzed for the presence of various phytochemical constituents.

A.CARBOHYDRATE TESTS

1.Molisch's test:

To the test solution, few drops of α-naphthol was added, then few drops of concentrated sulphuric acid was added through the sides of test tube, purple to violet colour ring appeared at the junction, indicates the presence of carbohydrates.

2.Fehling's test:

To the test solution, equal quantity of Fehling's A and B were added and heated on water bath, brick red precipitate was formed, indicates the presence of carbohydrates.

3.Benedict's test:

To the test solution, 5ml of Benedict's reagent was added and heated on water bath, red precipitate was formed, indicates the presence of carbohydrates.

4.Barfoed's test:

To 1 ml of the test solution, add 1ml of Barfoed's reagent was added and heated on a water bath, red cupric oxide was formed, presence of monosaccharide.

5.Test for pentoses:

To the test solution, equal volume of hydrochloric acid and phloroglucinol was added and heated, no red colour was produced, indicates the absence of pentoses.

6.Selivanoff's test (test for ketones):

To the test solution, crystals of resorcinol and equal volume of concentrated hydrochloric acid were added and heated on a water bath, rose colour was produced, indicates the presence of ketones.

B.ALKALOIDS

1.Dragendroff's test:

To 1ml of the extract, Dragendroff's reagent (potassium bismuth iodine solution) was added, no reddish brown precipitate was formed, indicates the absence of alkaloids.

2.Wagner's test:

To 1ml of the extract, Wagner's reagent (iodine potassium iodide solution) was added, no reddish brown precipitate was formed, indicated the absence of alkaloids.

3.Mayer's test:

To 1 ml of the extract, Mayer's reagent (potassium mercuric iodine solution) was added, no cream colour precipitate was formed, indicates the absence of alkaloids.

4.Hager's test:

To 1 ml of the extract, Hager's reagent (saturated aqueous solution of picric acid) was added, no yellow coloured precipitate was formed, indicates the absence of alkaloids.

5.Tannic acid test:

To the extract tannic acid solution was added, no buff colour precipitate was produced, indicates the absence of alkaloids.

C) GLYCOSIDE TEST

1.Legal's test:

The extract in pyridine and sodium nitroprusside solution was added to make it alkaline, formation of pink to red colour showed the presence of glycosides.

2.Baljet's test:

To 1 ml of the extract, 1 ml of sodium picrate solution was added and the change of yellow to orange colour reveals the presence of glycosides.

3.Borntrager's test:

Few ml of dilute sulphuric acid was added to 1 ml of the extract solution. Boiled and filtered and then the filtrate was extracted with chloroform. The chloroform layer was treated with 1 ml of ammonia. The formation of red colour of the ammonical layer showed the presence of anthraquinone glycosides.

4.Keller killani test:

1. 1gm of powdered drug was extracted with 10ml of 70% alcohol for 2 minutes, filtered, the filtrate was added to 10 ml of water and 0.5 ml of strong solution of lead acetate and filter, and the filtrate was shaken with 5ml of chloroform.

2. The chloroform layer was separated in a porcelain dish and the solvent was removed by gentle evaporation. The cooled residue was dissolved in 3 ml of glacial acetic acid containing 2 drops of 5% ferric chloride solution.

The solution was carefully transferred to the surface of 2ml of concentrated sulphuric acid. A reddish brown layer formed at the junction of the two liquids and the upper layer slowly became bluish green, darkening with standing.

D.TEST FOR SAPONINS

1. Foam test:

Small quantity of alcoholic extract was taken and 20ml of distilled water was added and shaked in a graduated cylinder for 15 minutes length wise. No Layer of foam, indicates the absence of saponins.

E. TEST FOR FLAVANOIDS

1. Shinoda's test:

To the test solution, few magnesium turnings were added and concentrated hydrochloric acid was added drop wise, pink scarlet, crimson red or occasionally green to blue colour appeared after few minutes indicating the presence of flavanoids.

2. **Alkaline reagent test:**

To the test solution, few drops of sodium hydroxide solution was added, intense yellow colour was formed which turned colourless on addition of few drops of dilute acid indicating presence of flavanoids.

3. **Zinc hydrochloride test:**

To the test solution, a mixture of zinc dust and concentrated hydrochloric acid were added. It gave red colour after few minutes showing the presence of flavanoids.

F. **TEST FOR TANNINS**

1. Ferric chloride test:

To the test solution, ferric chloride solution was added, green colour appeared showing the presence of condensed tannins.

2. Phenazone test:

To the test solution, 0.5 grams of sodium phosphate was added, warmed and filtered. To the filtrate 2% phenazone solution was added, bulky precipitate was formed which was often coloured, indicating the presence of tannins.

3. Gelatin test:

To the test solution, 1% gelatine solution containing 10% sodium chloride was added. Precipitate was formed, indicating the presence of tannins.

4. Test for Catechin:

Match stick was dipped in the test solution, it was dried and lastly moistened with concentrated hydrochloric acid. Then the stick was warmed near to flame. The colour of the wood changed to pink due to phloroglucinol. (Phloroglucinol was formed when catechins were treated with acids), indicating the presence of tannins.

7.4 ACUTE TOXICITY STUDY

Organisation for Economic co-operation and Development (OECD) regulates guidelines for oral acute toxicity study. It is an international organisation which works with the aim of reducing both the number of animals and the level of pain associated with acute toxicity testing. To determine the acute oral toxicity OECD frames the following guideline methods.

OECD 401 – Acute Oral Toxicity

OECD 420 – Acute Oral Toxicity: Fixed Dose procedure

OECD 423 –Acute Oral Toxicity: Acute Toxic Classic method

OECD 425 – Acute Oral Toxicity: Up and own Procedure

In the present study the acute oral toxicity of *Ottelia alismoides* was carried out according to OECD 423 guideline (Acute Oral Toxicity: acute Toxic Classic Method).

ANIMALS:

Male Swiss albino mice weighing between 20 – 25 gm was used for the study. The animals were obtained from animal house, Nandha College of Pharmacy, Erode. The animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24±2^{o}C and relative humidity of 30 – 70 %. A 12:12 light: day cycle was followed. All animals were allowed to free access to water and fed with standard commercial pelleted rat chaw. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethics Committee of Nandha College of Pharmacy. (IAEC No: NCP/IAEC 2016-17-18) and were in accordance with the guidelines of the CPCSEA.

Acute Toxicity Studies:

Acute toxicity studies were performed according to OECD-423 (Organization of Economic and Cooperation Development) guidelines. Male Swiss mice selected by random sampling technique were employed in this study. The animals were fasted for 4h with free access to water. The Ethanolic extract of *Ottelia alismoides (EEOA)* was administered orally at a dose of 5 mg/kg initially and mortality if any was observed for first 24 hrs and after 72 hrs. If mortality was observed in two out of three animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in only one animal out of three animals then the same dose was repeated again to confirm the toxic effect. If no mortality was observed,

then higher (50, 300, 2000 mg/kg) doses of the plant extracts were employed for further toxicity studies.

OBSERVATION:

The following general behaviour was also observed during the acute toxicity study (Ecobichon DJ, 1997)[142].

1. Sedative
2. Hypnotics
3. Convulsion
4. Ptosis
5. Analgesia
6. Stupar reaction
7. Motor activity
8. Muscle relaxant
9. Pilo erection
10. Change in skin colour
11. Lacrimal secretion
12. Stool Consistency

Result:

There was no mortality with the extract of *Ottelia alismoides* after 24 & 72 hrs even at (2000mg/kg). The extract did not alter the general behaviour in mice. No lethality or toxic reactions were found during and after the study period.

7.5 EXPERIMENTAL INDUCTION OF DIABETES

Sterptozotocin Induced Diabetes:

A freshly prepared solution of STZ (55 mg/kg in 0.01 M citrate buffer, pH 4.5) was injected intraperitoneally to overnight-fasted rats. The rats exhibited hyperglycaemia within 48 h of STZ administration. The rats having fasting blood glucose (FBG) values of 250 mg/dl or above were considered for the study. The study of test compound and standard drugs were dissolved in distilled water and administered orally with the help of gastric oral tube.

7.5.1 EXPERIMENTAL DESIGN

The Diabetic Neuropathy activity was tested on a total of 30 rats (24 diabetic rats and 6 normal rats) and they were divided into five groups and each group consists of 6 animals as follows,

Group I - Diabetic control received Streptozotocin injection (55mg/kg; b.wt; i.p), freshly prepared in citrate buffer.

Group II- Streptozotocin injection (55mg/kg; b.wt; i.p) + insulin (5 IU/kg, i.m).

Group III- STZ+insulin+ Standard drug Pregabalin (15mg/kg) dissolved in 0.5% CMC.

Group IV- STZ+insulin+ plant extract low dose (200mg/kg, b.wt; p.o) suspended in 0.5% CMC.

Group V- STZ+insulin+ plant extract high dose (400mg/kg, b.wt; p.o) suspended in 0.5% CMC.

In the first four weeks of the study all the groups were left untreated. From week 4 onwards the drug treatment started and continued up to the week 8 after STZ injection. Body weight and Behavioural assessments like thermal hyperalgesia (hot plate, tail flick & Tail immersion), Mechanical hyperalgesia (Tail clip) thermal allodynia (cold plate) & (chemical hyperalgesia) formalin were performed on week 0^{th}, 4^{th}, 6^{th} & 8^{th} of the study. At the end of 8^{th} week the blood was collected by retro-orbital sinus puncture, rats were sacrificed by cervical dislocation organs collected and isolated sciatic nerves were analyzed for following Biochemical and histopathological study.

Blood Sample and Organs Collection:

The blood was collected by retro orbital under mild ether anaesthesia from overnight fasted rats, into tubes containing potassium oxalate and sodium fluoride as anticoagulant for estimation of fasting plasma glucose. Plasma and serum were separated by centrifugation. After centrifugation at 2,000 rpm for 10 minutes, the clear supernatant was u sed for the analysis of various biochemical parameters. After collection of blood, all the treated animals were sacrificed the Sciatic nerve and liver tissues were isolated and rinsed in ice- cold saline and kept in formalin solution (10%) for further histopathological studies & oxidative stress parameters.

Nerve collection and preparation of homogenate:

After 8 weeks of experiment, sciatic nerve was removed bilaterally from the inguinal ligament to its trifurcation and incubated in triton x 100 for 20 min to remove blood from sciatic nerve. Sciatic nerves were chopped into small pieces and then homogenized with phosphate buffer solution (pH 7.0) by using homogenizer. The homogenate was then centrifuged in a cold centrifuge (Thermo Scientific) at 4ºC, 4000 x g for 10 min. The resulting homogenate was passed through a cellulose filter to remove impurities and was aliquoted for the measurement of SOD, GSH, CAT, GPx, LPO, Na^+, K^+, ATPase activity.

7.6 EVALUATION OF PARAMETERS[143]

7.6.1 General parameters:

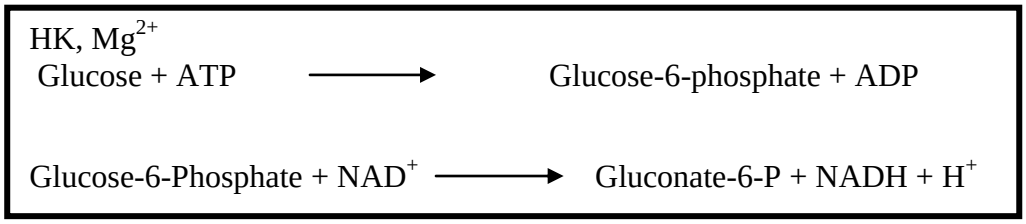
Body weight and Organ weight:

The body weight of rats was checked using a top loader weighing balance. The body weight is expressed in grams. The vital organs such as kidney and liver were collected. Blood was removed from the organs with the filter paper and their weights were assessed with an electric weighing balance. The organ weights were expressed in grams or milligrams.

7.7 EVALUATION OF BIOCHEMICAL PARAMETERS:

7.7.1 Estimation of blood glucose level:

The Blood glucose levels were estimated by Hexokinase method[144]. Glucose is phosphorylated by hexokinase (HK) in the presence of adenosine triphosphate (ATP) and magnesium ions to produce glucose-6- phosphate and adenosine diphosphate (ADP). Glucose-6-phosphate dehydrogenase (G6P-DH) specifically oxidises glucose-6-phosphate to gluconate-6-phosphate with the concurrent reduction of NAD^+ to NADH. The increase in absorbance at 340nm is proportional to the glucose concentration in the sample.



Reagents

Reagent 1:

- ✓ 0.05 M Tris HCl buffer, pH 8.0 with 13.3 mM $MgCl_2$
- ✓ 0.67 M Glucose in Tris-$MgCl_2$ buffer
- ✓ 16.5 mM ATP in Tris-$MgCl_2$ buffer
- ✓ 6.8 mM NAD in Tris-$MgCl_2$ buffer

Reagent 1A:

- ✓ 300 IU/ml Glucose-6-phosphate dehydrogenase in Tris-$MgCl_2$ buffer
- ✓ 300 IU/ml Hexokinase in Tris-$MgCl_2$ buffer

Procedure:

150 µl of reagent 1 was added with 30 µl of reagent 1A and to this 20 µl of suitable diluents was added and the contents were mixed thoroughly. To this mixture, 2 µl of serum sample was added. Then the contents were mixed and incubated at 37°C for 10 seconds. After zeroing the instrument with blank the absorbance of standard followed by the test sample was measured at 340 nm. The values were expressed as mg/dl.

7.8 EVALUATION OF NOCICEPTIVE PARAMETERS:

7.8.1 Tail Flick Method:

The tail flick test is a test of the pain response in animals, similar to the hot plate test. It is used in basic pain research and to measure the effectiveness of analgesics, by observing the reaction to heat. The quantitative measurements of pain threshold in animal against thermal radiation and for evaluation of analgesic activity. It was first described by D' Amour and Smith in 1941[145-146].

PROCEDURE:

The nociceptive response was evaluated regarding the latency to withdrawal of the tail in response to noxious radiant heating. Animals are

placed into individual restraining cages leaving the tail hanging out freely. The animals are allowed to adapt to the cages for 30 min before testing. The apparatus used is tail flick analgesiometer, the tip of tail of rat is placed on hot metal wire and latency of withdrawal is calculated manually by stop watch[R].

7.8.2 Tail Clip Method:

The method was described by Haffner[147].

PROCEDURE:

Six screened Wister rats were assigned to each of the 5 groups. The drug was administered 30 min prior testing. An artery clip was applied to the root of the tail (approximately 1 cm from the body) to induce pain. The animal quickly responds to this noxious stimuli by biting the clip or the tail near the location of the clip. The time between stimulation onset and response was measured by a stopwatch in 1/10 seconds increments. The length of time until response indicates the period of greatest activity after dosing [Schleyerbach 2002] in different period of treatments was recorded. The values of prolongation of latency time of experimental groups were compared with control group for statistical analysis.

7.8.3 Hot Plate Method:

The method originally described by Woolfe and MacDonald[148] (1944) .The paws of the rats are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping, withdrawal of the paws and

licking of the paws. The time until these responses occur is prolonged after administration of centrally acting analgesics.

PROCEDURE:

In this hot plate method animals from the each group were placed on the hot plate (Eddy′s hot plate) which is commercially available consists of an electrically heated surface. Temperature of this hot plate is maintained at 55° C- 56° C. This can be a copper plate or a heated glass surface. The observation is done up to the time until paw licking or jumping was noted the cut- off time was 10 sec. The reaction time was noted after the oral administration of the drugs and test compounds.

7.8.4 Formalin Test:

The formalin test in rats has been proposed as a chronic pain model which is sensitive to centrally active analgesic agents by Dubuisson and Dennis[149-150](1977).The formalin test uses a 10% formalin solution as a chemical noxious stimulus. By injecting the formalin solution into the paw of a rat, a model of persistent (chronic) pain caused by peripheral tissue injuries and inflammation is created.

PROCEDURE

At the end of the study, all the groups were subjected to the formalin test. Briefly, each rat was acclimatized to the observation box before the formalin test. After an adaptation period of 15 min, the right hind foot paw was injected with 50 mL of 2.5% formalin in the intraplantar region. Nociception

was evaluated by quantifying paw licking time during the first 10 min (acute phase) and at 20– 40 min (delayed phase) (Khalilzadeh et al., 2008; Luiz et al., 2007)

7.8.5 Cold Plate Method:

A cold plate apparatus was designed to test the responses of unrestrained rats to low temperature stimulation of the plantar aspect of the paw. At plate temperatures of 10 degrees C and 5 degrees C, rats with either chronic constriction injury (CCI) of the sciatic nerve or complete Freund's adjuvant (CFA) induced inflammation of the hind paw displayed a stereotyped behavior. Brisk lifts of the treated hind paw were recorded, while no evidence of other nociceptive behaviors could be discerned[151].

Procedure:

Cold-plate tests were conducted to evaluate the hyperalgesic responses to cold stimuli. For this test, the hot/cold-plate test device (Ugo-basile, 35100, Verase, Italy) was used. After setting the aluminium plate to 4 1C71 1C, the rats were placed in the Plexiglas compartment. The time that elapsed between placing the animals in the device and the time point that they quickly withdrew their paws was recorded (Rosellini et al., 2012). The steps taken by the animals when walking or the slow paw-withdrawal behavior related to locomotion were not considered (Ortega-Álvaro et al., 2012). Measurements were repeated three times for each rat, and the average of these three values was calculated (Rosellini et al., 2012). To avoid paw damage, the experiment was not conducted for more than 30 s.

7.8.6 Tail Immersion Method:

Hot and cold water Tail Immersion Test

This method was described by Sharma et al., 2006a[152].

Procedure:

The procedure was based on the reaction time of the typical tail-withdrawal reflex in rats induced by immersing the end of the tail in warm water of 52.5±1ºC and cold water of 10±0.5ºC. The lower portion of the tail, nearly 5 cm from tip, was marked and immersed in a beaker of freshly filled warm water and cold water. The rats reacted by withdrawing the tail in a few seconds. This reaction was measured two to three times, with an interval of at least 15min, in order to obtain two consecutive values that differed no more than 10%. After each determination, the tail was carefully dried. The cut-off time for warm water tail immersion was 15 s and cold water tail immersion 30s.

7.9 EVALUATION OF ANTIOXIDANTS PARAMETERS:

7.9.1 Estimation Superoxide dismutase(SOD)

This enzyme catalyzes the dismutation of superoxide anion ($O2^{-}$) to hydrogen peroxide and molecular oxygen in the following manner[153].

$$H_2O + 2O_2^{-\cdot} + 2H^{+} \rightarrow 2H_2O_2 + O_2$$

The enzyme activity was assayed by the method of Misra and Fridovich.

Reagents:

- ✓ 0.1 M Carbonate-bicarbonate buffer; pH 10.2.
- ✓ 0.6 mM EDTA solution
- ✓ 1.8 mM Epinephrine (prepared in situ)
- ✓ Absolute ethanol.
- ✓ Chloroform

Procedure:

0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml ethanol and 0.15 ml chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant, added 0.5 ml of 0.6 mM EDTA solution and 1 ml of 0.1 M carbonate-bicarbonate (pH 10.2) buffer. The reaction was initiated by the addition of 0.5 ml of 1.8 mM epinephrine (freshly prepared) and the increase in absorbance at 480 nm was measured. One unit of the SOD activity was the amount of protein required to give 50% inhibition of epinephrine autoxidation.

7.9.2 Estimation of Catalase (CAT)

This enzyme catalyzes conversion of hydrogen peroxide into water and molecular oxygen[154].

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The enzyme activity was assayed by the method of Sinha.

Reagents:

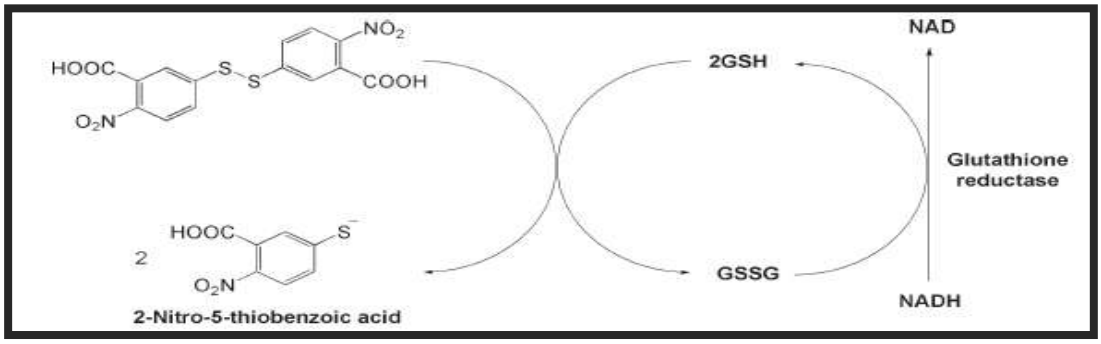
- ✓ Dichromate-acetic acid reagent: 5% potassium dichromate in water was mixed with glacial acetic acid in the ratio of 1:3 (v/v).
- ✓ 0.01 M Phosphate buffer; pH 7.0.
- ✓ 0.2M Hydrogen peroxide

Procedure:

0.1 ml of the tissue homogenate was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0) pre-warmed to 37°C, 0.4 ml of distilled water and the mixture was incubated at 37°C. The reaction was initiated by the addition of 0.5 ml of 0.2 M hydrogen peroxide and the reaction mixture was incubated at 37°C for one minute. The reaction was terminated by the addition of 2 ml of dichromate-acetic acid reagent after 15, 30, 45, and 60 seconds. Standard hydrogen peroxide in the range of 4-20 µ moles were taken and treated in the same manner. All the tubes were heated in a boiling water bath for 10 minutes, cooled and the green colour that developed was read at 590 nm against blank containing all components except the enzyme. Catalase activity was expressed in terms of µ moles of H2O2 consumed/min/mg protein.

7.9.3 Estimation of Reduced glutathione (GSH):

The total reduced glutathione was determined according to the method of Ellman[155].



The assay procedure is based on the reduction of Ellman´s reagent [5, 5´- dithio dis (2- nitrobenzoic acid)] (DTNB) by SH groups of glutathione to form 2-nitro-S-mercaptobenzoic acid per mole of glutathione. The product is measured spectrophotometically at 412 nm.

Reagents

- ✓ 0.2 M Phosphate buffer; pH 8.0.
- ✓ 0.6 mM DTNB reagent.
- ✓ 5%TCA

Procedure

0.1 ml of tissue homogenate was precipitated with 5% TCA. The contents were mixed well for complete precipitation of proteins and centrifuged. To 0.1 ml of supernatant, 2 ml of 0.6 mM DTNB reagent and 0.2 M phosphate buffer (pH 8.0) were added to make up to a final volume of 4 ml. The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series of standards treated in a similar way also run to determine the glutathione content. The amount of glutathione was expressed as nmoles/g tissue.

7.9.4 Estimation of Glutathione peroxide (GPx):

Principle:

GPx activity was assessed by modified method of Flohe and Gunzler (1984)[156].

Reagents:

- ✓ Sodium phosphate buffer 0.3 M, pH 7.0
- ✓ Sodium azide 1Omhl
- ✓ Reduced glutathione 4mM
- ✓ Hydrogen peroxide 2.5 mM
- ✓ TCA lPh
- ✓ Phosphate solution 0.3 M disodium hydrogen phosphate.
- ✓ DTNB 40 mg/100 ml of 1% sod~um cihate
- ✓ EDTA 0.8 mM 9. Standard 20 mg of reduced glutathione in 100 rnl distilled water. This solution contained 20 pg of glutathione/O. 1 ml.

Procedure:

GPx activity was assessed by modified method of Flohe and Gunzler (1984). For the enzyme reaction, 0.2 ml of the post-mitochondrial supernatant was placed into a tube and mixed with 0.4 ml reduced glutathione and the mixture was put into an ice bath for 30 min. Then the mixture was centrifuged for 10 min at 3000 rpm, 0.48 ml of the supernatant was placed into a cuvette, and 2.2 ml of 0.32 M Na2HPO4 and 0.32 ml of 1.0 mmol/L DTNB were added for color development. The absorbance at wavelength 412 nm was measured

on spectrophotometer (LKB-Pharmacia, Mark II, Ireland) after 5 min. The enzyme activity was calculated as nmol/mg protein.

7.10.EVALUATION OF OXIDATIVE STRESS PARAMETER

Estimation of Lipid Peroxidation (LPO):

Lipid peroxidation (LPO) was assayed by the method of Ohkawa et al[111], in which the malondialdehyde (MDA) released served as the index of LPO. The extent of LPO in the hepatic tissue was assayed by measuring one of the end products of this process, the thiobarbituric acid-reactive substances (TBARS). As 99% TBARS is Malondialdehyde (MDA), thus this assay is based on the reaction of 1 molecule of MDA with 2 molecules of TBARS at low pH (2- 3) and at a temperature of 95°C for 60 min. The resultant pink chromogen can be detected spectrophonometrically at 532nm[157].

Reagents

- ✓ Standard: 1, 1, 3, 3-tetra ethoxypropane (TEP).
- ✓ 8.1% Sodium dodecyl sulphate (SDS)
- ✓ 20%Acetic acid
- ✓ 0.8%Thiobarbituric acid (TBA)
- ✓ 15:1 v/v n-butanol: pyridine mixture

Procedure

To 0.2 ml of tissue homogenate, 0.2ml of 8.1% SDS, 1.5ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA were added. The mixture was made up to 4 ml with water and then heated in a water bath at 95.8ºC for 60 min using glass ball as a condenser. After cooling, 1ml of water and 5ml of n-butanol: pyridine (15:1 v/v) mixture were added and shaken vigorously. After centrifugation at 4000rpm for 10min, the organic layer was taken and its absorbance was measured at 532nm. The level of lipid peroxides was expressed as n moles of MDA formed/mg of protein.

7.11.OTHER PARAMETER:

Estimation of NA^+ , K^+ , ATPase activity[158-159].

Principle:

Na^+/K^+-ATPase activity was measured according to the method of Suhail and Rizvi 1987 The final assay mixture contained 0.5–1.2 mg membrane protein/mL, 20 mmol/L KCl, 140 mmol/L NaCl, 3 mmol/L $MgCl_2$, 30 mmol/L imidazole (pH 7.24), with or without 5×10^{-4} mol/L ouabain and 6 mmol/L ATP. Assay mixture was incubated for 30 min at 37 °C and the reaction was stopped by the addition of 3.5 mL of a solution-A (0.5% ammonium molybdate, 0.5 mol/L H_2SO_4, and 2% sodium dodecyl sulphate). The amount of liberated phosphate (Pi) was estimated according to the method of Fiske and Subbarow (1925).

REAGENTS

- ✓ 0.5–1.2 mg membrane protein/mL,
- ✓ 20 mmol/L KCl, 140 mmol/L
- ✓ NaCl, 3 mmol/L
- ✓ $MgCl_2$, 30 mmol/L
- ✓ imidazole (pH 7.24), with or without 5×10^{-4} mol/L ouabain
- ✓ f) nd 6 mmol/L ATP.

Assay mixture was incubated for 30 min at 37 °C and the reaction was stopped by the addition of 3.5 mL of a solution-A (0.5% ammonium molybdate, 0.5 mol/L H_2SO_4, and 2% sodium dodecyl sulphate). The amount of liberated phosphate (Pi) was estimated and the values are recorded (Fiske and Subbarow(1925). Further by adding tissue extract to the above same assay mixture and incubated for 30 min at 37 °C and the Na^+/K^+-ATPase activity was measured in a spectrophotometer (Star 21 plus USA) and the activity expressed as nmol pi released/mg protein per hour at 37 °C.

7.12 HISTOPATHOLOGICAL STUDIES

Hematoxylin, a basic bye is oxidized to hematein with a mordant, a metallic ion suchas the salts of aluminium. The positively charged aluminium-hematein complex combines with the negatively charged phosphate groups of the nucleic acids (DNA and RNA) forming blue/purple colour, which is characteristic of hematoxylin stains. Eosin is an acidic dye, which is

considered to have a selective affinity for the basic parts of the cell, i.e., the cytoplasm. Thus, the hematoxylin and eosin (H & E) stain is used to demonstrate different structures of the tissue[160].

The various steps involved in the preparation of pancreatic tissues for histological studies are as follows:

❖ **Fixation:**

In order to avoid tissue by the lysosomal enzymes and to preserve its physical and chemical structure, a bit of tissue from each organ was cut and fixed in bouin's fluid immediately after removal from the animal body. The tissues were fixed in bouin's fluid for about 24 hours. The tissues were then taken and washed in glass distilled water for a day to remove excess of picric acid.

❖ **Dehydration:**

The tissues were kept in the following solutions for an hour each; 30%, 50%, 70% and 100% alcohol. Inadequately dehydrated tissues cannot be satisfactorily infiltered with paraffin. At the same time over dehydration results in making the tissues brittle, which would be difficult for sectioning. So, careful precautions were followed while performing the dehydration process

❖ **Clearing:**

Dealcoholization or replacement of alcohol from the tissues with a clearing agent is called as clearing. Xylene was used as the clearing agent for

one or two hours, two or three times. Since, the clearing agent is miscible with both dehydration and embedding agents, it permits paraffin to infilterate the tissues. So, the clearing was carried out as the next step after dehydration to permit tissue spaces to be filled with paraffin. The tissues were kept in the clearing agent till they become transparent and impregnated with xylene.

❖ **Impregnation:**

In this process the clearing agent xylene was placed by paraffin wax. The tissues were taken out of xylene and were kept in molten paraffin embedding bath, which consists of metal pots filled with molten wax maintained at about 50^{o}C. The tissues were given three changes in the molten wax at half an hour intervals.

❖ **Embedding:**

The paraffin wax used for embedding was fresh and heated upto the optimum melting point at about 56–58^{o}C. A clear glass plate was smeared with glycerine. L-shaped mould was placed on it to from a rectangular cavity. The molten paraffin wax was poured and air bubbles were removed by using a hot needle. The tissue was placed in the paraffin and oriented with the surface to be sectioned. Then the tissue was pressed gently towards the glass plate to make settle uniformly with a metal pressing rod and allowed the wax to settle and solidity room temperature. The paraffin block was kept in cold water for cooling.

❖ **Section Cutting:**

Section cutting was done with a rotatory microtome. The excess of paraffin around the tissue was removed by trimming, leaving ½ cm around the tissue. Then the block was attached to the gently heated holder. Additional support was given by some extra wax, which was applied along the sides of the block. Before sectioning, all set screws holding the object holder and knife were hand tightened to avoid vibration. To produce uniform sections, the microtome knife was adjusted to the proper angle in the knife holder with only the cutting edge coming in contact with the paraffin block. The tissue was cut Sin the thickness range of about 7µm.

❖ **Flattening and Mounting of Sections:**

The procedure was carried out in tissue flotation warm water bath. The sections were spread on a warm water bath after they were detached from the knife with the help of hair brush. Dust free clean slides were coated with egg albumin over the whole surface. Required sections were spread on clean slide and kept at room temperature

❖ **Staining of Tissue Sections:**

The sections were stained as follows; deparaffinization with xylene two time search for five minutes

Dehydration through descending grades of ethyl alcohol

- ✓ 100% alcohol (absolute) - 2 minute
- ✓ 90% alcohol - 1minute
- ✓ 50% alcohol - 1 minute

Staining with Ehrlich's Haemaoxylin was done for 15-20 minutes. Then the sectioned tissues were thoroughly washed in tap water for 10 minutes. Rinsed with distilled water and stained with Eosin. Dehydration again with ascending grades of alcohol.

- ✓ 70% alcohol - 2 minute
- ✓ 90% alcohol - 2 minute
- ✓ 100%alcohol - 1 minute

Finally the tissues were cleared with xylene two times, each for about 3 minutes interval.

❖ **Mounting:**

On the stained slide, DPX mountant was applied uniformly and micro glass cover slides were spread. The slides were observed in Nikon microscope and microphotographs were taken.

7.13 STATISTICAL ANALYSIS

The data of all the results were represented as Mean ± S.E.M. on statistically analysed by one-way ANOVA followed by Duncun's Multiple range test was used for statistical analysis $p<0.05$ was considered significant[161].

RESULTS

Appearance and percentage yield of *EEMP*

Table No: 2

Drug	*Ottelia alismoides (L.,)* PERS
Solvent	Ethanol
Colour	Dark green
Consistency	Semi solid
Percentage yield	19.5 % w/w

PRELIMINARY PHYTOCHEMICAL SCREENING

Table No-3: Results of the Preliminary Phytochemical Constituents present in Ethanolic extract of *Ottelia alismoides(L)* PERS.

Table no: 3 Preliminary phytochemical constituents present in *EEPS*

S. No	Constituents	*Ottelia alismoides* Ethanolic extract
1.	Alkaloids	+
2.	Carbohydrates	_
3.	Protein	_
4.	Terpinoids	+
5.	Phenols	+
6.	Tannins	+
7.	Flavanoids	+
9.	Glycosides	+
10.	Saponins	_

+ve indicates the presence –ve indicates the absence

RESULTS:

The phytochemical constituents present in the Ethanolic extract of *Ottelia alismoides (L.)* PERS were Phenols, Tannins, Flavanoids, Glycosides, Alkaloids and Terpenoids.

Estimation of Blood glucose level:

The Effect of the different doses of Ethanolic extract of *Ottelia alismoides* Pers on Blood Glucose level.

Groups	Blood glucose level(mg/dl)	
	0^{th} week	8^{th} week
Group I STZ(55mg/kg)+Vehicle	330.15± 17.76	368.0± 59.05
Group II STZ (55mg/kg)+ Insulin (5 IU/kg)	341.10 ±22.31	160.10± 20.22***
Group III STZ+ insulin+ Pregabalin (15mg/kg)	319.15 ±13.18	140.01± 17.02** a
Group IV STZ+ insuln+ *EEOA* (200mg/kg)	336.10 ±15.46	120.08 ±13.15ns aa
Group V STZ+ insulin+ *EEOA* (400mg/kg)	317.10 ±12.18	115.21 ±11.06ns aa

Table no 4: Effect of *EEOA* on Blood Glucose level in experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*,**,***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when compared to Insulin Diabetic control.

a, aa indicates P < 0.05 & P < 0. 01 Significance between diabetic control group

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT).

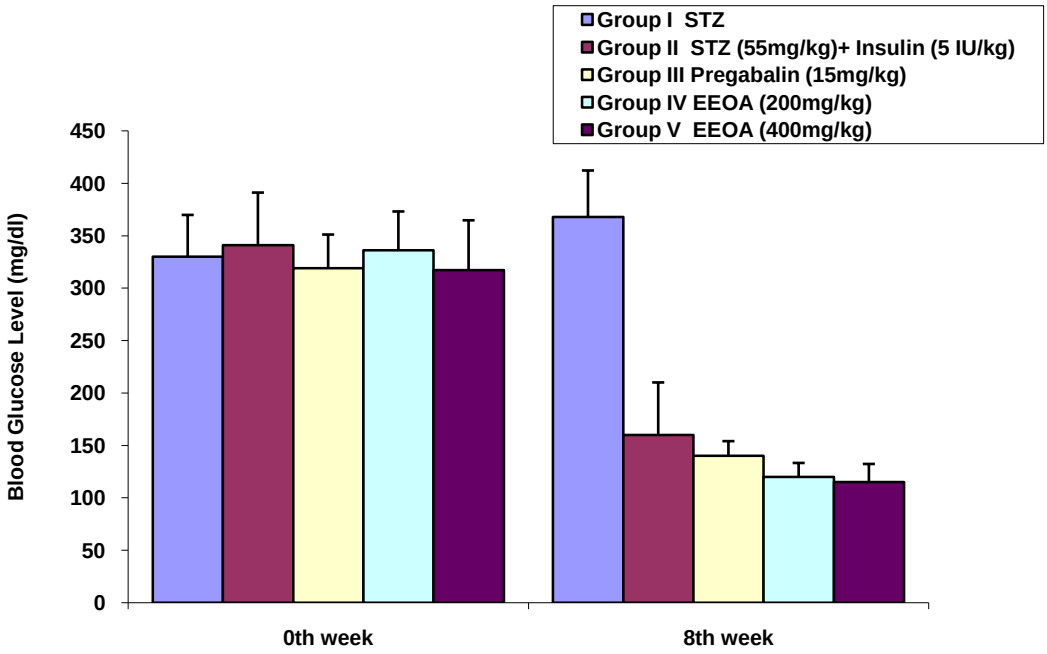


Fig.No. 16 Effect of *EEOA* on Blood Glucose Level in Experimentally induced Diabetic rat model

Estimation of Body weight & Organ weight:

The Effect of the different doses of Ethanolic extract of *Ottelia alismoides* Pers on Blood Glucose level.

Groups	Body weight	Organ weight	
		Liver	Kidney
Group I STZ(55mg/kg)+Vehicle	204.16±6.93	4.55±0.45	1.93±0.05
Group II STZ +Insulin (5 IU/kg)	249.06±5.31***	3.09±0.34*	1.83±0.05*
Group III STZ+ insulin+ Pregabalin (15mg/kg)	248.31±5.30*	2.84±0.31ns	1.62±0.07ns
Group IV STZ+ insuln+ *EEOA* (200mg/kg)	240.56±5.29*	2.30±0.30ns	1.60±0.04ns
Group V STZ+ insulin+ *EEOA* (400mg/kg)	245.10±5.30a	3.08±0.50ns	1.56±0.04ns

Table no 5: Effect of *EEOA* on Body weight & Organ weight in Experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*,**, ***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when compared Insulin diabetic control

a, aa indicates P < 0.05 & P < 0. 01 Significance between diabetic control group

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT).

RESULTS:

The Blood Glucose, Body weight and Organ Weight were measured and showed in Table no-3 & 4.

The Diabetic control (Group 1) showed significant increase in Blood Glucose, Kidney weight & liver and whereas the body weight were decreased when compared insulin diabetic control (Group 2).

Standard (Group 3) and *EEOA* (200 mg/kg& 400 mg/kg) showed statistically significant decrease in Blood Glucose and organ weight and increase in body weight when compared to Diabetic control (Group1).

ESTIMATION OF ANTIOXIDANT PARAMETERS

Effect of *EEOA* on Antioxidant level (SOD,CAT,GPx,GSH)

Groups	SOD (units per mg protein)	CAT (units per mg protein)	GPx (units per mg p rotein)	GSH (units per mgprotein)
Group I STZ(55mg/kg)+Vehicle	1.627±1.06	1.08±0.09	1.13±0.05	1.26±0.54
Group II STZ +Insulin (5 IU/kg)	2.155±1.03***	2.08±0.09***	3.08±0.31***	2.86±0.54***
Group III STZ+ insulin+ Pregabalin (15mg/kg)	2.654±1.84**	2.16±0.11**	2.65±0.63**	2.86±0.54**
Group IV STZ+ insuln+ *EEOA* (200mg/kg)	2.431±1.86*a	2.24±0.31ns a	2.98±0.74ns a	3.14±0.31ns a
Group V STZ+ insulin+ *EEOA* (400mg/kg)	2.956±2.31 ns aa	2.49±0.26 ns aa	3.24±0.46 ns aa	3.89±0.48 ns aa

Table no 6: Effect of *EEOA* on Antioxidant level(SOD, CAT,GPx & CAT) in Experimentally induced Diabetic rat model

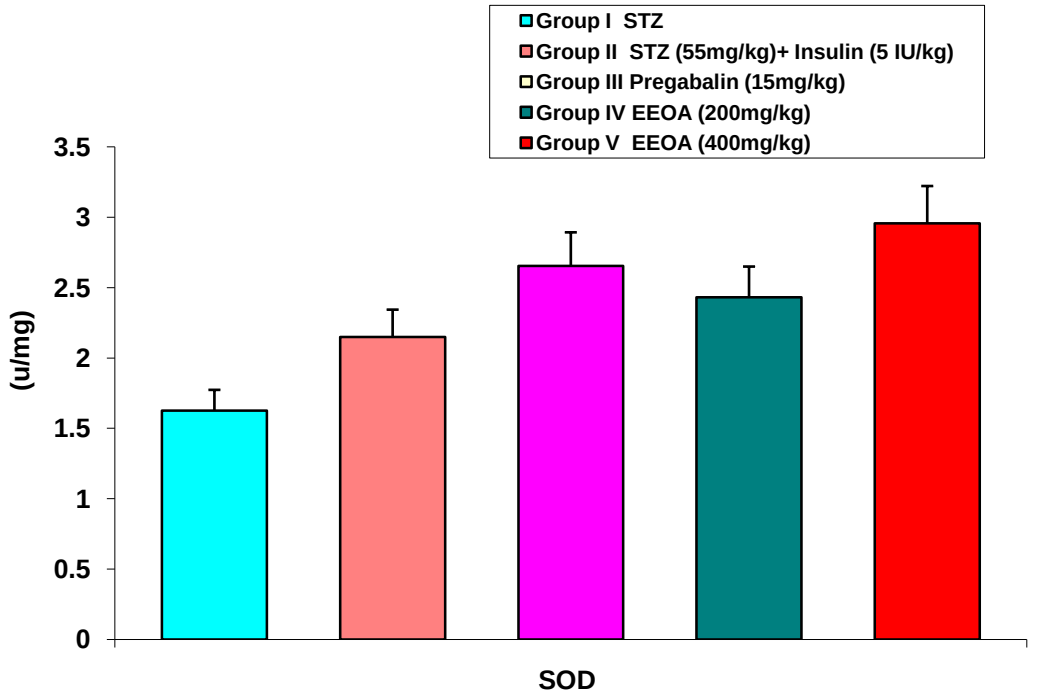
The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

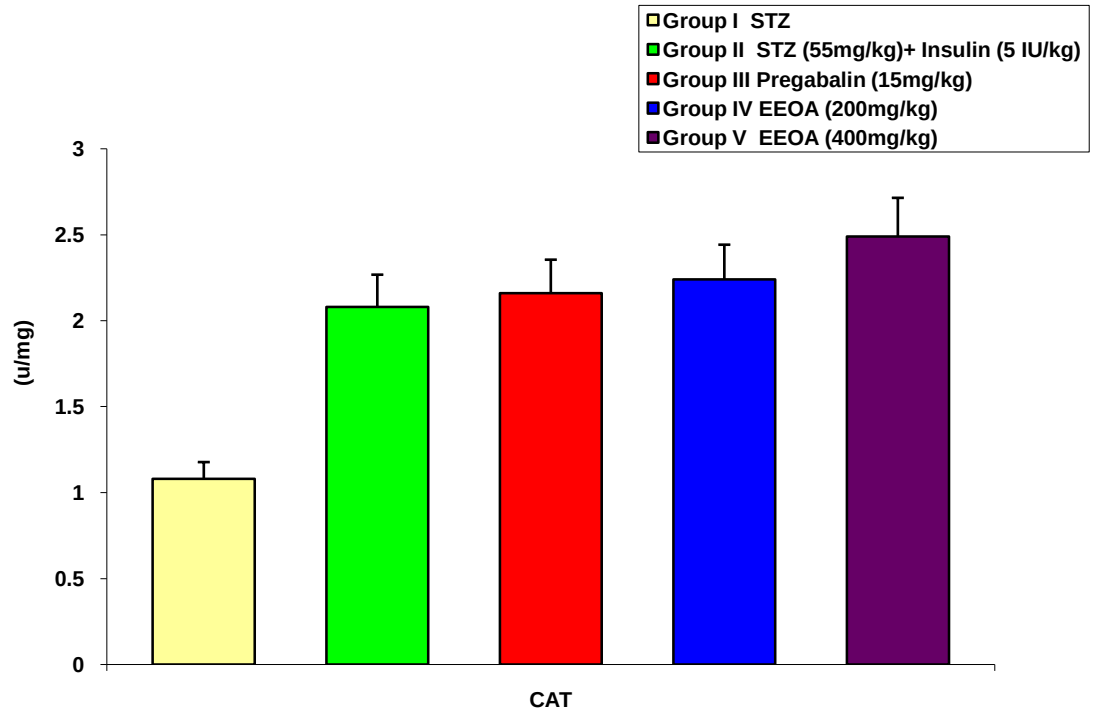
*,**,***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when compared to insulin diabetic control

a, aa indicates P < 0.05 & P < 0. 01 Significance between diabetic control

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT).


Group I STZ
Group II STZ (55mg/kg)+ Insulin (5 IU/kg)
Group III Pregabalin (15mg/kg)
Group IV EEOA (200mg/kg)
Group V EEOA (400mg/kg)
3.5
3
2.5
2
1.5
1
0.5
0
(u/mg)
SOD


Group I STZ
Group II STZ (55mg/kg)+ Insulin (5 IU/kg)
Group III Pregabalin (15mg/kg)
Group IV EEOA (200mg/kg)
Group V EEOA (400mg/kg)
3
2.5
2
1.5
1
0.5
0
(u/mg)
CAT

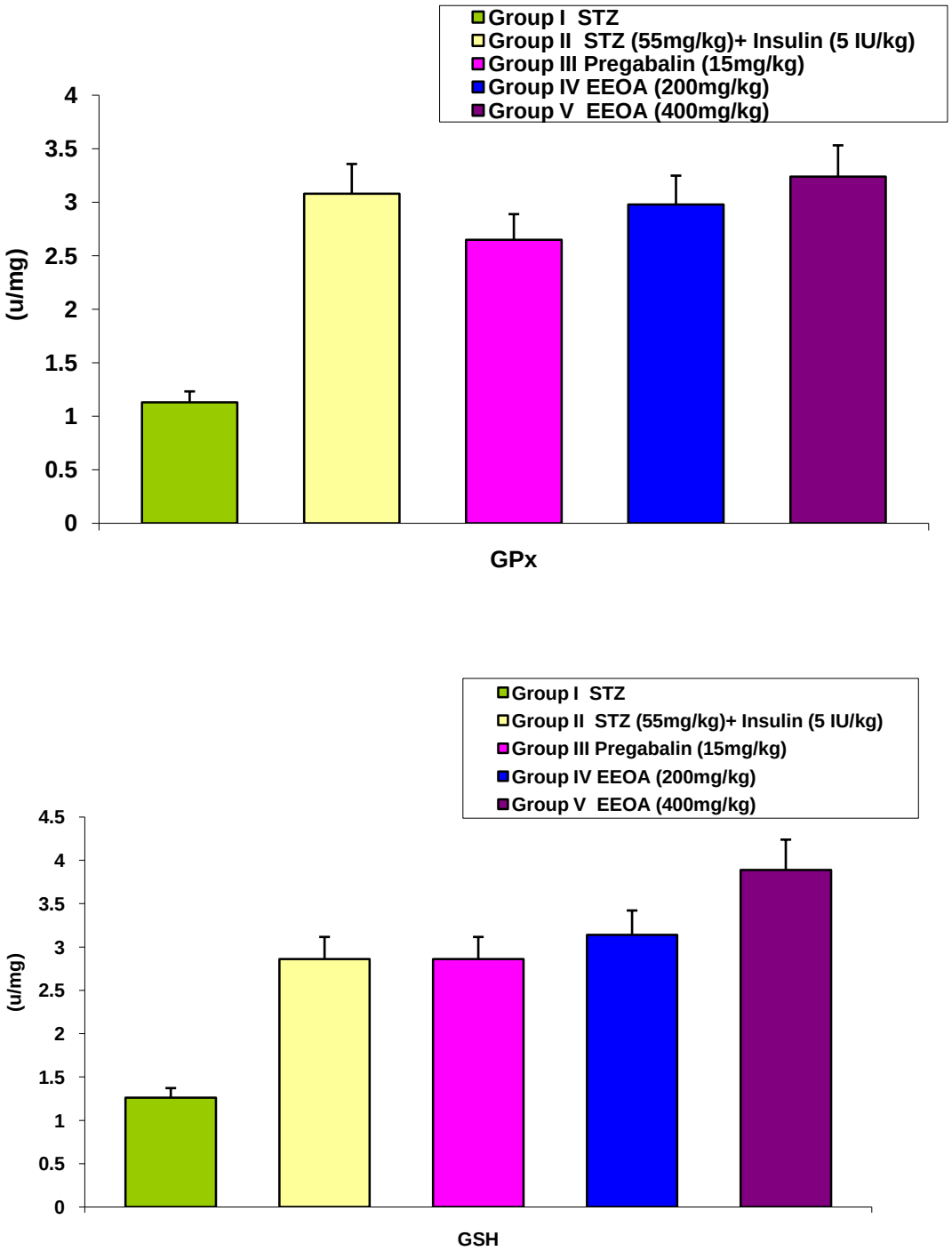


Figure no 17: Effect of *EEOA* on Antioxidant level(SOD, CAT,GPx & GSH) in Experimentally induced Diabetic rat model

RESULTS:

The Superoxide dismutase (SOD), Catalase (CAT), Reduced glutathione (GSH), Glutathione peroxides (GPx) levels were measured and showed in Table no-6 and Fig. no-(17).

The Diabetic control (Group 1) showed significant decrease in Superoxide dismutase (SOD), Catalase (CAT) and Reduced Glutathione (GSH) level when compared to the insulin diabetic control (Group 2).

Standard (Group 3) and *EEOA* (200 & 400 mg/kg) showed statistically significant increase in Glutathione peroxidase (GPx), Superoxide dismutase (SOD), Catalase (CAT) and Reduced Glutathione (GSH) level when compared to diabetic control (Group 1).

ESTIMATION OF OXIDATIVE STRESS PARAMETER

Effect of *EEOA* on Malondialdehyde (MDA) level

GROUPS	LPO(units per mg protein)
Group I STZ(55mg/kg)+vehicle	4.188±2.03
Group II STZ +Insulin (5 IU/kg)	3.86±2.03***
Group III STZ+ insulin + Pregabalin (15mg/kg)	3.256±1.84**
Group IV STZ+ insulin+*EEOA* (200mg/kg)	2.416±1.13ns a
Group V STZ+ insulin+ *EEOA* (400mg/kg)	2.136±1.39 ns aa

Table no 7: Effect of *EEOA* on Malondialdehyde (MDA) level in Experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*,**,***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when compared diabetic control group

a, aa indicates P < 0.05 & P < 0. 01 Significance between insulin diabetic treated group

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT).

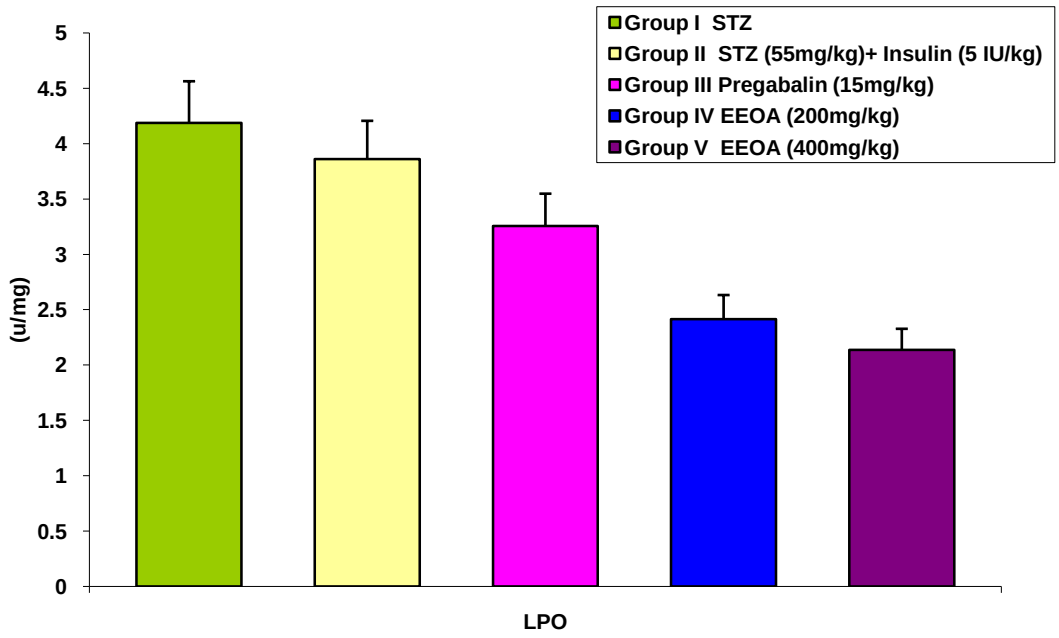


Figure no 18: Effect of *EEOA* on Malondialdehyde (MDA) level in Experimentally induced Diabetic rat model

RESULTS:

The Liver Malondialdehyde (MDA) levels were measured and showed in Table no-7 and Fig. no-(17). The Diabetic control (Group 1) showed significant increase in Malondialdehyde (MDA) level when compared to the normal control (Group 2).

Standard (Group 3) and *EEOA* (200&400 mg/kg) showed statistically significant decrease in Malondialdehyde (MDA) level when compared to Diabetic control (Group 1).

ESTIMATION OF Na^+ K^+ ATP ase ACTIVITY:

Effect of *EEOA* on Na^+ K^+ ATP ase Activity

Group	Na^+/K^+-ATPase activity (nmol pi released/mg protein per hour)
Group I STZ(55mg/kg)+vehicle	2.634 ± 0.206
Group II STZ+ Insulin (5 IU/kg)	5.924±0.314***
Group III STZ+ insulin + Pregabalin (15 mg/kg)	3.929±0.321** ns
Group IV STZ+insulin+ *EEOA* (200mg/kg)	5.026±0.234ns a
Group V STZ+ insulin *EEOA* (400mg/	6.117±0.249ns aa

Table no 8: Effect of *EEOA* on Na^+ K^+ ATP ase Activity in Experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*,**,***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when diabetic control group

a, aa indicates P < 0.05 & P < 0. 01 Significance between Insulin diabetic treated group

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT)

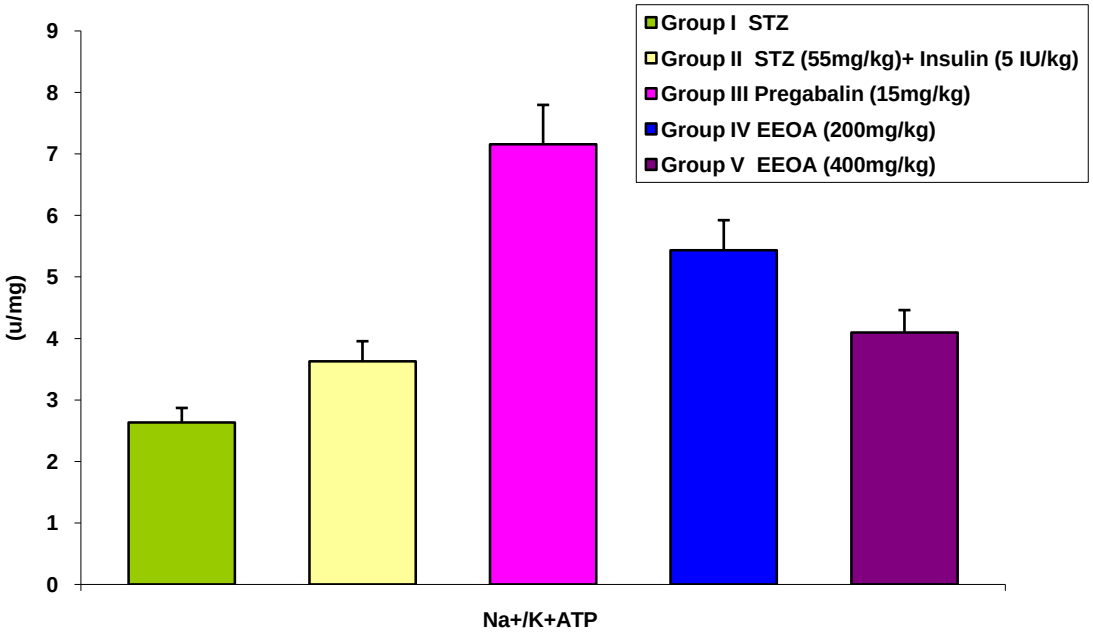


Figure no 19 : Effect of *EEOA* on in Na^+ K^+ ATP ase Activity Experimentally induced Diabetic rat model

RESULTS:

The Na^+, K^+ , ATPase activity were measured and showed in Table no-8 and Fig. no-(19).The diabetic control (Group 1) showed significant decrease in Na^+, K^+ , ATPase activity when compared to insulin diabetic control (Group1).

Standard (Group 3) and *EEOA* (200&400 mg/kg) showed statistically significant increase in Na^+, K^+ , ATPase activity when compared to Diabetic control (Group 1)

BEHAVIOURAL ASSESMENT

EFFECT OF EEOA ON HOT PLATE TEST:(Thermal Hyperalgesia)

Groups	Reaction Time in Sec			
	0 Week	4th Week	6th Week	8th Week
Group I STZ (55mg/kg)+vehicle	5.05 ± 0.84	4.46 ± 0.73	4.20 ± 0.27	3.91± 0.17
Group II STZ+ Insulin (5 IU/kg)	5.05 ± 0.31 ***	4.31 ± 0.67***	4.11 ± 0.37***	4.03 ± 0.54***
Group III STZ+ insulin + Pregabalin (15 mg/kg)	5.05 ± 0.56** ns	4.56 ± 0.16**	4.96 ± 0.56**	9.31 ± 0.16**
Group IV STZ+insulin+ *EEOA* (200mg/kg)	5.05 ± 0.31* a	4.60 ± 0.37*	4.70 ± 0.84ns a	9.54 ± 0.22ns aa
Group V STZ+ insulin *EEOA* (400mg/	5.05 ± 0.31ns a	5.20 ± 0.18ns a	8.81 ± 0.16ns aa	10.87 ± 0.12ns aa

Table no 9: Effect of *EEOA* on Hot plate test (Thermal hyperalgesia)

in Experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*, **, ***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when compared to insulin diabetic control

a, aa indicates P < 0.05 & P < 0. 01 Significance between diabetic control

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT)

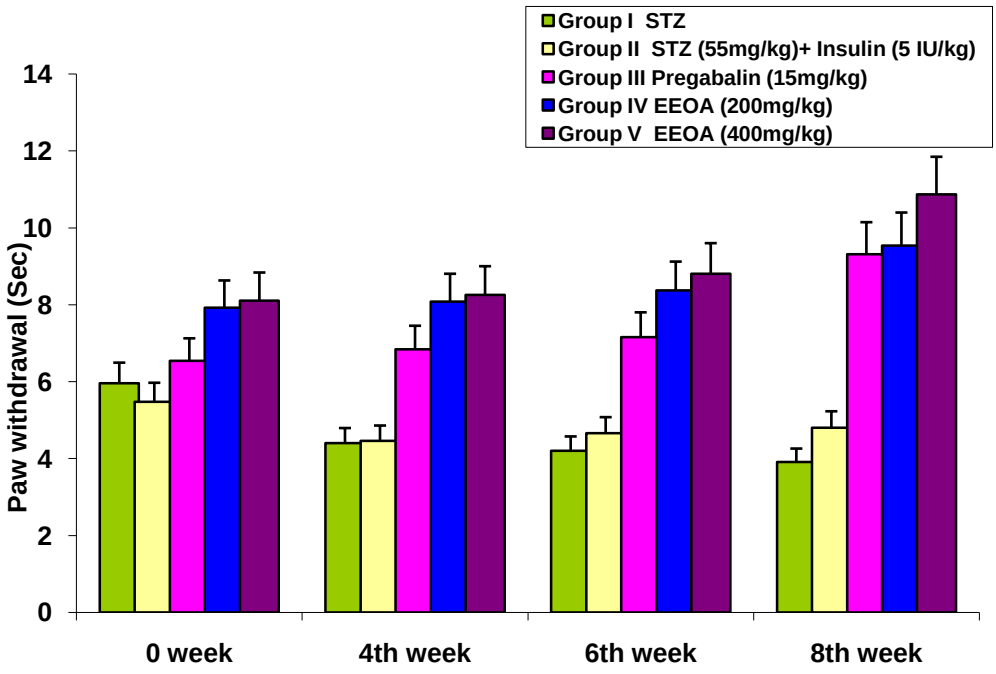


Figure no 20 : Effect of *EEOA* on Hot plate test (Thermal Hyperalgesia) in Experimentally induced Diabetic rat model

RESULTS:

The Paw Withdrawal latency were measured and showed in Table no-9 and Fig. no-(20). The diabetic control (Group 1) showed significant decrease in Paw withdrawal latency when compared to the insulin diabetic control (Group 2).

Standard (Group 3) and *EEOA* (200& 400 mg/kg) showed statistically significant increase in Paw withdrawal latency when compared to Diabetic control (Group 1).

EFFECT OF *EEOA* ON FORMALIN TEST: (Chemical hyperalgesia)

Groups	FORMALIN TEST(Acute phase)	FORMALIN TEST(Delayed phase)
Group I STZ (55mg/kg)+vehicle	32.93±1.08	30.11±1.86
Group II STZ+ Insulin (5 IU/kg)	58.54±2.16	60.88±1.96***
Group III STZ+ insulin + Pregabalin (15 mg/kg)	47.84±0.95	50.55±1.31aa
Group IV STZ+insulin+ *EEOA* (200mg/kg)	46.92±0.73	55.15±1.06aa
Group V STZ+ insulin *EEOA* (400mg/	53.65±1.18	62.56±1.18aa

Table no 10: Effect of *EEOA* on Formalin (chemical hyperalgesia) in Experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*,**, ***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when compared to insulin diabetic control

a, aa indicates P < 0.05 & P < 0. 01 Significance between diabetic control group

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT)

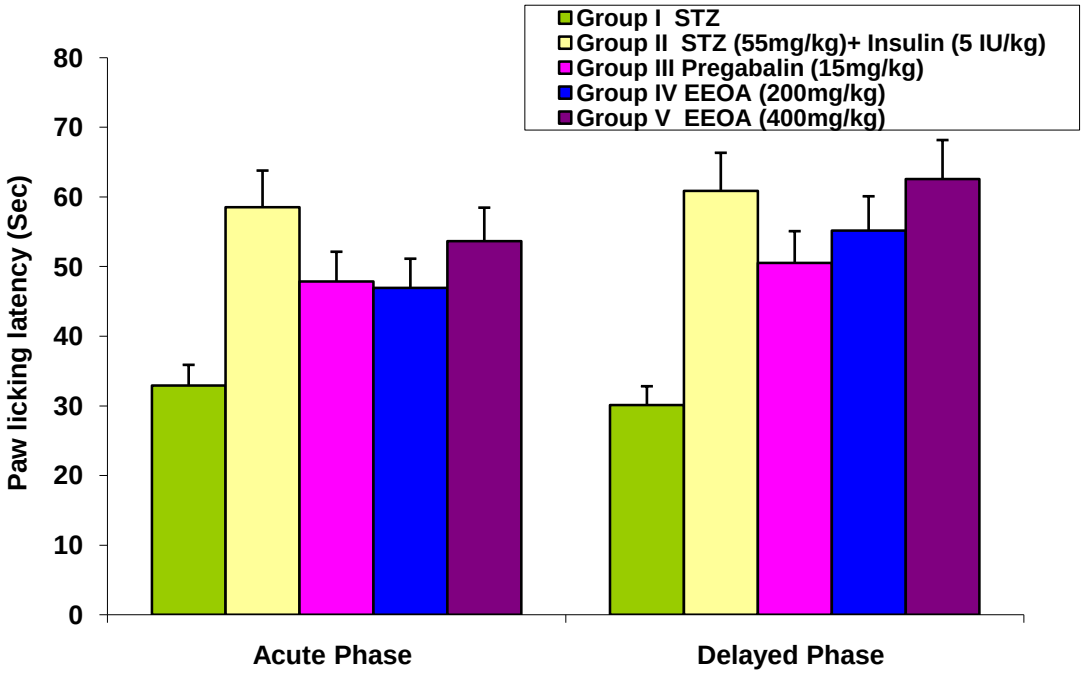


Figure no 21 : Effect of *EEOA* on Formalin test (Chemical Hyperalgesia) in Experimentally induced Diabetic rat model

RESULTS:

The response were measured and showed in Table no-10 and Fig. no-(21). The Diabetic control (Group 1) showed significant decrease in intensified nociceptive response and analgesic response when compared to the insulin diabetic control (Group 2).

Standard (Group 3) and *EEOA* (200& 400 mg/kg) showed statistically significant increase in intensified nociceptive response and analgesic when compared to Diabetic control (Group 1).

EFFECT OF *EEOA* ON TAIL FLICK TEST: (Thermal Hyperalgesia)

GROUPS	Reaction Time In Sec			
	0th week	4th week	6th week	8th week
Group I STZ (55mg/kg)+vehicle	4.23 ± 016	4.21± 0.27	4.13± 0.15	4.09 ± 0.627
Group II STZ+ Insulin (5 IU/kg)	9.22 ± 0.57***	4.36 ± 0.19***	4.21 ± 0.15***	4.45 ± 6.27***
Group III STZ+ insulin + Pregabalin (15 mg/kg)	5.93 ± 0.27** ns	6.16 ± 0.31** ns	7.93 ± 0.96** ns	9.54 ± 0.73** ns
Group IV STZ+insulin+ *EEOA* (200mg/kg)	5.16 ± 0.16*	5.91 ± 0.22 ns	7.11 ± 0.54aa	9.6 ± 0.56aa
Group V STZ+ insulin *EEOA* (400mg/	5.39 ± 0.23 *	6.11 ± 0.56a	8.27 ± 0.22aa	9.93 ± 0.73aa

Table No11: Effect of *EEOA* on Tail Flick test (Thermal hyperalgesia) in experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*,**,***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when insulin diabetic control

a, aa indicates P < 0.05 & P < 0. 01 Significance between diabetic control group

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT)

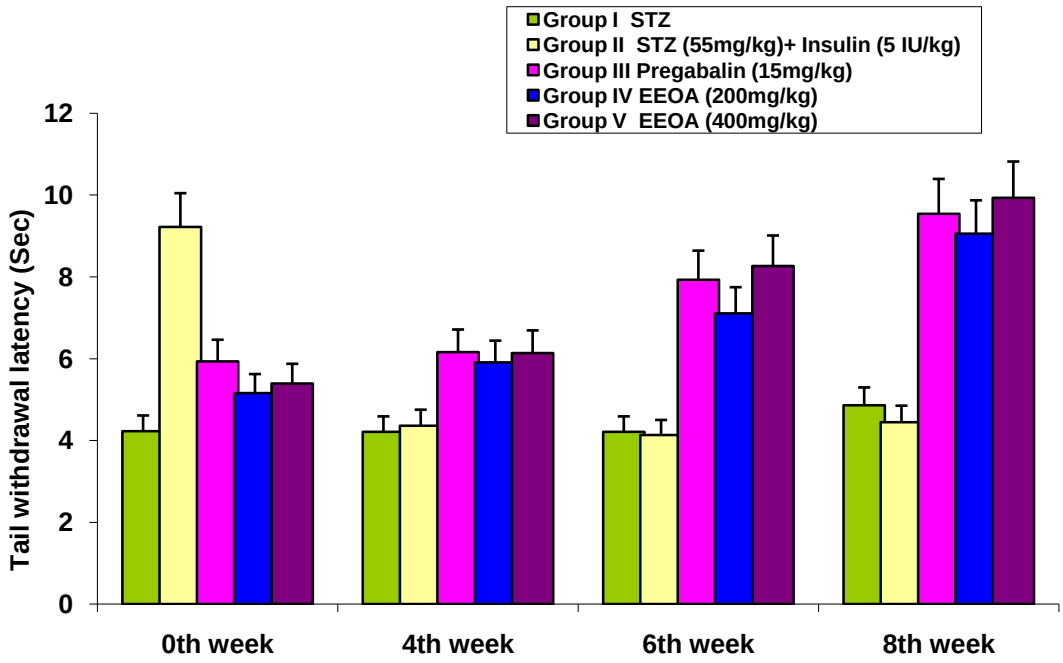


Figure no 22 : Effect of *EEOA* on Tail Flick test (Thermal Hyperalgesia) in Experimentally induced Diabetic rat model

RESULTS:

The Tail withdrawal latency (Sec) were measured and showed in Table no-11 and Fig. no-(22). The Diabetic control (Group 1) showed significant reduction in tail withdrawal latency when compared to insulin diabetic control(Group 2).

Standard (Group 3) and *EEOA* (200& 400 mg/kg) showed statistically significant increase in reaction time when compared to the Diabetic control (group 1).

EFEECT OF TAIL IMMERSSION TEST: (Thermal hyperalgesia)

Effect of *EEOA* on Hot waterTail Immerssion test (Thermal hyperalgesia)

GROUPS	Reaction Time in Sec			
	0th week	4th week	6th week	8th week
Group I STZ (55mg/kg)+vehicle	10.31 ± 0.93	4.80 ± 0.24	4.98± 0.39	4.86 ± 0.29
Group II STZ+ Insulin (5 IU/kg)	5.56 ± 0.73***	7.49± 0.27***	6.13± 0.57***	6.29 ± 0.11***
Group III STZ+ insulin + Pregabalin (15 mg/kg)	5.96 ±0.16**ns	8.11 ± 0.56** ns	11.54 ± 0.67** ns	3.08 ± 0.42** ns
Group IV STZ+insulin+ *EEOA* (200mg/kg)	6.03 ± 0.18*	6.94 ± 0.013 a	7.93 ± 0.63 aa	8.93 ± 0.56aa
Group V STZ+ insulin *EEOA* (400mg/	6.13 ± 0.31*	7.11 ± 0.015 aa	8.76 ± 0.23 aa	11.57 ± 0.27aa

Table no 12 : Effect of *EEOA* on Hot waterTail Immerssion test (Thermal hyperalgesia) in Experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*,**,***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when compared insulin diabetic control

a, aa indicates P < 0.05 & P < 0. 01 Significance between diabetic control

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT)

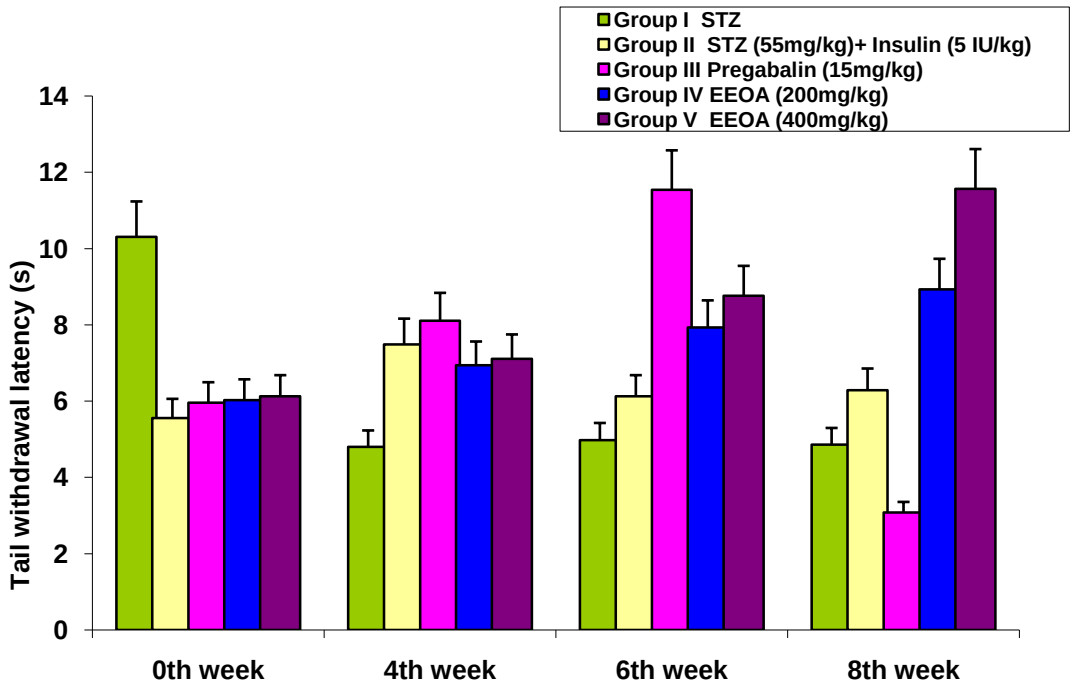


Figure no 23 : Effect of *EEOA* on Hot water Tail Immerssion test (Thermal Hyperalgesia) in Experimentally induced Diabetic rat model

RESULTS:

The Tail withdrawal latency (Sec) were measured and showed in Table no-12 and Fig. no-(23). The Diabetic control (Group 1) showed significant reduction in tail withdrawal latency when compared to the diabetic control (Group 2).

Standard (Group 3) and *EEOA* (200& 400 mg/kg) showed statistically significant increase in reaction time when compared to the Diabetic control (group 1).

Effect of *EEOA* on Cold waterTail Immerssion test (Thermal allodynia)

GROUPS	Reaction Time In Sec			
	0th week	4th week	6th week	8th week
Group I STZ (55mg/kg)+vehicle	9.86 ± 0.67	5.36± 0.34	4.76 ± 0.13	1.13 ± 0.27
Group II STZ+ Insulin (5 IU/kg)	6.11 ± 0.27**	7.58 ± 0.16***	6.53 ± 0.14***	5.32 ±0.21***
Group III STZ+ insulin + Pregabalin (15 mg/kg)	6.34±0.18** ns	8.56 ± 0.27** ns	11.36 ±0.18**ns	13.24 ±0.18**ns
Group IV STZ+insulin+ *EEOA* (200mg/kg)	6.46 ± 0.13*	7.67 ± 0.16a	8.54 ± 0.13aa	10.16 ± 0.93aa
Group V STZ+ insulin *EEOA* (400mg/	6.51 ± 0.15*	9.08 ± 0.53aa	10.56 ± 0.38aa	12.31 ± 0.84aa

Table no 13 : Effect of *EEOA* on Cold waterTail Immerssion test (Thermal allodynia) in Experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*,**,***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when compared insulin diabetic control

a, aa indicates P < 0.05 & P < 0. 01 Significance between diabetic control group

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT)

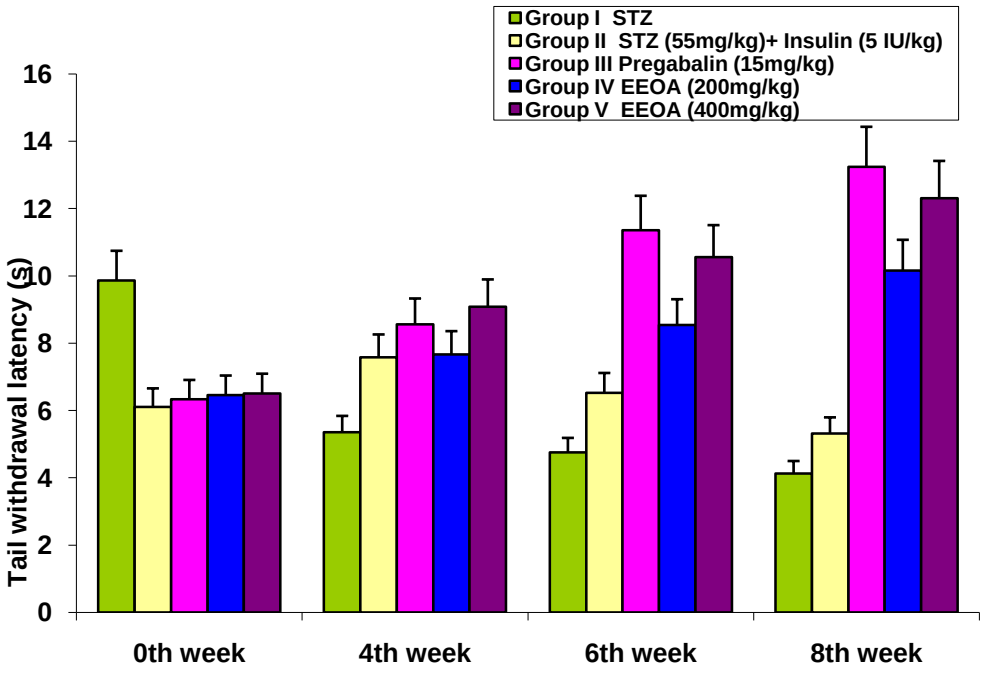


Figure no 24 : Effect of *EEOA* on Cold water Tail Immersion test (Thermal allodynia) in Experimentally induced Diabetic rat model

RESULTS:

The Tail withdrawal latency (Sec) were measured and showed in Table no.13 and Fig. no-(24). The Diabetic control (Group 1) showed significant reduction in tail withdrawal latency when compared to the insulin diabetic control(Group 2).

Standard (Group 3) and *EEOA* (200& 400 mg/kg) showed statistically significant increase in reaction time when compared to the Diabetic control (group 1).

Effect of *EEOA* on Cold Plate test (cold allodynia)

Experimental Croups	Reaction Time in Sec			
	0 Week	4th Week	6th Week	8th Week
Group I STZ (55mg/kg)+vehicle	11.54 ± 0.27	4.96 ± 0.28	4.48 ± 0.27	4.13 ± 0.12
Group II STZ+ Insulin (5 IU/kg)	6.23 ± 0.28 ***	6.5 ± 0.92***	4.46 ± 0.21***	4.56 ± 0.38***
Group III STZ+ insulin + Pregabalin (15 mg/kg)	6.78 ± 0.14** ns	6.98 ± 0.94**	8.56 ± 0.26**	9.78 ± 0.96**
Group IV STZ+insulin+ *EEOA* (200mg/kg)	6.82 ± 0.27* a	8.78 ± 0.24*	9.17 ± 0.34ns a	9.87 ± 0.34ns aa
Group V STZ+ insulin *EEOA* (400mg/	6.35 ± 0.78ns a	8.31 ± 0.39ns a	9.26 ± 0.76ns aa	10.13 ± 0.38ns aa

Table no 14 : Effect of *EEOA* on Cold plate test (cold allodynia) in Experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*,**,***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when compared insulin diabetic control

a, aa indicates P < 0.05 & P < 0. 01 Significance between diabetic control.

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT)

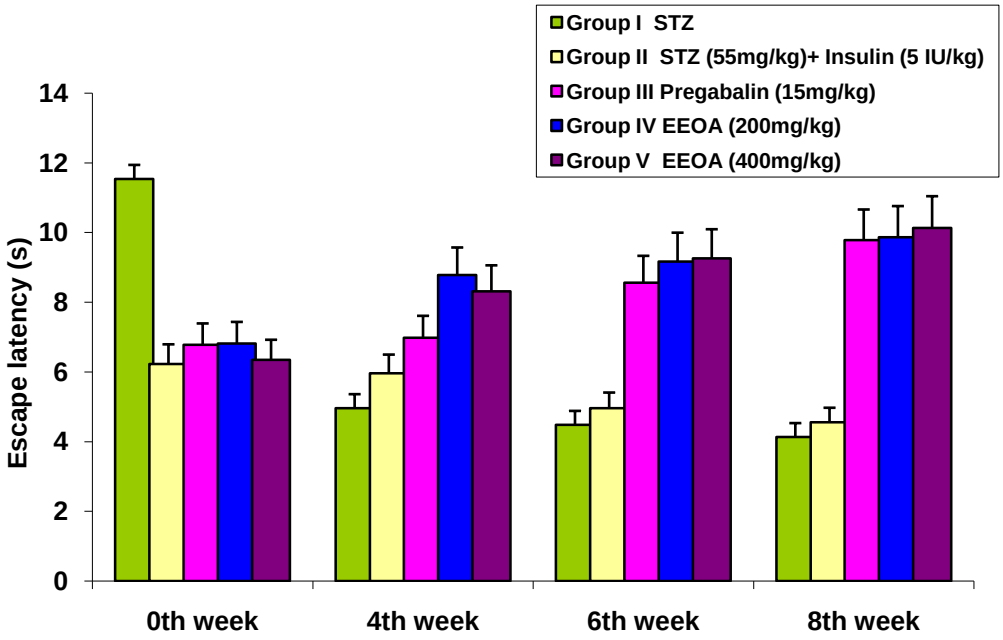


Figure no 25 : Effect of *EEOA* on Cold plate test (cold allodynia) in Experimentally induced Diabetic rat model

RESULTS:

The paw escape latency (Sec) were measured and showed in Table no.14 and Fig. no-(25). The Diabetic control (Group 1) showed significant reduction in tail withdrawal latency when compared to the normal control (Group 1).

Standard (Group 3) and *EEOA* (200& 400 mg/kg) showed statistically significant increase in reaction time when compared to the Diabetic control (Group 1).

Effect of *EEOA* on Tail clip test (mechanical hypralgesia)

Experimental Groups	Reaction Time in Sec			
	0 Week	4th Week	6th Week	8th Week
Group I STZ (55mg/kg)+vehicle	6.93 ± 0.22	11.23 ± 0.56	11.87 ± 0.53	11.94 ± 0.73
Group II STZ+ Insulin (5 IU/kg)	11.67 ± 0.64 ***	10.26 ± 0.56***	10.56 ± 0.53***	10.62 ± 0.73***
Group III STZ+ insulin + Pregabalin (15 mg/kg)	7.39 ± 0.13** ns	7.98 ± 0.61**	8.19 ± 0.36**	10.22 ± 0.86**
Group IV STZ+insulin+ *EEOA* (200mg/kg)	8.73 ± 0.87* a	8.92 ± 0.25*	9.16 ± 0.84ns a	10.58± 0.22ns aa
Group V STZ+ insulin *EEOA* (400mg/	8.98 ± 0.54ns a	8.98 ± 0.11ns a	10.56 ± 0.58ns aa	11.15± 0.18ns aa

Table no 15 : Effect of *EEOA* on Tail clip test (mechanical hypralgesia)

in Experimentally induced Diabetic rat model

The values were expressed as Mean ± S.E.M (n= 6 animals in each group)

*,**, ***, indicates significance P < 0.05 , P < 0.01 & P <0.001 when compared insulin diabetic control

a, aa indicates P < 0.05 & P < 0. 01 Significance between diabetic control

NS indicates Non-significant

Data was analysed by one-way ANOVA followed by Duncun Multiple range test (DMRT)

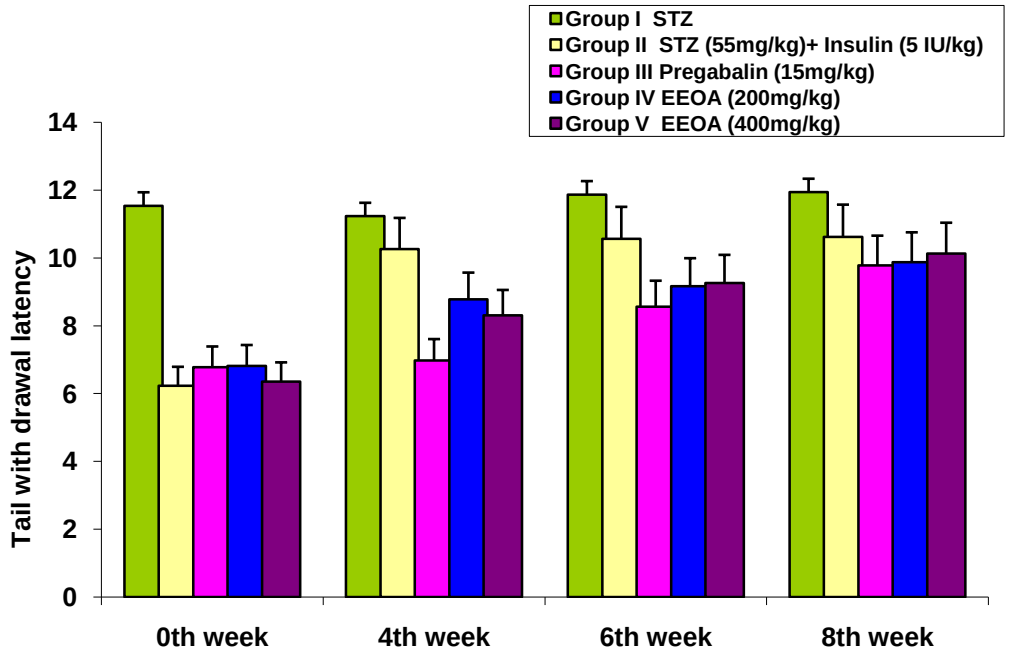


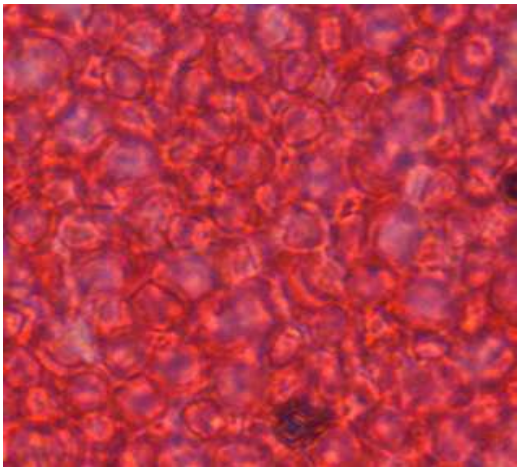
Figure no 26 : Effect of *EEOA* on Tail Clip test (Mechanical Hyperalgesia) in Experimentally induced Diabetic rat model

RESULTS

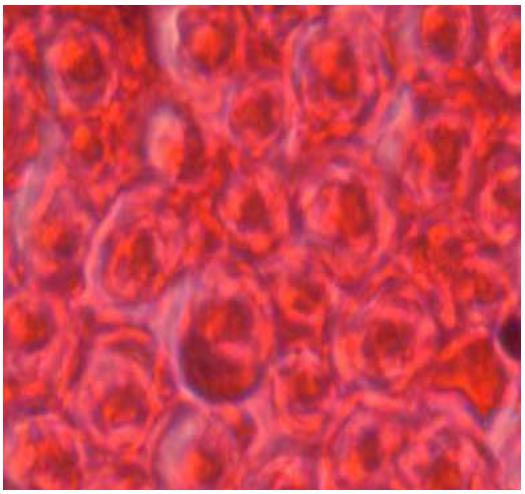
The Tail withdrawal latency (Sec) were measured and showed in Table no-15 and Fig. no-(26). The Diabetic control (Group 2) showed significant reduction in tail withdrawal latency when compared to the diabetic control(Group 1).

Standard (Group 3) and *EEOA* (200& 400 mg/kg) showed statistically significant increase in reaction time when compared to the Diabetic control (group 1).

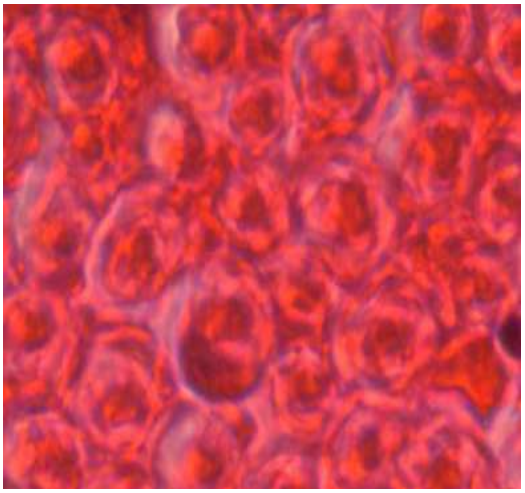
Histopathology studies



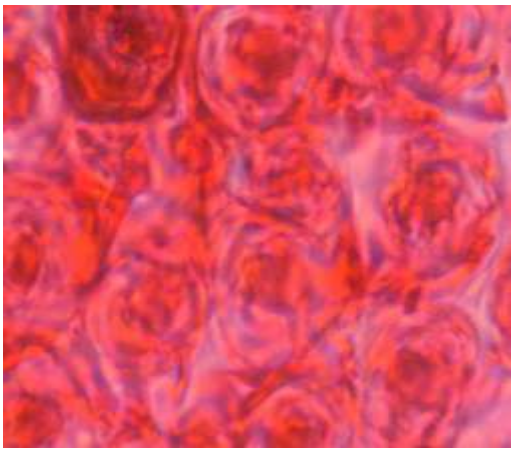
GROUP 1: (Diabetic control): The nerve fascicle is surrounded by relatively thick perineurium. The nerve axons are shrunken. Myelin sheath appears fragmented and separated in some fibers. Edema is also noticed in between the nerve fibers Other field shows marked thickening of less dense myelin sheath in large myelinated fibers with loss of their axons.

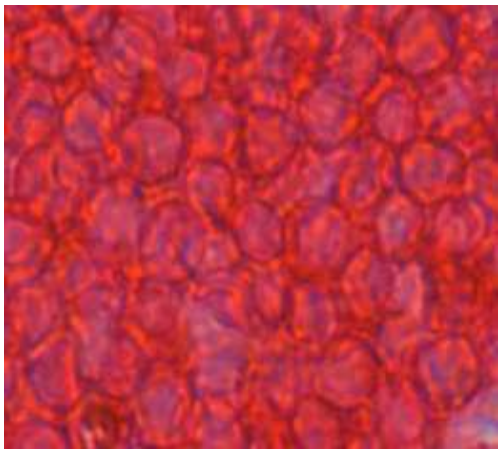


GROUP II: (STZ+ insulin): sciatic nerve is surrounded by the dense connective tissue epineurium. Each nerve fascicle is enclosed in the specialized connective tissue perineurium. The axons appear clear with variable diameters . The myelin is represented by dark ring surrounding the axons.



GROUP III: (STZ+ pregabalin()): Thick myelinated nerve fibers with intact axons (A) surrounded by hypertrophied Schwann cells . The thin myelinated nerve fibers have normal appearance ,edema partly reduced in the endoneurium .





GROUP IV:(STZ+*EEPS*(200mg/kg) GROUP V:(STZ+*EEPS*(400mg/kg))

Group IV and V receiving group specimen showed most nerve fibers normal, with normal nucleus, normal myelin thickness and maintenance of basement membrane of Schwann cells.

DISCUSSION

Hyperglycemia must be the originator in the pathogenesis of biabetic complications. Diabetes is a heterogeneous set of disorders with a range of pathologies and one of the most frequent complications of diabetes ids peripheral neuropathy[162]. Diabetic neuropathy is characterised by progressive chronic neuropathic pain that is tingling and burning in nature with hyperesthesia(excessive physical sensitivity, especially of the skin) and paresthesia(an abnormal sensation,typically tingling or pricking(pins and needels), caused chiefly by pressure on or damage to peripheral nerves) with deep aching and it is increased by touch [163]. Neuropathic pain is defined as a form of chronic pain that results from damage or abnormal function of central or peripheral nervous system. The clinical presentation of neuropathic pain induces hyperalgesia, allodynia and spontaneous pain[164,165]. Hyperglycemia leads to the toxicity of neurons due to increased glucose oxidation , leading to increased reactive oxygen species that may be controlled by the treatment with antioxidant.[166-168.]

In this study, *Ottelia alismoides*(*L*) PERS., extract was given for the treatment of neuropathic pain in STZ induced diabetic rats.

The preliminary phytochemical analysis of *EEOA* revealed the presence of Glycosides, Alkaloids, Flavonoids, Tannins, Terpenoids, phenolic compounds.The tannins, phenols and flavonoids present in the plant *Ottelia alismoides* shows anti-diabetic action. The alkaloids and glycosides present in

the herbal drugs are proved to be potent antioxidants as well as nephroprotective agent[169].

Acute toxicity studies revealed the non-toxic nature of the *EEOA* there was no lethality or any toxic reactions found with high dose (2000 mg/kg body weight) till the end of the study. According to the OECD 423 guidelines (Acute Oral Toxicity: Acute Toxic Classic Method), an LD_{50} dose of 2000 mg/kg and above was considered as unclassified so the *EEOA* was found to be safe.

In the present study STZ was used for induction of diabetes neuropathy in rats. The development of neuropathy was observed at 0 week after STZ induction, which was consistent with previous reports[170]. STZ induced diabetic animal models are most accepted animal models of diabetic neuropathy owing to their reproducibility and reversible diabetes[171,172]. STZ action involved pancreatic DNA alkylation through GLUT2 transporter mechanism. Which intern triggers multiple biochemical pathways such as polyol pathway, hexosamine pathway, protein kinase C pathway(PKC), advance glycation end (AGE) product and poly adipose ribose polymerase (PARP) pathway all of these pathways contribute towards oxidative stress by generating ROS in a mitochondria results in nerve damage and neuropathy[173-175]. Rats injected with 55mg/kg showed significant increase in blood glucose levels and decreased nociceptive thresholds. Similar thermal hyperalgesia, mechanical and formalin evoked pain in STZ induced rats have been demonstrated earlier[176,177]. Pregabalin a selective Ca 2.2 (α2-δ subunit) channel anatagonist and it has has anti neuropathic pain, anticovulsant and anxiolytic activities[178]. Treatment with *EEOA* extract along with insulin in

diabetic rats significantly increased the nociceptive threshold, decreased oxidative stress in a dose dependent manner.

Before recording the findings on the kidney and liver the effects of STZ on the plasma glucose levels and body weights were noted. The observations and results of the present study demonstrated that STZ was effective in producing severe hyperglycaemia in experimental animals. The animals Treated groups II,III & IV appeared ill-looking with loss of their body weights because of injurious effects of STZ which caused alkylation of DNA and produced hyperglycaemia and necrotic lesions[179,180]. Our *in vivo* results showed that *EEPS* extract (200 and 400 mg/kg) treatment had an attenuating effect on the serum glucose of the diabetic animals with significant improvement in body weight it may be due to *EEPS* persisting insulin secretogoge action like sulfonylureas.

Increase in the weight of kidney (hypertrophy) in STZ treated rats. The mechanism of renal hypertrophy is unknown, evidence suggest that local alterations in the production of one or more growth factors and/or their receptors are crucial to this process[181]. An increase (hypertrophy) in the weight of liver was compared with animals in treated groups. It could be attributed to increased triglyceride accumulation leading to enlarged liver which could be due to the increased influx of fatty acids into the liver induced by hypoinsulinemia and the low capacity of excretion of lipoprotein secretion from liver resulting from a deficiency of apolipoprotein B synthesis[182]. The *EEOA* treated animals were found to have significant increased in organ

weights when compared with that of animals with diabetes induced neuropathy.

Oxidative stress is believed to be a biochemical trigger for sciatic nerve dysfunction and reduced endoneurial blood flow in diabetic rats. Persistent hyperglycemia disturbs the endogenous antioxidant defense mechanism and prevents the free radicals scavenging activity[183]. In this study the activities of various antioxidant enzymes SOD, CAT, GPx, LPO had been found to be modulated in diabetic animals. The hyper activities of CAT, SOD could be a response of overproduction of H_2O_2 and free radicals in the diabetic sciatic nerve. The significant different in the activities of various biomarkers in diabetic versus treated animals indicated the augmented oxidative stress and tissue response during diabetes. Moreover nervous tissue is considered to be rich in polyunsaturated fatty acids mainly arachidonic acid and docosahexaenoic acids which are highly sensitive to LPO and depleted antioxidants status. The diabetic animals exhibited the similar biochemical imbalance in term of increased LPO levels and decreased GSH levels. Hence, these levels revealed the magnitude of oxidative stress and extent of structural and functional damage to the nervous system[184].

Diabetes causes increases in the LPO products such as malondialdehyde (MDA) or conjugated dienes in sciatic nerves. Scavenging free radicals reduced the above effects of sciatic pain models[185]. In the present study *EEOA* exhibited significant decrease in endogenous antioxidant enzyme. This antioxidant activity of the *Ottelia alismoides* extract could also have influenced its activity in diabetic neuropathic pain.

Acute hyperglycemia induced by STZ elicits reduction of Na^+, K^+, ATPase activity. Decrease in sciatic nerve Na^+, K+, ATP ase activity could alter the normal membrane axon repolarisation after the depolarization induced by an action potential. *EEOA* treatment restored of Na^+ K^+ ATPase activity. It might be possibly improved by inhibition of oxidative stress and also by amelioration of vascular function[186s].

Behavioral studies in diabetic rats often focus on the response to a painful or non-painful sensory stimulus, there by measuring hyperalgesia and allodynia respectively. The simplest of such tests measures the time to withdrawal of a limb such as the tail or a paw from a noxious heat source, with a faster withdrawal time being interpreted as hyperalgesia and a slower one as hypoalgesia[187]. In the present study diabetic neuropathy using animal models such as thermal hyperalgesia (tail flick, hot water tail immersion, hot plate) mechanical hyperalgesia (tail clip method) cold allodynia (cold plate, cold water tail immersion) and chemical hyperalgesia (formalin test)[188].

These models are supposed to be behavioural biomarkers of diabetic neuropathy. The hot plate and test involves two types of responses paw licking and jumping. Both responses integrate at supraspinal structures with the C and Aδ type I and II sensitive fibres participating in this model. The tail withdrawal (tail flick, tail clip, tail immersion) respons after thermal stimuli is elicited by the spinal motor reflex most probably via endogenous release of substance P in the spinal cord[189]. *EEOA* extract showed antinociceptive effect in the diabetic rats in both hot plate and tail immersion models, which indicate that *EEOA* may act via central mechanism. Cold allodynia (cold plate) might

be caused by disproportionate loss of Aδ fibers and sensitization of cold receptors in peripheral neuropathic pain. Impaired blood flow also seems to contribute to allodynia[190]. Vasodilator treatment has been demonstrated to reduce allodynia in diabetic rats. *EEOA* may act on improved neuronal blood flow by its direct vasorelaxant properties.

The nociception induced by formalin is associated with injured tissue. It characterised by two phases acute & delayed. The acute phase(0-10min) is short lived and initiates immediately after injection and is characterised by C-fibre activation due to peripheral stimuli. The delayed phase(20-40min) is a longer, persistent period caused by local tissue inflammation and also by functional changes in the dorsal of the spinal cord. Therefore, this phase is inhibited both both by opioids and analgesic agents. Substances that act primarily as central analgesic inhibit both phases while peripherally acting drugs inhibit only the delayed phase. In the present study the *EEOA* extract exhibited analgesia only in the delayed phase which suggests that the extract would possibly prevent the inflammatory markers induced pain perception.

The H&E stained lateral section of diabetic control sciatic nerve (A) showed nerve fascicle is surrounded by relatively thick perineurium. The nerve axons are shrunken. Myelin sheath appears fragmented and separated in some fibers. Edema is also noticed in between the nerve fibers Other field shows marked thickening of less dense myelin sheath in large myelinated fibers with loss of their axons compared to (B). Treatment of Pregabalin and *Ottelia alismoides* (400 mg/kg body weight) in diabetic rats was shown to improve the thick myelinated nerve fibers with intact axons (A) surrounded by

hypertrophied Schwann cells . The thin myelinated nerve fibers have normal appearance ,edema partly reduced in the endoneurium in dose dependent manner.

Therefore, *Ottelia alismoides* extract was proposed that in addition to its antidiabetic, the antioxidant properties is the prominent features in attenuation of diabetes induced neuropathy and its generating pain.

10. SUMMARY AND CONCLUSION

The present study was to investigate the Neuroprotective effect of *EEOA* in experimentally induced diabetic neuropathy in rats.

The phytochemical investigation reveals that the presence of tannins, phenols & flavanoids are proved to be effective in diabetic treatment.

Diabetogenic effect of STZ is more reproducible and the metabolic changes are much closer to those existing in human diabetes. Hence STZ is considered the drug of choice for the induction of experimental diabetes.

The Blood glucose level significantly decreased with treatment of *EEOA* proves that it has anti-diabetic activity.

The study shows marked inhibit in oxidative stress by enhancing reduced activity of antioxidant enzyme SOD, CAT, GSH, GPx, Na^+, K^+, ATPase & markedly elevate the level of LPO in liver compare to untreated diabetic control animals, these findings further strengthen beneficial effect of *Ottelia alismoides* as it showed production against STZ induced diabetic neuropathic pain in rats.

After 4^{th} week, diabetic control has shown signs of neuropathy while treated group has showed significant improvement in their condition. From this results, it is evident that *EEOA* has beneficial effect in protective against STZ- induced diabetic neuropathy in rats.

Histopathological assessment also showed that the damage caused by STZ to the sciatic nerve is also markedly reduced by the administration of *Ottelia alismoides.*

Finally these findings suggest that *Ottelia alismoides* treatment must be beneficial to treat pain in diabetic animals.

11. FUTURE PROSPECTIVES

Further study is required.,

1. To isolate and separate the active phytochemicals present in the ethanolic extract of the *Ottelia alismoides (L.)* pers.

2. Formulation of the isolated lead molecule can be designed.

3. Clinical trial of the formulated molecule in healthy human volunteers or diseased persons.

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