EXPLORING ETHANOLIC EXTRACT OF *ALPINIA CALCARATA ROSCOE* RHIZOME FOR ITS PERIPHERAL NEUROPROTECTIVE ACTIVITY IN WISTAR RATS

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For the award of the degree of

DOCTOR OF PHILOSOPHY

In the Faculty of

PHARMACY

by

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(Reg No: KLEU/Ph.D./2020-21/DO1220034)

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February 2025

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_____

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LIST OF ABBREVIATIONS

AC:	Alpinia calcarata
ACR:	Alpinia calcarata Roscoe
AgNPs:	Silver nanoparticles
AMs:	Alveolar macrophages
ANOVA:	Analysis of variance
ATP:	Adenine triphosphate
AVG:	Average
b.w.:	Body Weight
BBC:	Blood-brain barrier
CAT:	Catalase
CDDP:	Cis-diamminedichloroplatinum
CIPN:	Cisplatin-induced peripheral neuropathy
Cm:	Centimeter
CPCSEA:	Committee for Purpose of Control and Supervision of Experiments on Animals
DC:	Diseased control
DI:	Deionized
DIPN:	Diabetes-induced peripheral neuropathy
DM:	Diabetes mellitus
DN:	Diabetes neuropathy

DNA: Deoxyribonucleic acid

DPN: Diabetic peripheral neuropathy

DRG: Dorsal root ganglion

EAC: Ehrlich-Ascites Carcinoma

EEAC: Ethanolic extract of Alpinia calcarata

EEACR: Ethanolic extract of Alpinia calcarata Roscoe

ELISA: Enzyme-Linked Immuno Sorbent Assay

FBS: fasting blood sugar

GHb: Glycosylated haemoglobin

gm: Gram

GSH: Glutathione

H&E: Hematoxylin and Eosin

HD: High dose

HDM: House dust mites

HEE: Hot ethanolic extraction

HPLC: High-performance liquid chromatography

HPTLC: High-performance thin-layer chromatographic

HRPA: Horseradish peroxidase

Hrs: Hours

Hs-CRP: High sensitivity c reactive protein

HWE: Hot water extraction

Hz: Hazards

i.p. Intraperitoneal

ICH: International Conference on Harmonization

IDF: Inter-national diabetes federation

IL-6: Interleukin 6

K+: Potassium

Kg: Kilogram

Kpal: Kulchitsky-Pal

LD: Low dose

LOD: Limit of dryness

LOQ: Limit of quantification

LPS: Lipopolysaccharides

MAPK: Mitogen-activated protein kinase

MDA: Malondialdehyde

mg: Miligram

Min: Minutes

ml: Millileter

mm: Millimetre

MT: Masson trichrome

MW: Molecular Weight

Na+: Sodium

NC: Normal control

NCV: Nerve conduction velocity

NF-kB: Nuclear factor-kappa B

NGF: Nuclear growth factor

nm: Nanometer

ns: Non-significant

^{o}C: Degree Celsius

Pg: Picogram

pH: Potential of Hydrogen

PKC: Protein Kinase C

PN: Peripheral neuropathy

p-value: Probability value

RA: Rheumatoid arthritis

ROS: Reactive oxygen species

rpm: Resolution per minute

RSD: Relative standard deviation

RT: Retention time

SAG: Sigma Aldrich, Germany

SC's: Schwann cells

SD: Standard deviation

Sec: Seconds

SEM: Standard error mean

SOD: Superoxide dismutase

SST: System suitability testing

STZ: Streptozotocin

TGF-ß: Transforming growth factor beta

TMB: Tetramethylbenzidine

TNF-α: Tumour necrosis factor-α

TRP: Transient receptor potential

USA: United States of America

UV: Ultraviolet

V: Volts

Vs: Verses

µg: Microgram

µl: Microleter

µm: Micrometer

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ABSTRACT

Alpinia calcarata Roscoe (ACR) is a member of the Zingiberaceae family, and its rhizome contains bioactive compounds such as flavonoids and terpenoids, as well as phenolic compounds with antioxidant, anti-inflammatory, antidiabetic, antifungal, antibacterial, analgesic, and anticancer properties. This study explores the neuroprotective potential of the Ethanolic Extract of *Alpinia calcarata Roscoe* (EEAC) using Wistar albino rat models of peripheral neuropathy. HPLC analysis confirmed the presence of stigmasterol in EEAC, which may contribute to its therapeutic effects. The rats were maintained in suitable laboratory conditions (12 hours light/dark cycle, 25ºC ± 2ºC temp) with a nutritional diet and water ad libitum and the models prepared and used for the experimental study were diabetic-induced peripheral neuropathy (DIPN) and chemotherapy-induced peripheral neuropathy (CIPN).

DIPN was induced through a single intraperitoneal injection of streptozotocin (STZ) at 55 mg/kg, dissolved in ice-cold 0.1 M citrate buffer (pH 4.4). EEAC treatment significantly ameliorated mechanical allodynia, thermal hyperalgesia, and cold allodynia in a dose-dependent manner. Biochemical analysis showed that the treatment significantly reduced oxidative stress and increased antioxidant enzyme activities, such as superoxide dismutase, catalase, and glutathione peroxidase, in the sciatic nerves of treated rats. Histopathological evaluation revealed reduced nerve damage and degeneration in the EEAC-treated groups and improved myelin sheath and axonal integrity indicating the effectiveness of the treatment in DIPN models.

CIPN was induced with cis-diamminedichloroplatinum II (Cisplatin) administered intraperitoneally at a dose of 2 mg/kg per week for five weeks. The intervention groups were treated orally with EEACR daily during the induction period. Treatment with EEAC showed significant attenuation of neurotoxicity as evidenced by behavioural improvements in mechanical allodynia, thermal hyperalgesia, and cold allodynia. Biochemical assessments revealed a significant decrease in oxidative stress markers and elevated antioxidant enzyme activities, including superoxide dismutase, catalase, and glutathione peroxidase, in the nervous tissues of EEACR-treated rats. Histopathological examination indicated reduced nerve damage and enhanced preservation of myelin and axonal structures in the treatment groups. The findings from this study suggest that EEAC, enriched with stigmasterol, offers promising neuroprotective effects against cisplatin-induced neuropathy in rats.

The results suggest that bioactive compounds in *Alpinia calcarata Roscoe* rhizome may serve as potential therapeutic agents for treating and managing diabetic and cisplatin peripheral neuropathy. However, further research is warranted to elucidate the precise molecular mechanisms underlying the protective effects of the extract.

Key-words: *Alpinia calcarata*, cisplatin, Diabetic, peripheral neuropathy, cis diamminedichloroplatinum II, neuroprotection, oxidative stress, stigmasterol, chemotherapy

1. INTRODUCTION

1.1. BACKGROUND

Neuropathic pain is the most common, but it can cause various ailments. Usually, painkillers suppress peripheral neuropathic pain as well as the central nervous system. Patients suffering from some affectionate pain, numbness, and weakness, including but not limited to it, are called neuropathic pain and nociceptive.[1] Neural aches are generally not simple to cure due to underlying pathophysiology complexity and since the causes of the disease are heterogeneous. Neuropathic pain is more prevalent in females and the elderly population over 50 years old and more common in the 50-64 age group.[2] Neuropathy is damage or degradation in nerve functionality leading to pain and unusual numbness or tingling. It generally happens in the hands and legs but can lead to other areas.

Peripheral neuropathy is a commonly found illness in patients with diabetes and also ones undergoing chemotherapy. Apart from limbs, other areas control nerves and various involuntary functions like urination, and pulse rate are affected. This type of illness is stated as Autonomic neuropathy.[3] Occasionally, neuropathy serves as a symptom of an underlying disorder. Other forms of neuropathy, such as damage to a single nerve, are referred to as mononeuropathies. When multiple nerves affecting all limbs are involved, it is termed polyneuropathy.[4] Polyneuropathy is a condition in which nerve fibres far from the spinal cord or brain are affected. Sometimes, more than two isolated nerves in different areas of the body are affected, a condition known as Mononeuritis multiplex.[5]

Peripheral Neuropathy (PN)

Signal carrier nerves between the brain or spinal cord and other body organs or areas are called as peripheral nerves. Damaging or diseasing of peripheral nerves is called PN. Peripheral means beyond the spinal cord and brain; neuro means with regards to nerve and pathy means illness.[6] Peripheral nerves are mostly unmyelinated and some are encapsulated

by myelin sheath formed by Schwann cells (SC's), connective tissue element (lymphatic vessels and blood, endoneurium sheath, epineurium sheath, and connective tissue sheath) involved differently; Wallerian deterioration and axonal lesion is usual in PN and can be consideration for the underlying cause. PN may be considered a heterogeneous and complicated illness type.[7] PN is a prevalent neurological disorder affecting Peripheral nerves, which help the proper functioning of voluntary movements and involuntary actions. Identifying true PN from various other ill conditions with similar symptoms is essential.[8]

Figure 1 shows the conditions in which Peripheral neuropathy is induced in the human body. Metabolic and endocrine diseases such as Kidney failure, liver failure, and diabetes mellitus are some of the most common reasons behind PN. Leprosy, Lyme disorder, and sarcoid are a few of the Infectious diseases causing peripheral neuropathy. Physical trauma is the severe injury to a body due to an accident or any impact. It can also result in peripheral neuropathy. Deficient condition of vitamins also causes PN. Sometimes, cancer treatment induces PN, which is termed is termed as CIPN (Chemo or Cisplatin-induced PN).

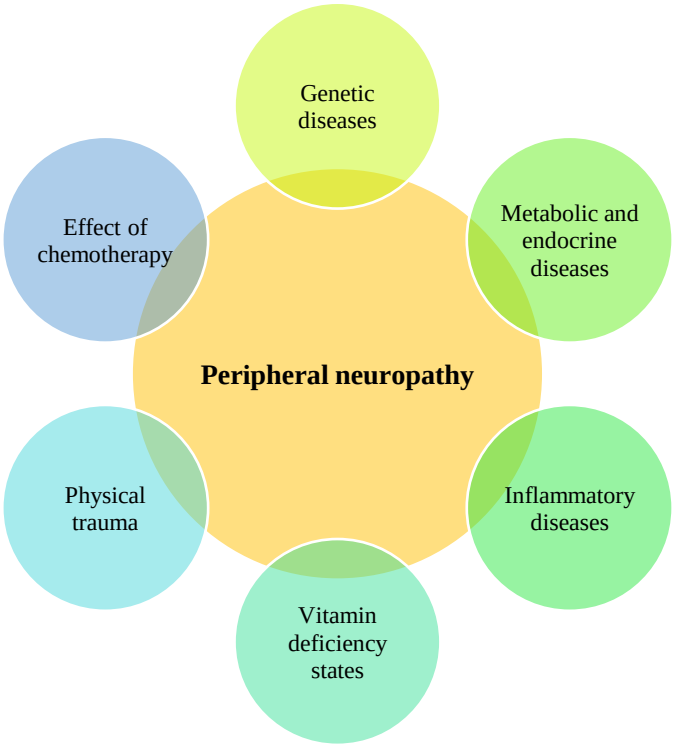


Figure 1: Etiology of Peripheral Neuropathy

Diabetic Peripheral Neuropathy (DPN)

Neural-related aches are a usual symptom of the PN state in diabetes (DPN), which is also a common diabetes mellitus (DM) complexity.[9] DM is a metabolic disorder, a general phenotype of hyperglycaemia, and it arises from insulin shortfall (Type-1) or resistance which is also called as type two diabetes. Increased glucose levels are generally noticed in diabetic patients as skeletal muscle and liver cannot store glycogen, and tissues are unable to utilise glucose. DM is a severe health problem in developed nations. Microvascular and macrovascular complications are the side effects of uncontrolled DM. Microvascular complications are the most common type of DPN.[10] DPN accelerates nerve degradation and is irreversible. Gradual damage of sensory fibres leads to a decline in the robustness of nerves. Diabetic neuropathy consists of autonomic, sensory and motor nerves. DPN involves initial ill-effects that occur in un-myelinated 'C' fibres, resulting in sensitivity degradation, deep muscular pain, physical sensitivity, severe numbness, weakness, continuous burning and allodynia, breakdown of tendon reflexes caused by axonal deterioration in distal body parts (it primarily impact limbs, all four or any of them).[11,12] In 2017, IDF (International Diabetes Federation) reported a global diabetes count of 451 million with year 2045 population projection of 693 million.[13] Almost about half of people with diabetes also suffer from neuropathy, with painful neuropathy affecting approximately 10-20% of diabetic patients.[14] Individuals having diabetes may face nerve complications, which may elevate with obesity, time and age.[15] DPN is a widespread illness that causes dysfunction of nerve fibres and significantly affects diabetic patients' daily activities. The USA alone spends annually about 10 billion USD on DPN and related conditions.[16] DPN may also lead to severe conditions such as dejection feeling, bone ache hypersensation, and mental and physical hypersensitivity.[17]

Determinants for diabetes PN

Diabetes Peripheral Neuropathy

- Advanced age
- Hypertension
- Peripheral vascular disease
- Dyslipidemia
- Poor glucose control
- Long-standing diabetes
- Obesity
- Positive *HLA-DR3/4* genotype
- Excessive alcohol consumption
- Smoking

Pathophysiology/Mechanism for diabetes neuropathy (DN)

Increased blood glucose levels result in insensitivity to insulin and abnormal levels of fat in the blood. It also leads to mitochondrial oxidative stress. As a result, reactive oxygen species start gathering, leading to cell-level damage. Damage to nerve fibre and inflammatory activities are elevated due to chemokine and cytokine generation in peripheral nerve tissues.[18] Diabetes leads to Hyperglycemia which promotes oxidative stress, production of glycation end products and inflammatory cytokines, and epigenetic changes. All these collectively result in neural inflammation and damage to neural transmitters and receptors of which peripheral pain is one of the key symptoms besides numbness and tingling.[19]

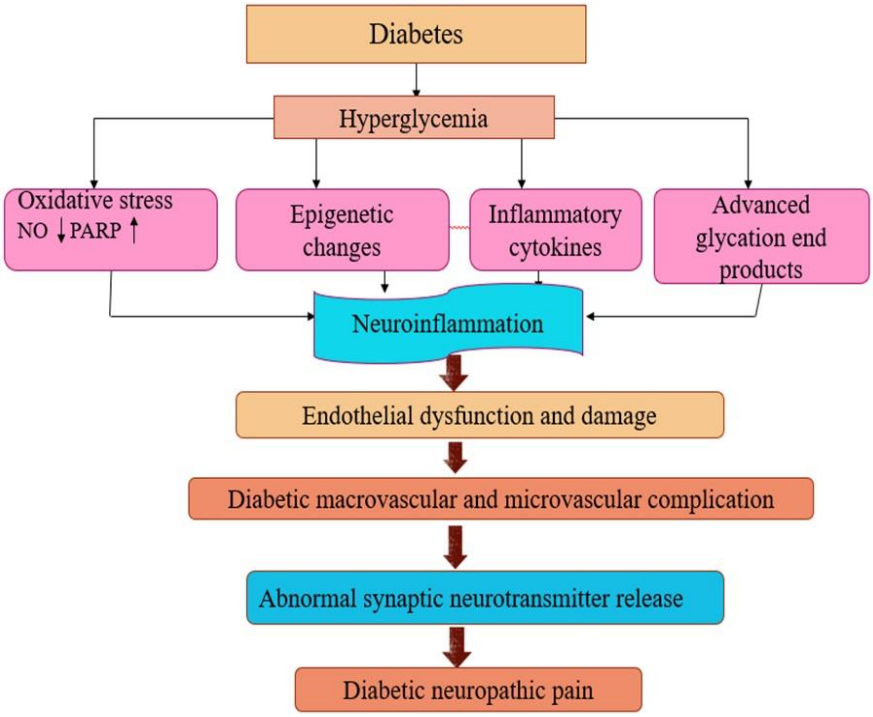


Figure 2: Pathophysiology of Diabetic Peripheral Neuropathy

Chemotherapy-induced Peripheral Neuropathy (CIPN)

CIPN points to unwanted alteration in the peripheral nervous system due to exposure to neurotoxic chemotherapic agents. It is a common and significant non-hematologic ill-effect of chemotherapy. CIPN interferes with delays in optimal treatment and sometimes premature ending of chemotherapy.[20] It can limit motor activities, and increase morbidity with degrading well-being. CIPN can exist anywhere between 30 to 80 %, most of which show severe symptoms during treatment. It results in abnormal somatosensory functioning of the nervous system. Typical illness starts with paresthesia and dysesthesia in the toe or finger area.[21] Many CIPN patients take years to recover, while more than 50% take at least six months post-chemotherapy treatment.[22] Pain and impaired sensory perception can exist for several months to years post-chemotherapy. As a result, cancer may be suppressed leaving back PN. This is particularly significant with platinum-type cancer treatment compounds, as CIPN can continue several months to years' post-treatment.[23] The platinum-based

chemotherapeutic agent cisplatin or cis-diamminedichloroplatinum (CDDP) is widely used to treat several solid tumors. Cisplatin is an anti-tumour used commonly for treating various malignancies including lungs, ovaries, testicles, brain, uterus, and bladder.[24] Cisplatin can react with mitochondrial DNA, generating reactive oxygen compounds affecting axonal transmission due to the axonal localisation of mitochondria, leading to chronic neural weakness.[25] Due to CDDP's severe adverse effects, including peripheral neuropathy, its usage is severely restricted. CDDP Neurotoxicity, hepatotoxicity, ototoxicity, nephrotoxicity, and gastrointestinal toxicity, including peripheral and central neuropathy, have been widely reported. However, it is challenging to pre-identify patients with CIPN because risk factors include prior treatment with neurotoxic drugs such as Metabolic and endocrine diseases such as Kidney failures, liver failure, and diabetes mellitus are some of the most common reasons behind peripheral neuropathy (PN). Leprosy, Lyme disorder, and sarcoid are a few of the Inflammatory diseases causing PN. Physical trauma is the severe injury to a body due to an accident or any impact. It can also result in peripheral neuropathy. Vitamin deficiency, especially Vitamin B1, B12, E, and A, also causes PN. Sometimes, cancer treatment induces PN, which is termed Chemotherapy-induced Peripheral Neuropathy.[26,27]

Pathophysiology/Mechanism for CIPN

The causes of PN induced due to chemotherapy (CIPN) from platinum-based medications: Platinum (Pt)-containing medications stimulate glial cells, and Cisplatin exerts its effects by attracting and activating immunity cells, leading to elevation in inflammation-promoting cytokines levels. The initiation of TRPV1 and TRPA1 channels leads to increased sensitivity and overactivity of peripheral neurons. Along with reactive oxygen species (ROS), these end up harming the blood-brain barrier (BBB). The phenomenon ultimately contributes to the activity of neural inflammation, exacerbating pain and sensory dysfunction. Pt-based drugs cause mitochondrial damage, producing higher reactive oxygen species (ROS). This turn, damages proteins, enzymes and neural lipids disturbing calcium balance, triggering autonomic PN changes along with dorsal root ganglion (DRG) cells. Pt-containing drugs can change the function of Na and K ions, and transient receptor potential(TRP) ionic tracks, leading to increased excitability in peripheral neurons. The processes described above can all modify the responsiveness of peripheral neurons.[28,29]

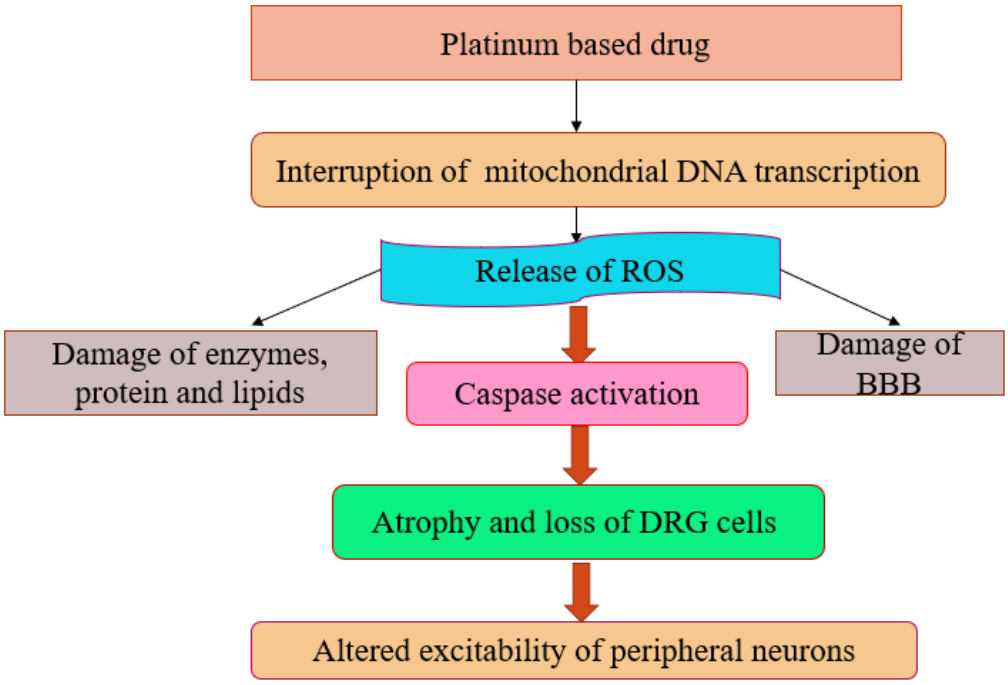


Figure 3: Pathophysiology of CIPN

The development of neuropathy depends on the level of risk involved, with continuous chemotherapy treatments with prolonged dosage being high risk. Pain from anticancer drugs-induced neuropathies is challenging. These neuropathies may degrade an individual's daily activities. They are considered to be relatively resistant to traditional pain-relieving medications such as opioids.[30]

Several agents belonging to various chemical categories were introduced in medical experiments to find cures for motor issues of DPN and CIPN. However, very selective of them have been authorized to be used further.[31]

***Alpinia calcarata Roscoe* :**

Botanical name- *Alpinia calcarata Roscoe* (ACR), **Family**- *Zingiberaceae*

Common name- Snap ginger, Cardamon ginger

Hindi- Kulanjan

Kannada- Chikkadumparaasme

Sanskrit- Rasna, Sugandhamoola, Kulanjana

Traditional medicine is highly regarded across several Asian countries, including Bangladesh, India, Indonesia, Vietnam, Thailand, Malaysia, Sri Lanka, Taiwan, and Cambodia. *Alpinia calcarata Roscoe* (ACR), is from the family *Zingiberaceae*, a rhizomatous perennial plant cultivated abundantly all over India and throughout the oriental countries. It's been widely utilised in Indian Ayurveda and also in Unani traditions in Asian regions. Research also supports traditional plant medicinal use for formulating new drugs with comparatively lesser ill effects.[32,33] ACR's utility is well-known in preservative, spice, and herbal medicine, boasting a rich phytochemical composition that includes flavonoids, glycosides, phenols, phytosterols, and tetracyclic terpenoids.





Figure 4: a) *Alpinia calcarata* inflorescence **b)** Rhizome of *Alpinia Calcarata* Roscoe

Rhizome- The rhizomes are brown coloured 2-6 cm long and width of around 2 cm, branched and high density in nature. The rhizomes are cylindrical and have a single-layered epidermis.

Leaves- Leaves of the plant are simple, distichous, linear, glabrous, margin entire, and apex long acuminate, different 25- 32 cm length and 2.5- 5 cm width. Its single-layered epidermis.

Petioles- Are enveloped structures, 10 to 44 cm, prominent and membranous,

Flowers- Dense with random shapes and bisexual with 8.5 cm in length. It is a graceful, fragrant plant found in the Indian region. [34,35]

Phytoconstituents: Previous phytochemical examination of this plant indicated Alkaloids, Steroids, Coumarins, Cardiac glycosides, Anthraquinones, tannins, and Polyphenols existance.[34,36]

- Essential oils of volatile constituents in roots, leaves and also in rhizomes of AC. Cineol, protocatechuic acid, beta-pinene, and Methyl cinnamate were identified in root and leaves oil, whereas root solids predominantly contain α-ethyl acetate. [37,38,39]

Among its bioactive metabolites, stigmasterol is particularly notable for its significant role in managing and treating conditions. Stigmasterol has already been reported in the literature to play an essential role in treating and managing cancer, diabetes, thyroid, osteoarthritis and other inflammatory disorders.[40,41,42] Plant-derived medicines are vital in proactive healthcare, emphasising disease prevention and promoting overall well-being rather than merely managing symptoms. If ethanolic extract of *Alpinia calcarata* (EEAC) rhizomes proves to be an effective remedy for peripheral neurotoxicity (PN), it could be regarded as gentler than synthetic medicines, offering lesser unwanted ill effects. Herbal medicines have gained popularity among patients seeking to improve their quality of life. This interest has also sparked significant attention from the scientific community, which is increasingly exploring the medicinal properties of plants to validate their effectiveness and discover new therapeutic compounds. This study evaluates physical, behavioural, and biochemical parameters, along with direct measurements of nerve conduction velocity (NCV) along with histopathological analysis of sciatic nerve fibre, to investigate neuroprotective effects of EEAC in DPN and

CIPN. Despite its importance, more literature should be on dedicated methods for quantifying stigmasterol in the EEAC. Therefore, the current exploration goal is to formulate and test high performance liquid chromatography (HPLC) procedure within ICH instructions,[43] an International Conference on Harmonization for analysing stigmasterol content in EEAC rhizomes and evaluating the neuroprotective potential in peripheral neuropathy in a rat model. This initiative continues our exploration of bioactive metabolites in AC, utilising HPLC for its simplicity, precision, and reliability in quantifying analytes.[44]

1.2. REVIEW OF LITERATURE:

Liao W et al., 2022: In the study, Alveolar macrophages (AMs) play a crucial role in respiratory infections and asthma remodelling. Calcaratarin D (CalD), derived from ACR, has proven significant anti-inflammation activities. The CalD role and effectiveness in the rat model of asthma induced by house dust mites (HDM) are explored. CalD's anti-inflammatory role was revealed, effectively modulating AM activity and offering potential therapeutic benefits.[45]

Erusappan T et al., 2022: In this study, anti-inflammatory properties in galangin were explored by analysing nitric-oxide along with the generation of cytokines in lipopolysaccharides (LPS) excited RAW cells. Additionally, qPCR and Western blot techniques were employed to investigate its mechanism by examining mRNA and protein expression levels. The findings suggest that galangin's anti-inflammatory effects are mediated by inhibiting key cell signaling regulators. Notably, this study is one of 1st identifying IRAK1 and JAK1 being targets by galangin, highlighting the potential for treating inflammation-related diseases.[46]

Devi Naorem Manglembi et al., 2023: This study investigated a green synthesis approach for producing spherical silver nanoparticles (AgNPs) using AC, addressing the demand for efficient, cost-effective, and eco-friendly antifungal and antioxidant agents. The fabricated AgNPs demonstrated activity attributed to the dual functionality of surface coverage and the small size of the silver particles. The bioactive components ACR and leaf were found herbal reducing and stabilizing substances, enabling the generation of AgNPs with enhanced antifungal and antioxidant properties. These findings suggest that AgNPs derived from AC represent a promising, eco-friendly solution for fungal control and antioxidant applications.[47]

Shanmugam Pugazhendhi et al., 2022: In this research, the aqueous extract from *Alpinia Calcarata* showed reducing and stabilising agents. The addition of the extract is crucial for gold nanoparticles even spread and also desired dimension is possible to get by adjusting the

extract amount used. The findings revealed that the formulated gold nanoparticles are suitable for optical applications, including optical switches and limiters. [48]

Premrajan P et al., 2021: This study is to investigate whether the phytoconstituent in AC, referred as "Snap Ginger" or "Indian Ginger," has been traditionally used in various medicinal practices, particularly in South and Southeast Asia. Recent studies have highlighted its significant anti-arthritic activity, providing scientific validation for its traditional use in the treatment of arthritis symptoms.[49]

Bhat G et al., 2021: The authors endeavoured to establish crucial oils from the rhizomes and leaves of *Alpinia calcarata Roscoe*. ACR, aside from its medicinal properties, is also valued in perfumery and fragrance formulations. These essential oils are used for their fragrance and as raw materials in producing various phytochemicals and traditional medicine. Additionally, the essential oils from *Alpinia calcarata* have demonstrated significant antimicrobial activity against several pathogenic bacterial strains.[50]

Ferdous M et al., 2020: The present study on *Alpinia calcarata* highlighted leaf's effectiveness in treating pains. This scientific evidence supports historical uses in treating arthritis. Moreover, this research provides evidence for anxiolytic with sedative role of AC leaf extract on central CNS of mice model. This dual action not only aids in pain relief but also helps in managing anxiety and chronic pain conditions such as arthritis.[51]

Raina A P., 2015: In this current investigation, ACR oils were reported to have shown chemical differentiation based on which part of ACR oil was extracted from. Revealed oil's utility as chemo-taxonomic markers in food flavouring, cosmetics, and pharmaceutical products.[52]

Upadhye AS et al., 2018: This research conclusively showed that the ACR develops HPTLC (high-performance thin layer chromatographic) fingerprinting of *Alpinia galanga*.[53]

Erusappan T et al., 2021: this research explored ACR as a potential therapy for rheumatoid arthritis, a chronic auto immune condition affecting polyarthritis in the human body; there is currently no permanent cure for RA. The study indicates that the existence of flavonoids in

ACR effectively moderated RA and Galangin and Quercetin may also have therapeutic use in RA conditions.[54]

Saba P., 2017: In this research, methanolic extracts from *Alpinia calcarata* leaf showed cytotoxic activity and free radical scavenging properties. Hexane extract of rhizome displayed cytotoxic role and the leaf extract showed effectiveness against oxidation.[55]

Singh Subhashree et al., 2020: In this research, Analysis using GC–MS of AC methanolic extract showed the presence of multiple compounds that could be linked to various biological activities. The plant contains several bioactive compounds that validate its traditional medicinal use. There is potential for drug development through subsequent research focused on isolating bioactive compounds, elucidating their structures, and conducting pharmacological activity screenings.[56]

Mathew S et al., 2020: The authors in the current study found that 5 major phytoconstituents are present in ACR methanol extract. Simultaneous classification of phytochemical and High-performance liquid chromatography assessment showed that ACRs contain sterols, terpenoids flavonoids, etc. Traditional use of ACR in treating fungal infections could based on these phytoconstituents. These phytoconstituents indicate that this plant is a good antimicrobial agent. It is observed that *Alpinia calcarata* rhizomes possess a potent anti-fungal activity.[57]

Melanathuru Vijayalakshmi et al., 2017: The water extracts from the rhizomes of *Alpinia calcarata* were reported to have been found in antioxidant and anticancer activity. The current study shows ACR extract exhibited more antioxidant activity. It conclusively found cell viability (effect on Hep G2 cell) of 73.59% with water extract of ACR.[58]

Randhawa Harmandeep K et al., 2016: In this research, flavonoids extracted from ACR showed the potential to inhibit the intrinsic efflux pump system in strains of Staphylococcus aureus.[59]

Tripathi P., 2016: In this investigation, the rhizome of *Alpinia calcarata* was found to demonstrate the potential to serve as a safer natural antioxidant source. The extracts can

minimise or prevent rancidity in edible and medicinal items by supressing oxidation activity which prevents food poisoning or similar conditions. It also preserves nutrition factors with increased shelf life.[60]

Arawwawalaa L D et al., 2012: This study's findings rationalise the medicinal use of ACR as in treating inflammation, using HWE (extraction process using heated water) and Ethanolic Extraction at 500 mg/kg b.w. doses. [37]

Arambewela LS et al., 2004: This research showed that *Alpinia calcarata* rhizome extracts demonstrated antinociceptive role and also confirmed its dose dependency by using hotplate and formalin response checks. However, tail flick testing showed negligible results. A marginally more substantial impact for HEE compared to the HWE was attributed to opioid mechanisms.[61]

Rajasekar R et al., 2014: The present research showed that *Alpinia calcarata* rhizome has antidiabetic and antihyperlipidemic effects, particularly 200 mg/kg b.w. dose in streptozotocin-induced diabetic models of mice. EEACR showed medicinal effects in managing glucose levels, metabolic regulation and potential to manage complications related to diabetes.[62]

Perveen R et al., 2012: In this investigation**,** EEAC rhizome of 8 mg/kg, displayed potent cytotoxic role to treat Ehrlich-Ascites Carcinoma(EAC) type of cancer affected swiss-albino rat. This revealed powerful anti-tumor role of the rhizome.[63]

Arambewela L et al., 2011: In this current investigation, the author and their research team endeavoured to use *Alpinia calcarata* of HWE and HEE, which stayed within acceptable limits of Reno toxicity and liver toxicity, which have been utilized in researching biological functions.[64]

1.3. JUSTIFICATION

Herbal substances' medicinal use has been seen since the ancient human age. Over the years, drug therapy has become one of the significant components of the current healthcare system. The current status of present therapy results from massive research over various years in medicine. Over the last few decades, Synthetic drugs have developed extensively, resulting in advanced formulations for treating multiple diseases. Although allopathic medicine provides quick relief, the relief comes with mild to moderate and severe side effects. Sometimes, side effects are so severe that they affect the everyday life and health of the patients. Hence, having medicine free of unwanted ill effects is important. The modern healthcare system is complete with synthetic and non-herbal drugs. However, these drugs have a systematic and scientific approach to use practice. Herbal medicines, despite their use since ancient times, often lack the advanced development approaches and methodologies applied to synthetic drugs.

Apart from qualified Ayurvedic doctors, practitioners, and healthcare professionals, many people in rural India use herbal medicines directly or indirectly. Therefore, this non-codified herbal preparation's clinical efficacy and safety must be established as a guideline for healthcare practices. Ayurveda has a rich history and growing interest worldwide. Standardising classical herbal medicine can help ensure its quality, safety, and efficacy for a global audience. The drug should not harm patients even if it does not help. Plant-derived medicines represent a crucial aspect of proactive medicine, which focuses on preventing diseases and enhancing overall well-being rather than merely treating symptoms. If EEAC is proven to be an effective remedy for PN, it is likely to be seen as a gentler alternative to synthetic drugs and produce fewer side effects. Herbal medicines have gained popularity among patients seeking to improve their quality of life.

This study uses a scientific approach to standardise herbal medicine. This interest has also sparked significant attention from the scientific community, which is increasingly exploring the medicinal properties of plants to validate their effectiveness and discover new therapeutic compounds. However, the Peripheral neuroprotective activity of EEAC wasn't explored with scientific methods. Thus, the demand for medicinal plants and related compounds is ever-

increasing for phytopharmaceutical studies. And the use of these studies for treating peripheral neuropathy is also growing globally. This study evaluates physical, behavioural, and biochemical parameters, the direct NCV and sciatic-nerve histopathology to identify the neuroprotective role of EEACR in PN treatment.

1.4. OBJECTIVES

PRIMARY OBJECTIVE:

Explore the peripheral neuroprotective role of *Alpinia calcarata Roscoe* rhizome ethanolic extract in the following models:

a) Diabetes-induced peripheral neuropathy

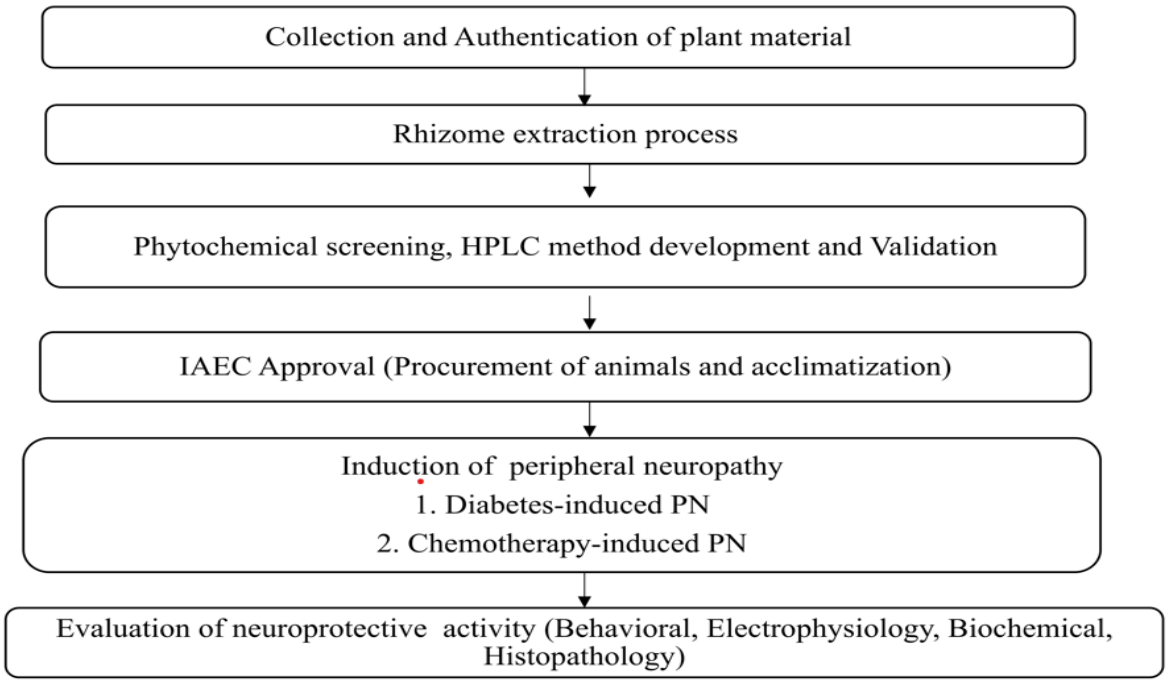
b) Chemo-therapy-induced peripheral neuropathy

SECONDARY OBJECTIVE:

a) Explore the effect of ethanolic extract of *Alpinia calcarata Roscoe* rhizome in Rat models of Peripheral neuropathy by measurement of the behavioural, Electrophysiological, biochemical and histopathological parameters.

b) To develop suitable analytical methods and their validation by chromatographic technique (HPLC).

PLAN OF WORK:


Collection and Authentication of plant material
Rhizome extraction process
Phytochemical screening, HPLC method development and Validation
IAEC Approval (Procurement of animals and acclimatization)
Induction of peripheral neuropathy
1. Diabetes-induced PN
2. Chemotherapy-induced PN
Evaluation of neuroprotective activity (Behavioral, Electrophysiology, Biochemical, Histopathology)

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Collection and Authentication of plant material:

Tender and fully grown rhizomes of ACR gathered from the Indian Institute of Horticultural Research, (Bengaluru) Karnataka, India, in January. The authentication was confirmed by Dr. P.E. Rajasekharan, Nodal Officer and Principal Scientist (GAC Division), recorded at the Department of Plant Genetic Resources, (Bengaluru) Karnataka, India, with specimen voucher# 375657 and verified *Alpinia calcarata* with http://www.theplantlist.org.



Figure 5: Raw material of *Alpinia calcarata* rhizome

2.1.2 Ethanolic extraction of *Alpinia calcarata*:

The freshly collected *Alpinia calcarata* (Haw.) *Roscoe* (ACR) rhizomes were fragmented and air-dried under shade for 2 weeks. 500g dried powder was extracted in 1.5 litres of ethanol via a 4 hrs Soxhlet process. It was then filtered, followed by concentration under diminished pressure at 55°C temp. The percentage yield was 18.5 dry w/w. Then 4°C preserved to use further. Then, Poly-vinyl-pyrrolidone (44,000 MW) coprecipitate in a 1:1 (w/w) ratio was

used for the administration. For subsequent HPLC analysis, the filtrate obtained was a sample solution. [38,65,66]



Figure 6 : Ethanolic extract of *Alpinia calcarata Roscoe* rhizome

Table 1: List of chemicals

S. No	Chemical	Supplier
1.	Streptozotocin	Sigma- Aldrich, Germany (SAG)
2.	Cisplatin injection IP -CISTERO-50 50mg/ 50ml	HETERO HEALTHCARE LIMITED
3.	Stigmasterol (99%)	SAG
4.	Insulin	Eli-Lilly
5.	Acetonitrile (HPLC grade)	Merck Ltd Mumbai
6.	Orthophosphoric acid (OPA) (HPLC grade)	Merck Ltd Mumbai
7.	Tris HCl	Thomas Baker
8.	5,5dithiobis(2-nitrobenzoic-acid) (DTNB)	SAG

9.	Trichloroacetic acid (TCA)	Fisher Scientific
10.	Reduced Glutathione (GSH)	SAG
11.	SOD Assay Kit	SAG
12.	Catalase Assay Kit	Cayman Chemical
13.	MDA (Malondialdehyde	Cayman Chemical
14.	Ferrous sulphate (FeSO4)	SAG
15.	Butanol	
16.	Thio-barbituric acid (TBA)	
17.	Tri-chloroacetic acid (TCA)	
18.	Acetic(acid)	
19.	Glycosylated hemoglobin	Prism Diagnostics Pvt. Ltd, Mumbai
20.	Rat high sensitivity C- reactive protein kit	Krishgen Biosystems Ltd.
21.	Tumor necrosis factor-α (TNF α)	Pierce Endogen, USA
22.	Interleukin 6(IL6)	Pierce Endogen, USA
23.	Other chemicals	S.D Fine Chem. Ltd

2.2 METHODS

2.2.1 Qualitative Phyto-chemical screening of ethanolic extraction of *Alpinia calcarata*:

Phytochemical screening was done using an ethanolic extract of Alkaloids, Terpenoids, Tannins, Steroids, Glycosides, Flavonoids, Saponins, Carbohydrates, and Proteins.[67, 68]



Figure 7: Phytochemical screening of EEAC

2.2.2 HPLC Studies

Steps to carry HPLC method:

- **Preparation of standard sample:**

10mg stigmasterol dissolved with 10ml diluent (1000µg/ml) for further dilutions prepared from these stock solutions, using acetonitrile: orthophosphoric acid (90: 10) to get the desired concentration. The standard stock was prepared in calibrated flasks.

- **Preparation of extract solution**

10mg of *Alpinia calcarata* extract was dissolved in 10 ml of diluent Acetonitrile: Orthophosphoric acid (90: 10). Then 30 minutes of sonication, followed by filtration.

- **Equipment**

An Agilent (1100) gradient HPLC equipment with an Ultra-Violet (UV) detector, an RP C-18 Agilent column with a G1310A pump, and Chemstation (10.01) software were used.

- **Chromatographic Condition**

The chromatographic column was an Agilent C-18 reverse phase with 4.6×250mm, 5um grain size. HPLC system with columns was maintained under ambient conditions. The mobile stage (700uL/min) with 0.1% acetonitrile combined with ortho-phosphoric acid in a 90:10 ratio. 20 µL was injected followed by eluent analysed using wavelength 220 nm with 8-minute runtime, ensuring good compound resolution.

This analytical procedure was cross-verified with ICH instructions (International Council for Harmonisation).[69]

HPLC Method Development and Optimization:

Various solvents tested for forming suitable HPLC procedures to analyse stigmasterol in a herbal extract. The appropriateness of the solvent system was evaluated based on its cost, sensitivity, and the time required to complete a single analysis. The mobile phases tested for this purpose included methanol: water, acetonitrile: water, and acetonitrile: orthophosphoric acid. The condition yielding the best resolution and peak symmetry was chosen.

Validation of method:

The developed method optimised, tested and ensured controllability. Analytical method for determining stigmasterol in the ACR rhizome ethanolic extract was validated by assessing various robustness, precision and repeatability standards per ICH Q2 (R1) regulatory guidelines.

Linearity

Stigmasterol dilutes (10-50 µg/mL conc.) prepared by 1mg/mL stock. Curvature calibration is done by graphing the concentration of stigmasterol against the area under the peak to evaluate linearity.

Sensitivity

Detection limit (LOD) and Quantification limit (LOQ)

These parameters are necessary for understanding the method sensitivity, and they are obtained by applying statistical formulas, LOD and LOQ determination

LOD =3.3×StdDev/Slope

LOQ =10×StdDev/Slope

'SD' represents the response standard deviation, and 'Slope' is of calibration curve.

Precision

Repeatability of measurements within same day and from day to day was checked. Repeatability was assessed by performing (6) replicate tests within 24 hrs, keeping solution and test conditions constant. Similar testing was also repeated on different days. The precision results were shown as the percentage relative SD (% RSD).

Specificity

The method's specificity was assessed by using a blank sample having no analyte and then examining any potential disturbance in analyte retention timing. The same blank solution was injected three times to determine any intervening material eluted at the same retention time.

Ruggedness (Reproducibility)

In HPLC validation, ruggedness measures an analytical method's ability to maintain performance when tiny changes occur in experimental conditions. Ruggedness was evaluated by having two different analysts perform the analysis using the same HPLC system but with other columns.

Robustness

Robustness refers to the method's potential to give precise and repeatable results even in small, intentional variations in chromatographic conditions; it measures a method's reliability. Small and intentional flow rate, wavelength, or temperature changes are made in

robustness studies. The method's robustness was studied by flow changing with 700uL/min**,** and the sample's wavelength (λ max) was 0.22um. Standard solution injection and the variations in RSD were monitored three times.

System suitability

System suitability (SST) testing verifies that a chromatographic system works appropriately before any sample analysis. System suitability testing (SST) is a critical component of many analytical procedures, as defined by ICH and USP (United States Pharmacopeia). System suitability was determined through six consecutive analyses of a standard solution of stigmasterol. Key parameters, including retention time, column efficiency, peak symmetry, and theoretical plates, were determined for the standard solution

Solution stability

Constant solution specimen is analysed at regular intervals to check the same.

2.2.3 Animals

Wistar albino male/female rats, weights ranging from 200 to 250gm maintained in clean polypropylene cages under suitable ambience of 12hrs day/night cycle and room temp ~27 ºC with food and water *ad libitum*. Commercially purchased rat edible pellets from VRK Nutritional Solutions, Pune, Maharashtra were used. Experimentation studies were done and maintained per the CPCSEA guidelines for the KLE College of Pharmacy, Bengaluru, India, animal facility, ensuring the highest ethical standards in our research.

The study was divided into two parts:

2.2.3.1 Part I: Diabetes-induced peripheral neuropathy

Experimental design

Single i.p. 55mg/kg streptozotocin (STZ) dissolved in 4.4pH 0.1M citrate buffer (0ºC) injection was used to induce diabetes in rats.[70,71] To inhibit the triphasic response of drug-

induced hypoglycaemia, a solution of 5% glucose was given to rats overnight. The plasma glucose levels were assessed three days after the injection to ensure diabetes condition. The rats exceeding 250mg/dl were identified as successful DIPN models for further study. Then four groups each consisting of (n=6) individuals were formed and the following treatment was given to animals of different groups:

Group-I – (Normal; NC): No ACR dose

Group-II – (Diseased; DC): i.p. STZ-55mg/kg

Group-III – (Low Dose; LD): i.p. STZ-55mg/kg + ACR 250mg/kg, b.w., for 4 weeks (STZ+ACR)

Group-IV – (High Dose; HD): i.p. STZ-55mg/kg + ACR 500mg/kg, b.w., for 4 weeks (STZ+ACR).[37,61]

Diabetic rats were given 3IU/day insulin for the survival of animals during the treatment and to maintain moderate levels of blood glucose for four weeks. Treatment commenced on the second day following STZ administration, alongside insulin administration (3 IU/kg, subcutaneously).

Neuropathy was assessed after 4 weeks using the following parameter.

1. Body weight and % Glycosylated haemoglobin (GHb)
2. Thermal and cold hyperalgesia by tail flick test
3. Motor co-ordination using rota rod apparatus
4. Grip strength by grip strength meter
5. Beam walk and wire hang

After this, the following parameters were studied**:**

6. Electrophysiological parameters
7. Biochemical parameters
8. Histopathological parameters

2.2.3.2 Part II: Chemotherapy-induced PN

PN is a dose-limiting, potentially long-lasting illness due to several critical neurotoxic medications used in chemotherapy. In particular, platinum agents (cisplatin, oxaliplatin), Taxanes (microtubule-directed) agents [taxols (paclitaxel, and docetaxel)], Vinca alkaloids (vincristine), are commonly used as antineoplastic agents and are known to induce peripheral neuropathy.[72] Development of peripheral neuropathy induced by cisplatin was studied and assessed in rats. Cisplatin (2mg/kg,i.p.) was administered (with 2mL normal saline water in sequence as nephrotoxicity prevention) to form CIPN rat models.[72,73]

Experimental design

After a week's acclimatisation, rats were subjected to a five-week experiment followed by analysis. And the following treatment was given to animals of different groups:

Group I – (Normal; NC): No ACR dose

Group II – (Diseased; DC): 2 ml Saline oral, CDDP 2mg/kg b.w., i.p. once a week, 5 doses.

Group III – (Low Dose; LD): Animals received the 2 ml Saline oral, CDDP 2 mg/kg b.w., i.p. weekly once + ACR 250mg/kg, b.w., orally for 5 weeks (CDDP+ACR)

Group IV – (High Dose; HD): Animals received the 2 ml Saline oral, CDDP 2 mg/kg b.w., i.p. weekly once + ACR 500 mg/kg, b.w., orally for 5 weeks (CDDP+ACR).[37,61]

Neuropathy was assessed after 5 weeks using the same parameters as diabetic peripheral neuropathy. Treatment was given along with the cisplatin for 5 weeks, after which the following parameters were studied**:**

1. Behavioural parameters
2. Electrophysiological parameters

3. Biochemical parameters
4. Histopathological parameters

2.2.4 Evaluation parameters

Physical

Body weight, as well as food/water consumption during the experiment period were monitored of all animals.

Retro-orbital sinus serum specimen gathered with partial anesthesia. Under mild anesthesia, blood was collected using glass capillaries. The serum was separated from blood after centrifugation, and the following parameters were estimated: fasting blood sugar (FBS) and glycosylated haemoglobin, using respective assay kits.[74]

Behavioural parameters

Hot hyperalgesia

Tail immersion testing has been done to assess hot/cold sensitivity. When the terminal part tail was immersed 1 cm in water at a temp. (46ºC), as a response to the thermal sensation, time it takes to remove the tail is considered discomfort or reflexive action, and the max duration was kept at 12sec. [75] Hot hyperalgesia was evident in both DIPN and CIPN, as animals of both models showed less time to flick the tail immersed in hot conditions.

Cold hyperalgesia

The tail immersion test has been performed to assess cold sensitivity. Recorded the tail retraction time when its terminal was immersed 1 cm in water at a temp. (4ºC). As a response to the cold sensation, tail retention time has been noted, and a max limit 10sec set to prevent prolonged exposure.[76] Cold hyperalgesia was evident in diabetic and cisplatin-treated rats, and both models showed less time to flick their tails while immersed in freezing conditions.

Rota-rod Performance (motor coordination)

The retention time was measured using a rotarod apparatus. Rats were put on the rotating rods and recorded the time for which they remained with the rod. The device consists of a rough metal rod, 80 cm wide and 8 cm in diameter, designed to prevent the rats from escaping. In the wooden chamber, the rod was positioned at a height of 50 cm using black-painted acrylic glass. It was mounted on a rotating drum which was partitioned into five, each measuring 10cm. All the rats had three trial runs with a minimum 3-hour gap. Animals were practised daily to learn rota-rod walking at gradually elevated speeds, ranging from 1-50rpm, over 5 minutes. The final test of 5 minutes was done in which the rat's withstand timing was recorded at speeds of 25 and 50 rpm.[77] Diabetic and cisplatin-treated rats exhibited impaired muscle coordination compared to normal rats.

Grip Strength

The rats were familiarised with the grip strength meter over a specified period. Each rat was suspended with its tail to measure grip strength, with horizontally oriented on the apparatus grid. Animals were suspended by their forelimbs. The retention timing of the animal on the wire was noted using a stopwatch, and the response force by the rat was recorded before leaving its grip. Recorded the force exerted by animals.[15] Cisplatin-treated and diabetic rats showed reduced grip strength compared to normal rats.

Wire hanging test

Metal hangers were fixed on top of a cage having bedding and animals were hanged to the hanger. They were released in a short time post their holding wire with forearms instinctively. During the five-minute hanging session, rats used their limbs and tails based on

their hanging ability. The total hanging time was recorded for analysis.[78] The wire hang of diabetic and cisplatin-treated rats and both models were impaired compared to normal rats.

Beam walk

A beam walking test was performed on a wood strip of 20 mm width. Concisely, the rats were required to pass a one-meter wooden pipe positioned at 1m elevation from the ground. Recorded slips count, travelled distance and turn count by test animal. Turns and slip counts are used to assess incoordination.[79] The beam walk of cisplatin and STZ-treated rats and both models were impaired compared to normal rats.

Electrophysiological parameters

Nerve conduction velocity (NCV)

A Power-Lab data-acquisition setup measured non-invasive NCV. An anaesthetic i.p. combination of Xylazine(5-10mg/kg) and Ketamine(80-100mg/kg) was administered during measurement. The sciatic nerve was stimulated using a bipolar electrode with an 8V frequency of 20Hz signal. It calculated the NCV by dividing the stimulating-recording electrode spacing by the difference in proximal and distal latencies. The stimulating electrode probed sciatic-notch and recorded to the 2nd interosseous muscle in the hindfoot and GND-electrode to the hindfoot lateral part. Stimulus latency is considered to be stimulus artefact and M wave top gap. After this similar measurement was done with the recording electrode

$$\text{Nerve conduction velocity (m/sec)} = \frac{\text{Distance between stimulation sites}}{\text{Latency of M wave (sciatic notch) - Latency of M wave (Achilles tendon)}}$$

on the tendon for recording latency. [80,81]

Biochemical parameters

In Diabetes, at the end of the experiments using the STZ-induced model, retro-orbital sinus blood samples were collected with partial anaesthesia. Under mild anaesthesia, blood was collected using glass capillaries. The serum samples were separated from the blood after centrifugation. The following parameters were estimated: serum insulin levels in diabetic neuropathy and C-reactive protein (hs-CRP) in cisplatin-treated and diabetic rats, using specific ELISA kits.[82] And Procured from Krishgen Biosystems Ltd.

Inflammatory Markers (Cytokine assay)

The cytokine TNF-α along with IL-6 assessment was done with commercially available ELISA kits (Spectramax i3x, Molecular devices), following manufacturer instructions from Pierce Endogen (Rockford, IL, USA) and the method used by Whiteside.[83]

Rat TNF-α ELISA

Reagent Preparation:

1. **Wash buffer: A**1x Working washing buffer developed by mixing 20X wash-buffer concentrate along deionised (DI-water).

2. **Standard formation:** Standards are serially diluted from 1000pg/mL to 31.25pg/mL using standard diluent.

Assay procedure:

1. 0.1 ml/well standard with sample poured in dish. The diluent (1X) assay used is 0 pg/ml. The dish kept covered for two hrs at RT.

2. Contents were aspirated followed by plate washing 4 times using 1X-washing-buffer. Residual was removed firmly. All the washes were performed similarly.

3. A volume of 100 µl of the dilution Detected Antibody A solution was dispensed into each well, after which content was air tight closed for one hr at RT.

4. Repeated the washing step in 2 above.

5. 0.1ml Streptavidin-HRPA dilute was introduced into every well and the plate was maintained covered at RT for 30min.

6. Repeated the washing step in 2 above.

7. 100µl TMB was mixed and allowed to mature for half hr in the dark at RT.

8. Terminate the procedure by adding 100µl Stop sol. to all wells and allowing +ve wells to turn yellow from blue.

9. Within half hr post-reaction termination, record 0.45um wavelength absorbance.

Rat IL-6 ELISA

Reagent formation:

1. **Wash buffer:** 20X concentrate diluted by DI water to get 1X working wash buffer solution.

2. **Standard Preparation:** Standards are serially diluted from 8000pg/mL to 125pg/mL using standard diluent.

Assay procedure:

1. Poured in wells 50µl Biotin attached antibody.

2. 100µl/well sample/standard mixed and closed, kept aside at 37°C for two hrs.

3. The contents were aspirated, and the plate underwent washing with washing-buffer(1X) four times. Future washes were performed similarly.

4. 100 µl of the Streptavidin-HRP dilute applied in every well, after which again covered for half hr at 37°C.

5. Repeated washing step as in #3 four times.

6. Then mix 0.1ml TMB Substrate dilute followed by incubation. (37°C, dark, half hr).

7. Each well-received 100µl of the Stop Solution. +ve wells turned yellow from blue.

8. Record 0.45um wavelength absorbance.

Biochemical (Oxidative stress parameters)

Sciatic nerves were isolated and homogenised with the help of probe homogeniser in icing cold 0.1M phosphate buffer with (pH-7.4). Then divided into aliquots to separate the "post-nuclear and post-mitochondrial" fractions.[80] Lipid-peroxidation assessment performed based on MDA levels.[84] Glutathione (GSH) reduction was also evaluated to identify tissue sulfhydryl groups.[85] The activity activity of superoxide dismutase (SOD) was assessed.[86] , and CAT significance was assessed based on the decomposition rate of H_2O_2.[87]

Histopathology of sciatic nerve

Outlayed sciatic nerves, submerged in 10% neutral formalin buffer (pH 7.4) for fixation. Then embedded using paraffin wax for making sections. Fine 5µm sections were obtained via microtome. H&E staining of sections was done to observe the general morphology., Masson trichrome (MT) and Kulchitsky-Pal methods.[88,89] Photographs of the stained sections were taken under a microscope for detailed examination. A pathologist, blinded to the study groupings, conducted a histopathological assessment of the slides to identify any pathological changes.

Hematoxylin and Eosin (H&E) stain

The Feature was noted and scored according to the proforma specially designed for histopathological evaluation of the nerve, emphasising possible diabetic and cisplatin-induced vascular, axonal, demyelinating, and inflammatory changes.

Masson's trichrome stain

This stain highlights collagen (blue). Epineurial, perineurial, and endoneurial collagen were assessed for increased fibrosis, an indirect sign of nerve fibre loss, and the presence or absence of endoneurial vascular hyalinisation, a feature of diabetic microangiopathy.

Kulchitsky-pal (K-pal) stain

This stain highlights large and small myelinated fibres. The sections were assessed for

1) Density of myelinated fibers and loss,

2) Presence of thin myelinated fibres, a feature of demyelination,

3) Several clusters of closely packed tiny myelinated fibres indicate regeneration, axonal sprouting, axonal swelling, and enlarged axons surrounded by stretched-out myelin sheaths.

2.2.5 Statistical analysis

The comparison across groups (normal control, disease control and treatments) was done using one-way variance data analysis (ANOVA). Following the ANOVA, post hoc comparisons were selected using Dunnett's multiple comparison tests. The probability value (p-value) of *0.05* assumed the threshold for statistical significance. Graph Pad-prism software (version 9) was used for ploys and data was analysed statistically to establish Mean±SEM. standard error of the mean.

3. RESULTS

3.1. QUALITATIVE PHYTO-CHEMICAL SCREENING :

Table 2: Qualitative phytochemical screening of Ethanolic extraction of *Alpinia calcarata*

Sr no	Chemical classes	Phytochemical moiety/test	Presence
1	Alkaloids	Mayer-reagent	++
		Dragendroff-reagent	++
		Wagner-reagent	
2	Cardiac glycosides	Keller-Killiani	+
3	Anthraquinone glycosides	Bontrager's test	++
4	Steroids (Triterpenoids)	Liebermann–Burchard test	+
		Salkowski Test	++
5	Saponin	Frothing test	-
6	Flavonoid	Lead-acetate	+
7	Carbohydrates	Molish	+
		Benedict's	++
		Fehling's	-
8	Proteins	Biuret	-

(++): MODERATE PRESENCE, (+): MILD PRESENCE, (-): ABSENCE

3.2. HPLC results:

HPLC Method Development and Validation

Table 3: Chromatographic parameters of developed method

Parameters	**Chromatographic condition**
Column	4.6X250 MM Agilent C-18, 5µm particle
Flow speed	0.7mL/min
Mobile-phase	Acetonitrile: 0.1% Orthophosphoric acid (90: 10)
Run time	8 minutes
Wavelength	220 nm
Temperature of column	Ambient
Injection volume	20 µl
Sample solvent	Acetonitrile:0.1% Orthophosphoric acid (90: 10)

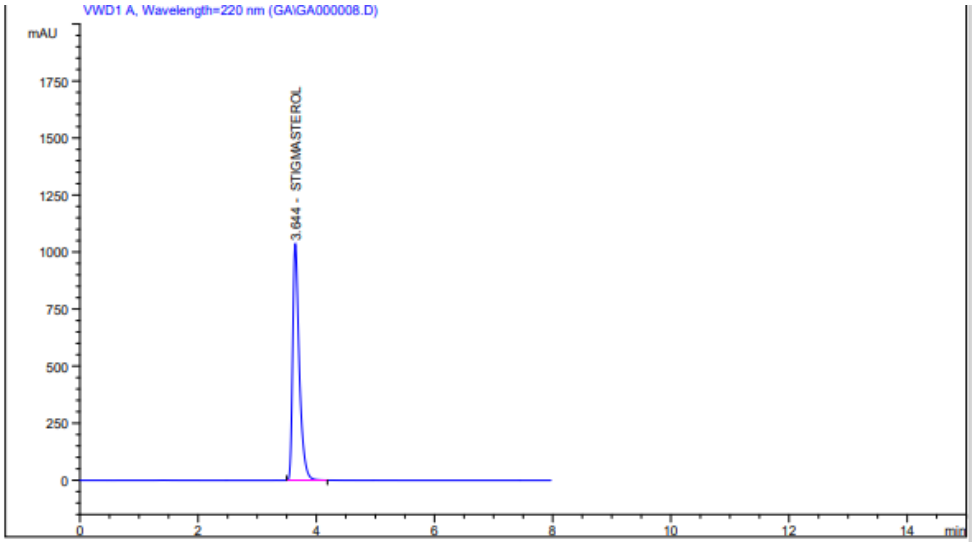


Figure 8: Stigmasterol HPLC chromatogram

Table 4: Linearity range of stigmasterol

CONC	AREA
10	3801.614
20	5060.931
30	6275.39
40	7623.19
50	9054.5

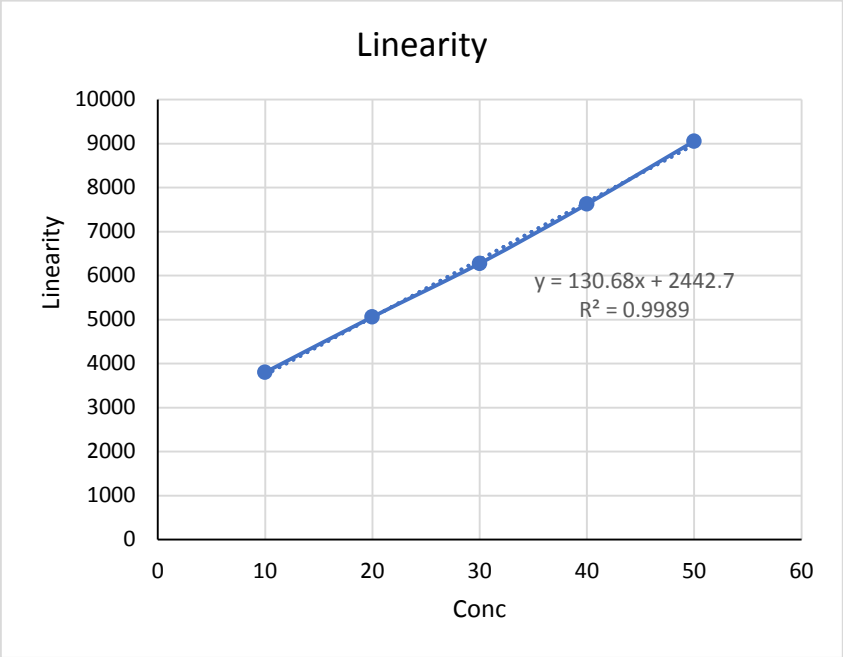


Figure 9: Linearity of stigmasterol

Table 5: Repeatability and intermediate precision

	Inter-day		Intraday 1day	2day	3 days
Sr No	**Area**	**RT**	**Area**		
1	5060.93	3.5	5083.56	5053.51	5023.12
2	5085.21	3.5	5106.27	5107.23	5116.95
3	5103.93	3.5	5112.34	5122.32	5122.12
4	5094.29	3.5	5078.38	5098.48	5068.38
5	5069.38	3.6	5094.59	5084.34	5094.12
6	5073.16	3.6	5073.12	5043.23	5043.12
Avg	5081.15		5091.377	5084.852	5077.968
%RSD	**0.319682**		**0.281779**	**0.556554**	**0.721081**

Table 6: Ruggedness

	Area by analyst 1	**Area by analyst 2**
	5060.9311	5013.32
	5085.211	5116.78
	5103.93	5102.67
	5094.29	5088.12
	5069.38	5034.43
	5073.16	5048.54
Avg	5081.15035	5067.31
SD	16.24350108	37.5921
%RSD	0.319681567	0.741855

Table 7: Robustness

Condition	**Modification**	**Area**	**Mean**	**SD**	**%RSD**	**Rt**	**SD**
Mobile Phase flow rate	6	5216.23	5260.667	52.67299	1.001261	3.81	0.077675
ml/min	6	5318.85				3.96	
	6	5246.92				3.92	
	7	5060.93	5074.953	16.16929	0.31861	3.51	0.05
	7	5071.29				3.56	
	7	5092.64				3.61	
	8	4822.16	4893.71	70.08881	1.432222	3.17	0.017321
	8	4896.73				3.2	
	8	4962.24				3.2	
Wavelength	219	5016.73	5019.493	12.93825	0.25776	3.56	0.020817
nm	219	5008.16				3.59	

	219	5033.59				3.6	
	220	5060.93	5074.907	16.05984	0.316456	3.65	0.081445
	220	5071.34				3.52	
	220	5092.45				3.5	
	221	5087.93	5062.66	26.73398	0.528062	3.6	0
	221	5034.67				3.6	
	221	5065.38				3.6	

Table 8: System suitability test for stigmasterol

Drug	area	Theoretical plate	Tailing factor	Rt
stigamasterol	5060.93SD	8123	0.99	3.6

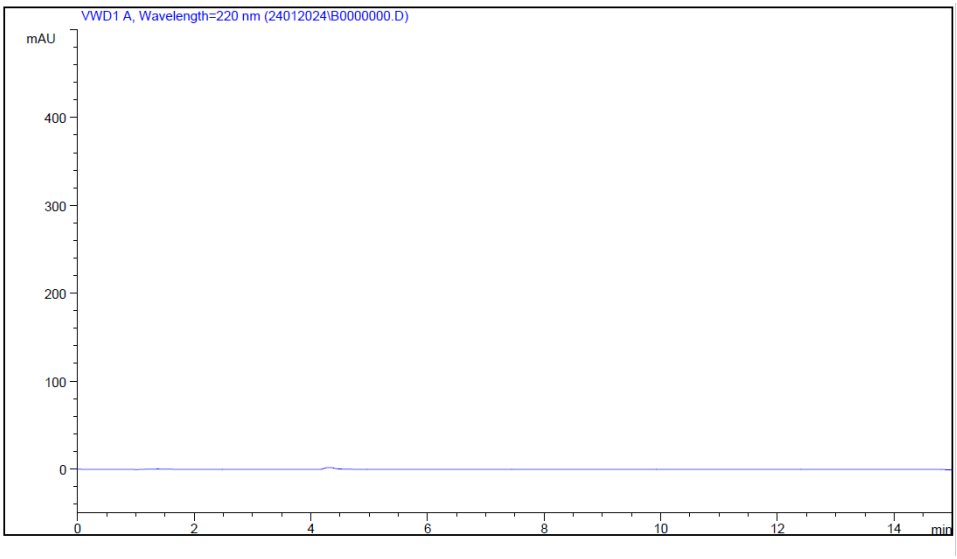


Figure 10: HPLC peak of stigmasterol

Table 9: Validation parameter results of simultaneous method

Parameters	**Stigmasterol**
Linearity	y=138.68X+ 2442.7
Range (µL/ml)	10-50 µg/ml
R^2	0.998
LOD(µg/mL)	02.080µg/ml
LOQ(µg/mL)	06.330µg/ml
Precision %RSD	
Precision Intra-day (%RSD)	
Area covered by function curve	0.721081
Precision Inter-day (%RSD)	
Area covered by function curve	0.319682
Ruggedness (%RSD)	0.741855
Robustness (%RSD)	
Mobile phase Flow rate (ml/min)	0.31861
UV detector wavelength (nm)	0.316456
System suitability	
SD	5060.93
Theoretical plate	8123
Tailing factor	0.99
Rt	3.6
Stability	72 hrs

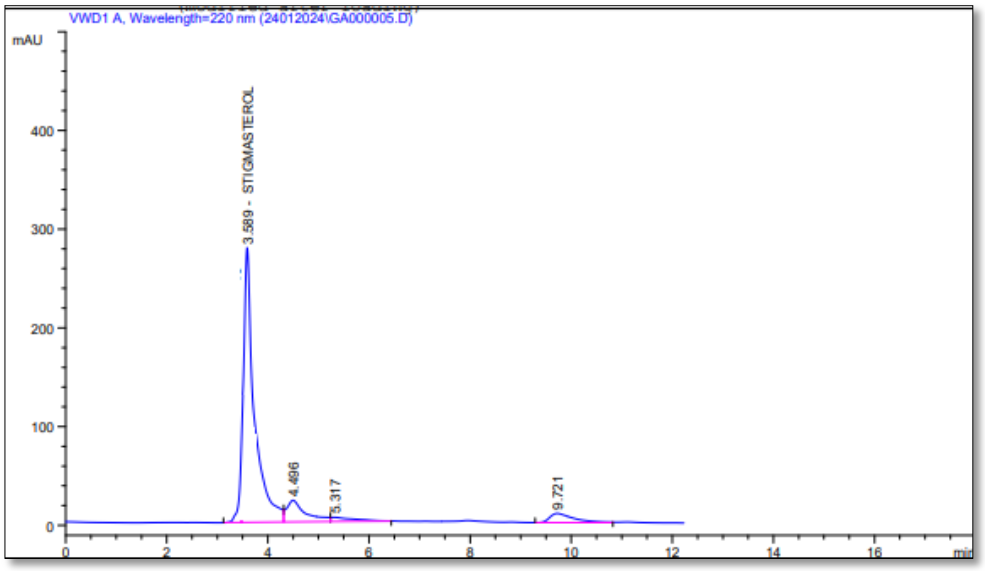


Figure 11: Chromatogram of stigmasterol in *Alpinia calcarata* ethanolic extract

3.3. Part I: Diabetes-induced peripheral neuropathy:

Table 10: Effect of EEAC on the consumption of water/food, weight, Plasma-glucose level, % Hba1C, behavioural parameters

Evaluation parameter	Group **I**-(Normal).	Group **II**-(STZ).	Group **III**-(STZ+ ACR 250mg/kg)	Group **IV**(STZ + ACR 500mg/kg)
Food consumption (gm/day/animal)	21.14±0.845	17.78±1.306$^{\#\#}$	20.75± 1.352*	24.23±0.1502**
Water Intake (ml/day/animal)	22.77±1.212	42.91±1.611$^{\#\#\#}$	33.10±1.539**	29.75±1.814***
Weight (gm)	229.3±10.72	145.4±6.154$^{\#\#\#}$	192.2±8.778**	211.1±8.825***
Plasma glucose level (mg/dl)	105.8±5.062	317.4±9.288$^{\#\#\#}$	278.7±13.30*	254.8±9.354***
Hba1C (%)	4.262±0.117	11.02±0.4254$^{\#\#\#}$	9.153±0.115***	10.01±0.129*
Hot Hyperalgesia (sec)	9.47±0.289	5.517±0.1815$^{\#\#\#}$	6.467±0.160*	8.467±0.274***
Cold Hyperalgesia (sec)	15.17±0.268	9.217±0.3260$^{\#\#\#}$	12.77±0.55***	13.79±0.530***
Motor coordination (sec)	204.83±6.71	28.5±4.41$^{\#\#\#}$	99.83±8.20***	176.61±10.50***

Grip strength (newton)	861.7±32.29	485.0±35.47$^{###}$	661.7±55.28*	775.1±38.06***
Wire hang (sec)	48.9±4.79	20.1±1.61$^{##}$	33.6±2.23**	42.0±3.77**
Beam Walk	58.50±7.136	120.3±1.33$^{##}$	96.40±5.50*	77.67±5.60**

###: P<0.001, ##: P<0.01 respect to normal, ***: P<0.001, **: P<0.01, *:P<0.05, ns: P>0.05 with respect to STZ group. Values in Mean ± SEM and n=6.

3.3.1. **Evaluation parameters:**

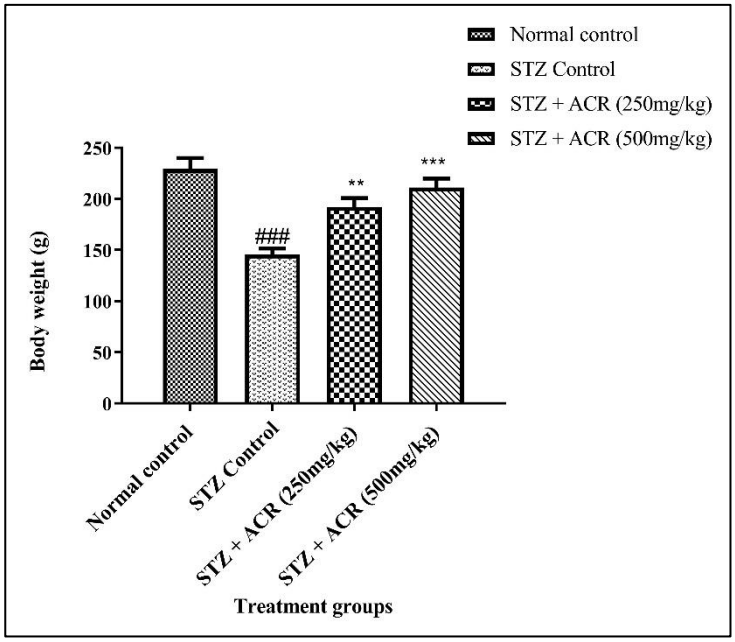


Figure 12: Effect of administration of ACR and STZ on weight. (numbers represented as mean ± SEM with n=6). ### P<0.001 against control, *P<*0.05*, **P<*0.01*, and *** P<*0.001* against STZ set.

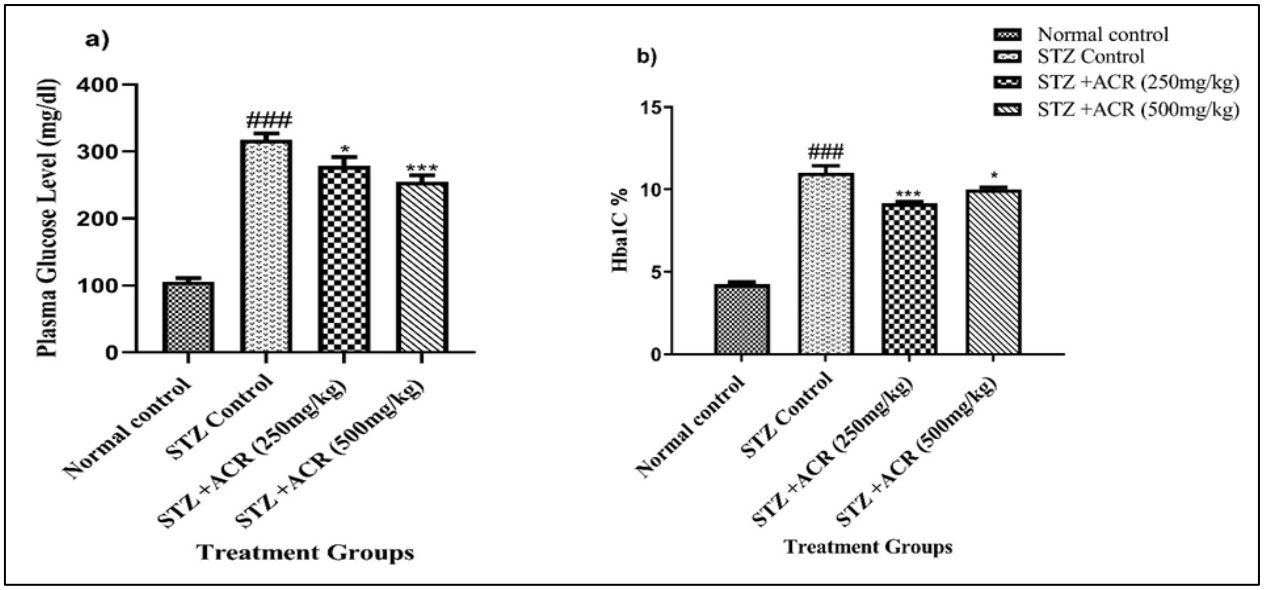


Figure 13: Effect of administration of ACR and STZ on **a)** Plasma glucose level and **b)** % Glycosylated haemoglobin levels in rats. (numbers expressed as mean ± SEM, n=6). ###:P<0.001 against control, *P<0.05,**P<0.01, and *** P<0.001 against STZ set.

3.3.2. **Behavioural parameters**

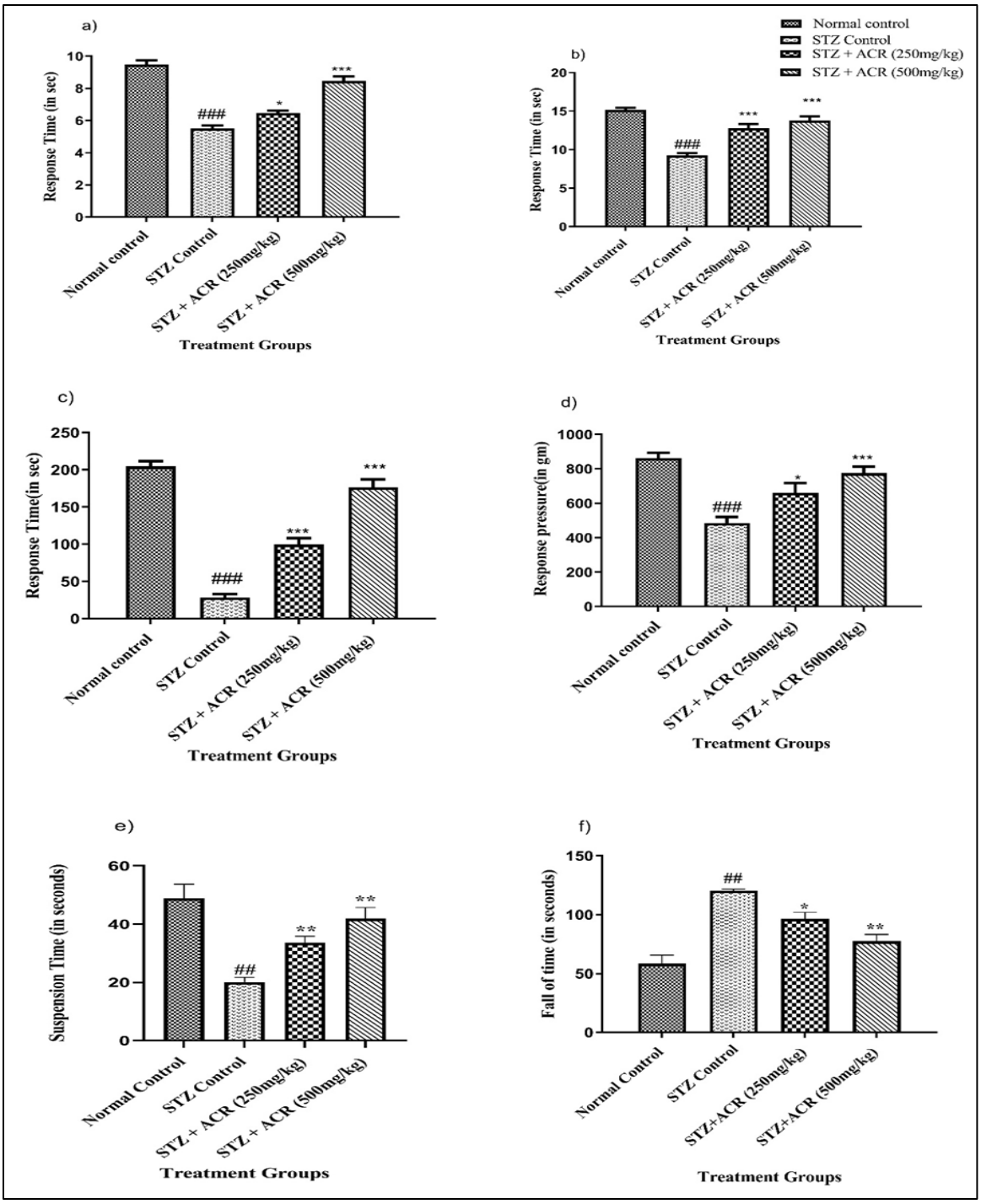


Figure 14: ACR treatment results in **a)** hot water, **b)** cold water, **c)** Rota-rod, **d)** Grip strength, **e)** Wire hang, **f)** Beam Walk. Numbers in Mean ± SEM, n=6. ### P<0.001, ##P<0.01 against normal; *P<0.05, **P<0.01, and *** P<0.001 against STZ.

3.3.3. **Electrophysiological parameters:**

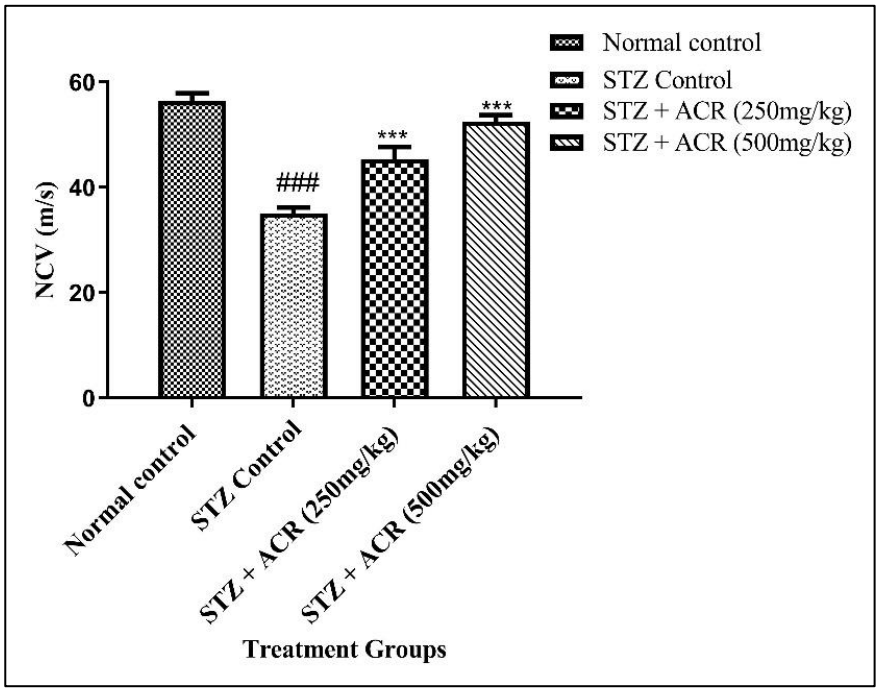


Figure 15: Effect of administration of ACR and STZ on NCV. (results represented in mean ± SEM, n=6). ###: P<0.001 against control; *** P<0.001 against STZ.

Table 11: EEAC treatment results on inflammatory markers and serum insulin parameters

	Evaluation parameter	Group **I**-(Normal).	Group **II**-(STZ).	Group **III**-(STZ+ ACR 250mg/kg)	Group **IV**(STZ + ACR 500mg/kg)
Inflammatory markers	hs-CRP	1.630±0.529	8.573±0.514$^{###}$	7.712±0.464^{ns}	3.413±0.319***
	TNF-α	29.32±5.575	523.5±28.82$^{###}$	262.8±11.21***	126.3±12.67***
	IL6	33.75±28.94	305.7±56.66$^{##}$	103.0±25.38**	67.42±12.92***
	Serum Insulin	16.82±1.196	7.722±0.209$^{###}$	10.82±0.364*	13.43±0.640***

Numbers in Mean± SEM, n=6. ###: P<0.001, ##: P<0.01 vs. normal, ***: P<0.001, **: P<0.01, *:P<0.05, ns: P>0.05 vs. STZ.

3.3.4. **Biochemical parameters:**

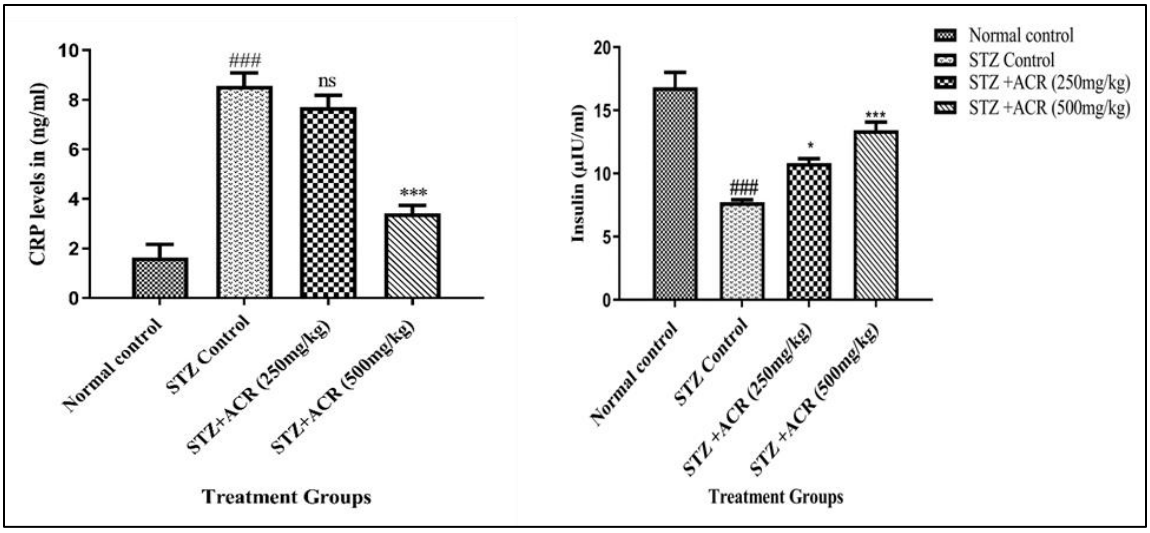


Figure 16: Effect of administration of ACR and STZ on CRP level and Serum Insulin. Numbers in mean ± SEM, n=6. ### indicates P<0.001 Vs. control; *P<0.05, *** P<0.001 and ns compared P>0.05 Vs. STZ.

3.3.4.1. **Inflammatory biomarkers:**

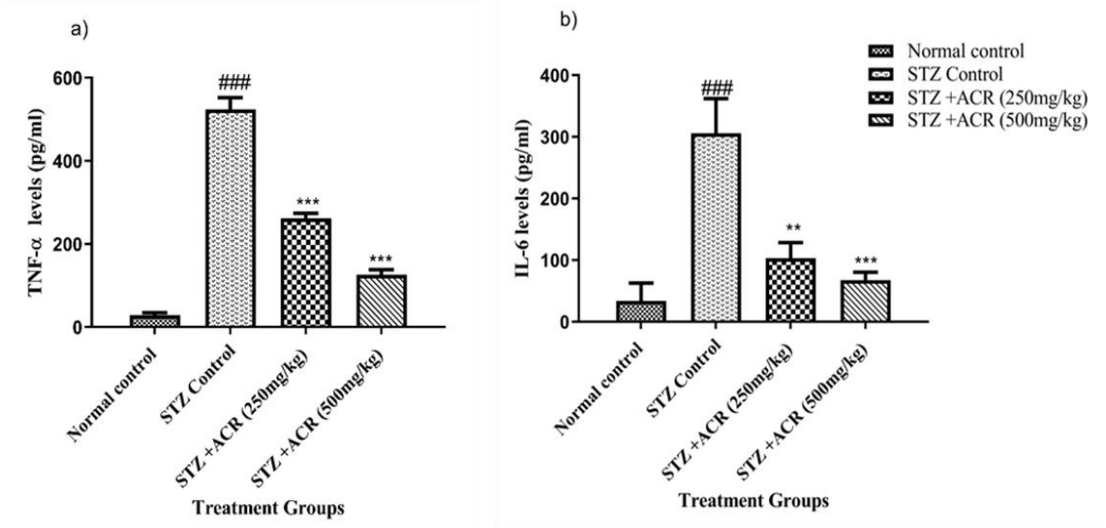


Figure 17: Effect of administration of ACR and STZ **a)** TNF-α and **b)** IL-6. (numbers in mean ± SEM, n=6). ### showed P<0.001 against control; **P<0.01, and *** P<0.001 against STZ.

3.3.4.2. **Oxidative stress markers:**

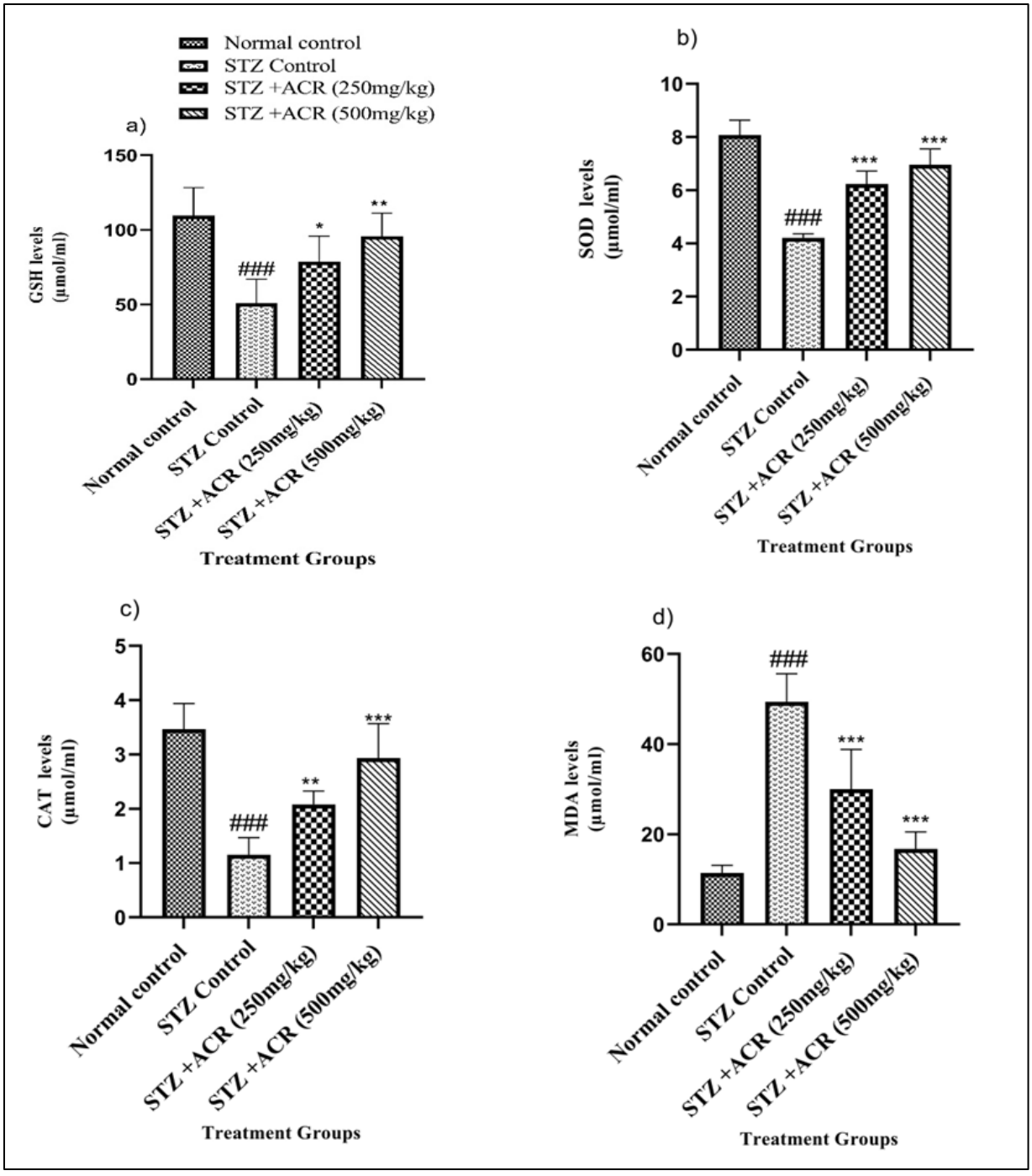


Figure 18: Effect of administration of ACR and STZ **a)** GSH, **b)** SOD, **c)** CAT, and **d)** MDA. Numbers in mean± SEM, n=6. ###: P<0.001 against control; *P<0.05, **P<0.01, and *** P<0.001 against STZ.

3.3.5. **Histopathological parameters of tissue:**

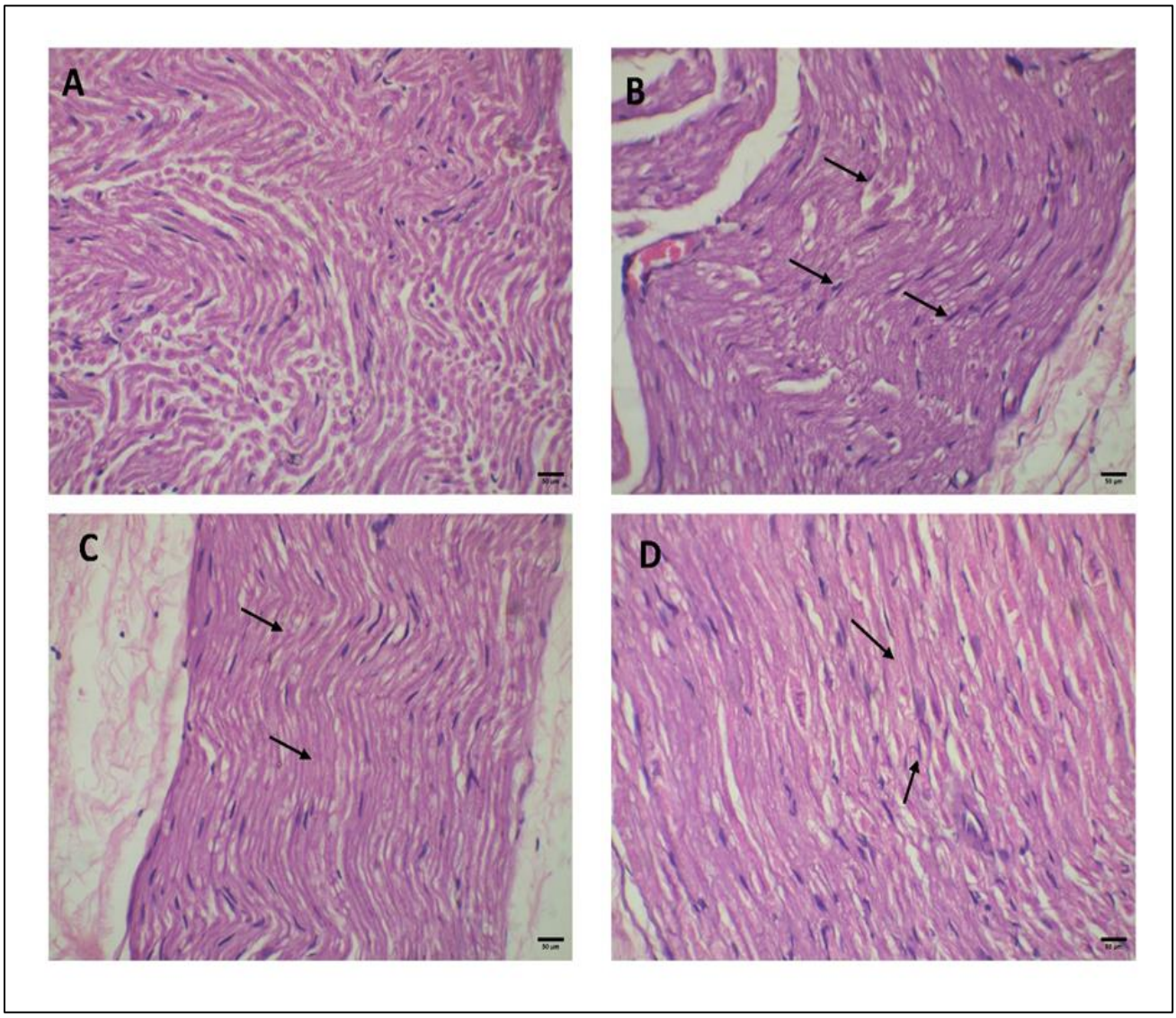


Figure 19: Effect of ACR treatment on the histopathological sections of rat sciatic nerve-(Haematoxylin-eosin (H & E) staining with 40X zoom): **a)** Control **(b)** STZ-group, **(c)** & **(d)** STZ + ACR, 250mg/kg and 500mg/kg.

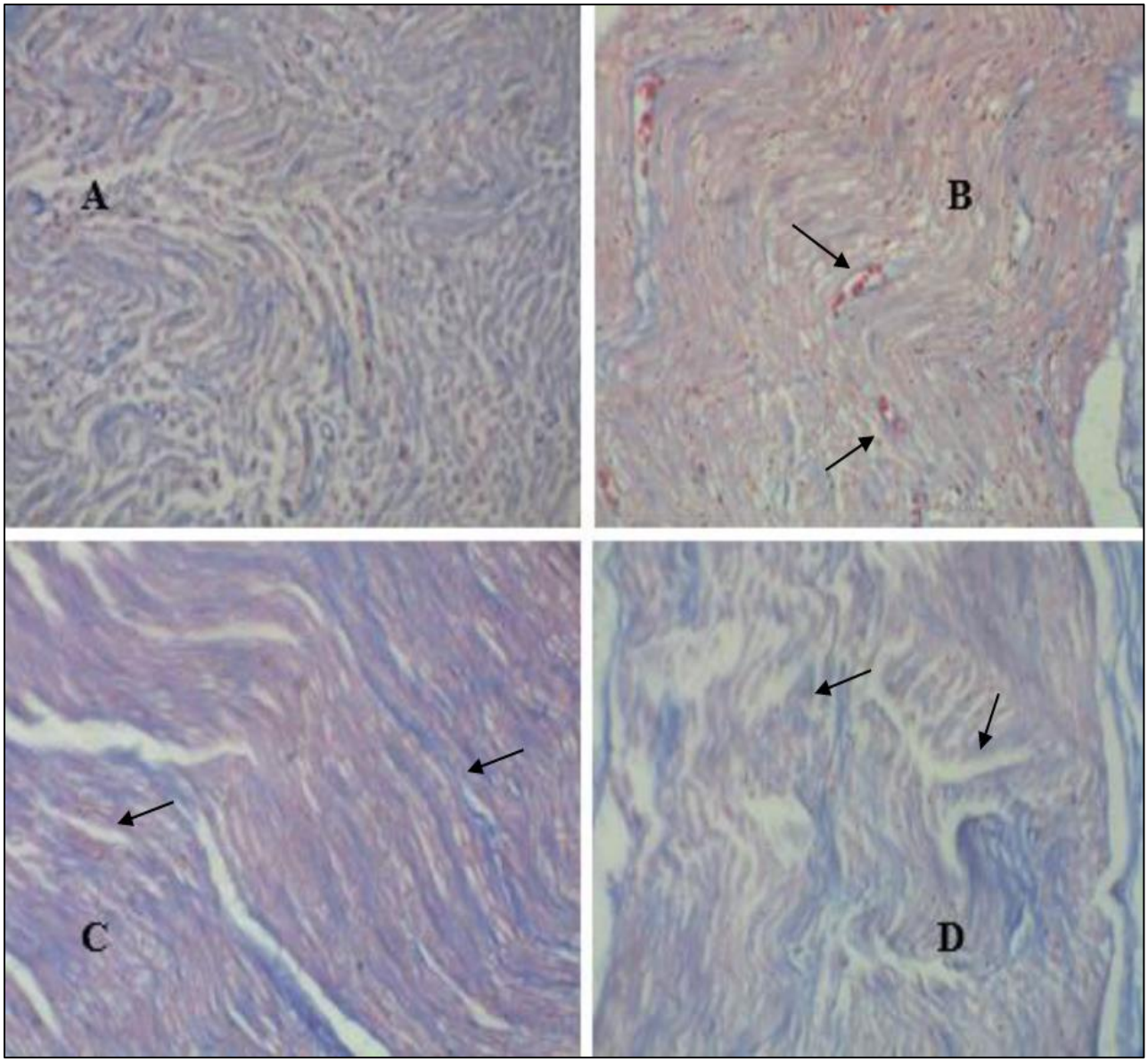


Figure 20: Effect of ACR treatment on the histopathological sections of rat sciatic nerve-Masson Trichome staining with 40x zoom: **a)** Control, **(b)** STZ-group, **(c)** & **(d)** STZ + ACR, 250mg/kg and 500mg/kg.

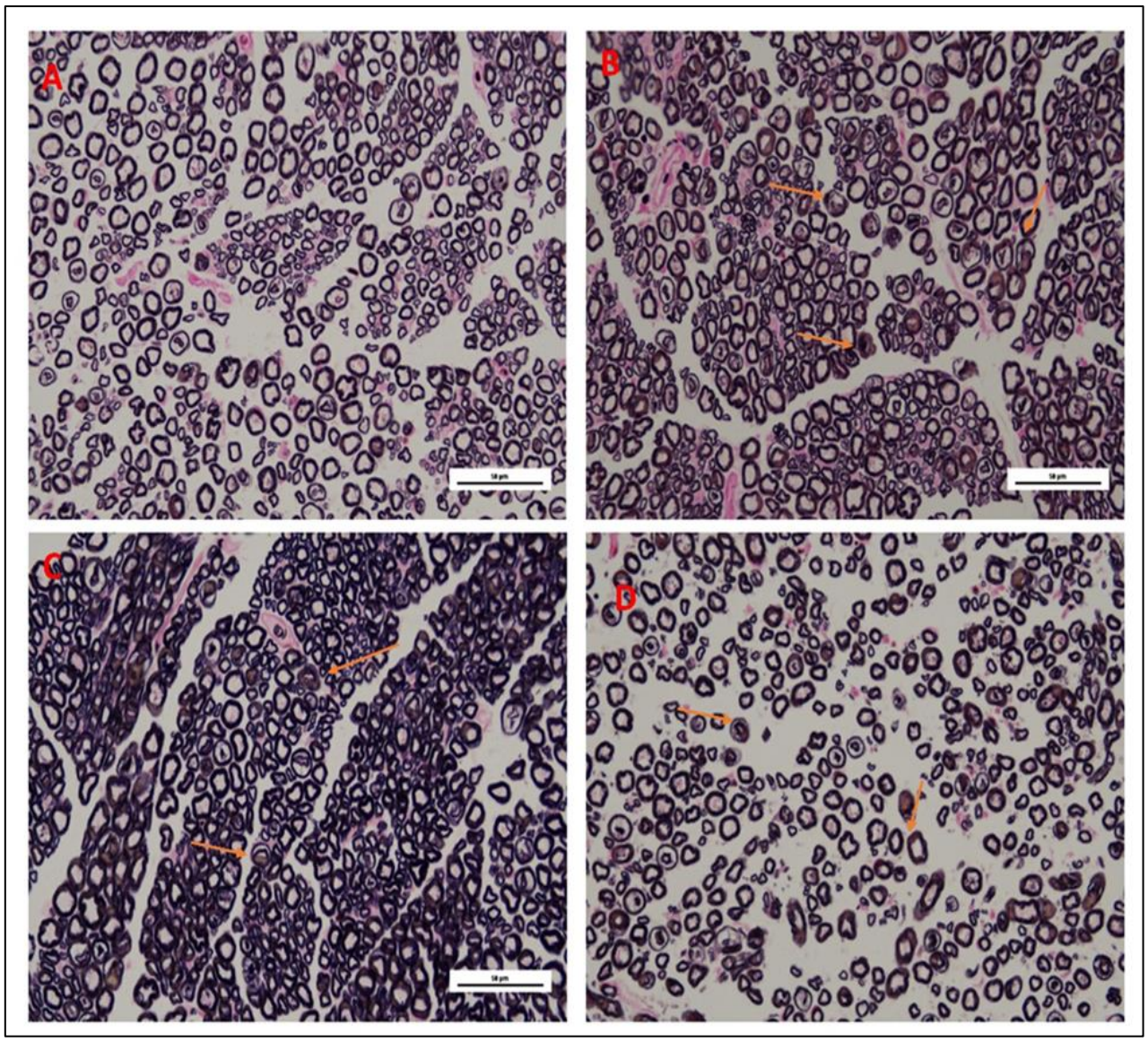


Figure 21: Effective ACR treatment on the histopathological sections of rat sciatic nerve-Kulchitsky-Pal (Kpal) staining with 40x zoom: **a)** Control, **(b)** STZ-group, **(c)** & **(d)** STZ + ACR, 250mg/kg and 500mg/kg.

3.4. Part II: Chemotherapy-induced peripheral neuropathy:

Table 12: Effect of ACR on food and water consumption, body weight, and behavioural parameters

Evaluation parameter	Group **I**-(Normal).	Group **II**-(STZ).	Group **III**-(STZ+ ACR 250mg/kg)	Group **IV**(STZ + ACR 500mg/kg)
Food consumption (gm/day/animal)	27.7 ± 0.570	21.0 ± 0.370$^{###}$	25.4 ± 0.557***	23.3 ± 0.640^{ns}
Water Intake (ml/day/animal)	28.0 ± 0.742	34.5±1.14$^{###}$	29.7 ± 0.595**	32.2 ± 0.926^{ns}
Weight (gm)	292 ± 10.5	198 ± 1.82$^{###}$	278 ± 11.9***	254 ± 17.7*
Hot Hyperalgesia (sec)	8.85 ± 0.44	3.8 ± 0.1$^{###}$	5.45 ± 0.191***	8.07 ± 0.09***
Cold Hyperalgesia (sec)	15.1 ± 0.8	8.87 ± 0.3$^{###}$	11.3 ± 0.2***	14.8 ± 0.2***
Motor coordination (sec)	185 ± 18.1	39.5 ± 6.04$^{##}$	92.8 ± 7.66**	191 ± 23.1**
Grip strength (newton)	702 ± 26.1	433 ± 24.4$^{###}$	535 ± 18.6***	677 ± 30.2***
Wire hang (sec)	49.6±4.74	18.9±2.40$^{##}$	32.9±2.62*	42.1±3.78**
Beam Walk	58±7.37	120±0.333$^{##}$	101±6.49^{ns}	78±6.03**
Distance travelled(cm)	110±10.2	42.0±4.81$^{##}$	59.8±3.90**	77.0±3.31**
Number of slips	4.50±0.563	1.17±0.477$^{##}$	3.83±0.601*	4.17±0.477*
Number of turns	4.83±0.792	1.16±0.477$^{#}$	3.83±0.654*	4.16±0.477**

Numbers in Mean± SEM, n=6. ###: P<0.001, ##: P<0.01 with respect to normal group, ***: P<0.001, **: P<0.01, *:P<0.05, ns: P>0.05 with respect to cisplatin group.

3.4.1. **Evaluation Parameters:**

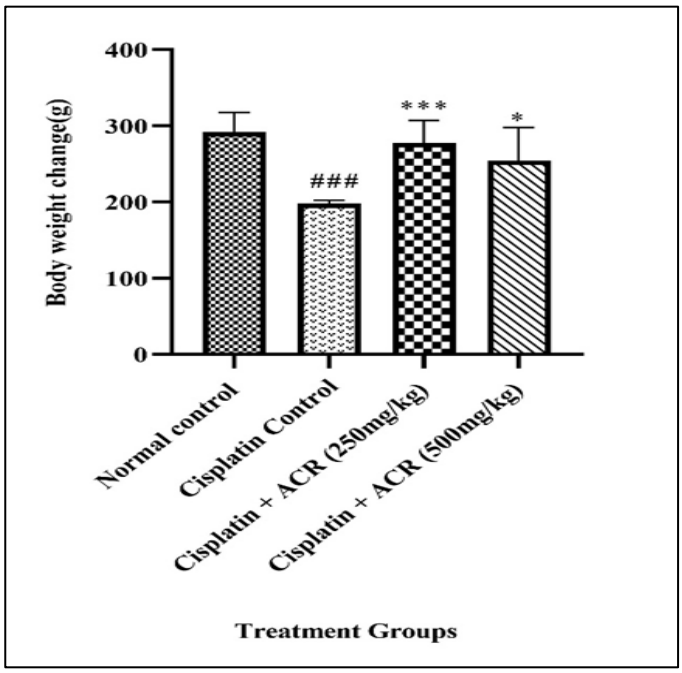


Figure 22: Effect of administration of ACR and Cisplatin on rat weight (numbers in mean ± SEM, n=6). ###: P<0.001 Vs. control; *P<0.05, **P<0.01, and *** P<0.001 Vs. Cisplatin.

3.4.2. **Behavioural Parameters:**

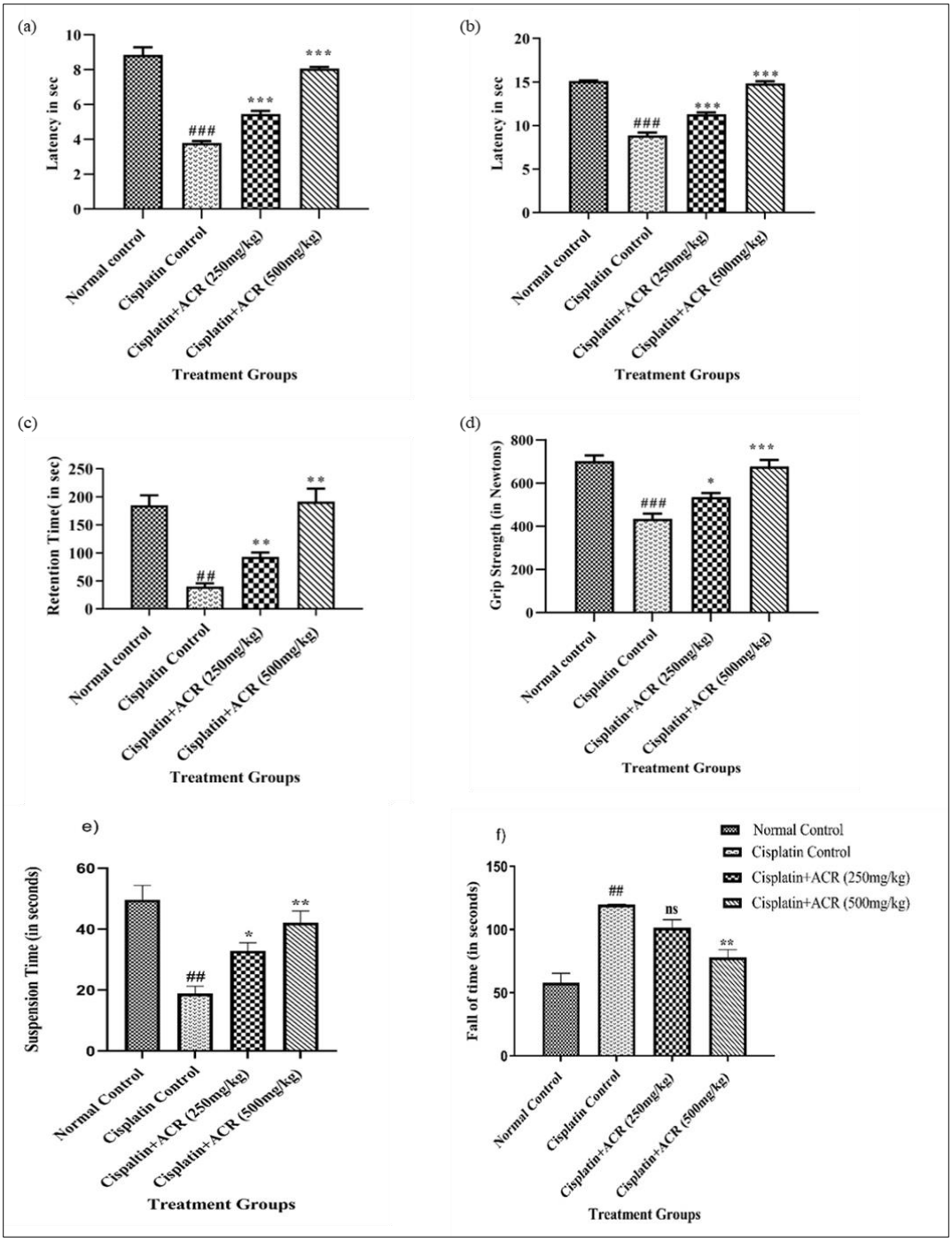


Figure 23: Effect of administration of ACR and Cisplatin on **a)** Hot water, **b)** Cold water, **c)** Rota-rod, **d)** Grip strength, **e)** Wire hang, **f)** Beam Walk in rats. (numbers in mean ± SEM, n=6). ### P<0.001 against control; *P<0.05, **P<0.01, and *** P<0.001 against Cisplatin.

3.4.3. **Electrophysiological Parameters:**

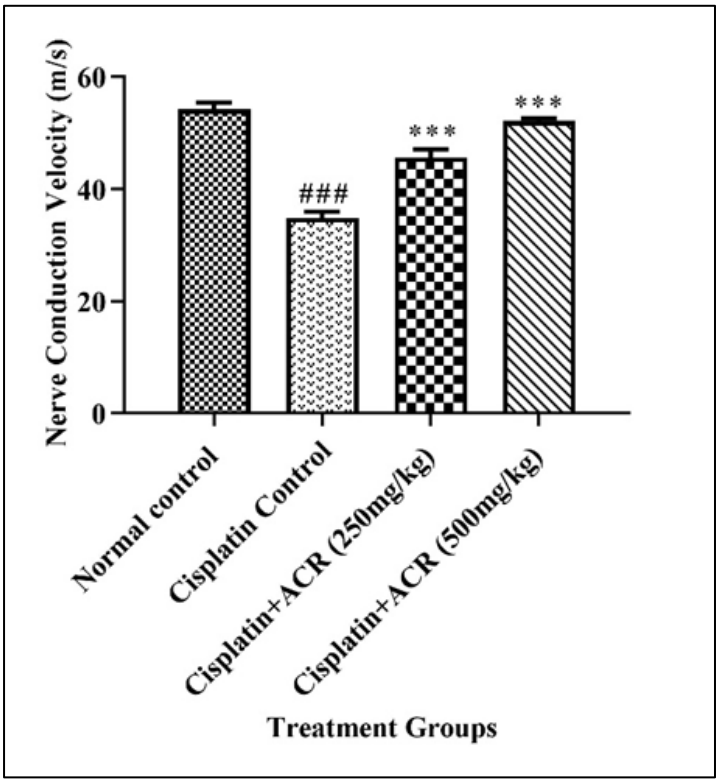


Figure 24 Effect of ACR and Cisplatin on NCV. (results in mean ± SEM, n=6). ###: P<0.001 against control; *** P compared <0.001 against Cisplatin.

Table 13: ACR treatment and Inflammatory markers and oxidative-stress parameters

	Evaluation parameter	**I** (Normal control)	**II** (Cisplatin control)	**III** (Cisplatin + ACR 250mg/kg)	**IV** (Cisplatin + ACR 500mg/kg)
Inflammatory markers	hs-CRP	1.55 ± 0.34	9.08 ± 0.41$^{###}$	8.1 ± 0.47^{ns}	3.91 ± 0.48***
	TNF-α	24.4 ± 9.52	510 ± 17.8$^{###}$	238 ± 11***	110 ± 7.82***
	IL6	27.1 ± 29.7	485 ± 42.4$^{###}$	342 ± 33.2*	142 ± 31***
Oxidative Stress Parameters	SOD	5.14 ± 0.17	3.74 ± 0.13$^{###}$	4.65 ± 0.19**	5.02 ± 0.19***
	CAT	2.24 ± 0.27	0.91 ± 0.13$^{##}$	1.7 ± 0.14*	2.14 ± 0.16***
	MDA	8.6 ± 1.05	37.4 ± 5.56$^{###}$	22 ± 3.27*	14.4 ± 3.11**
	GSH	109 ± 7.11	41.5 ± 3.83$^{###}$	67.3 ± 7.35*	92.2 ± 6.56***

Results in mean± SEM, n=6. ###: P<0.001, ##: P<0.01 with respect to normal group, ***: P<0.001, **: P<0.01, *:P<0.05, ns: P>0.05 with respect to cisplatin group.

3.4.4. **Biochemical Parameters:**

3.4.4.1. **Inflammatory Biomarkers:**

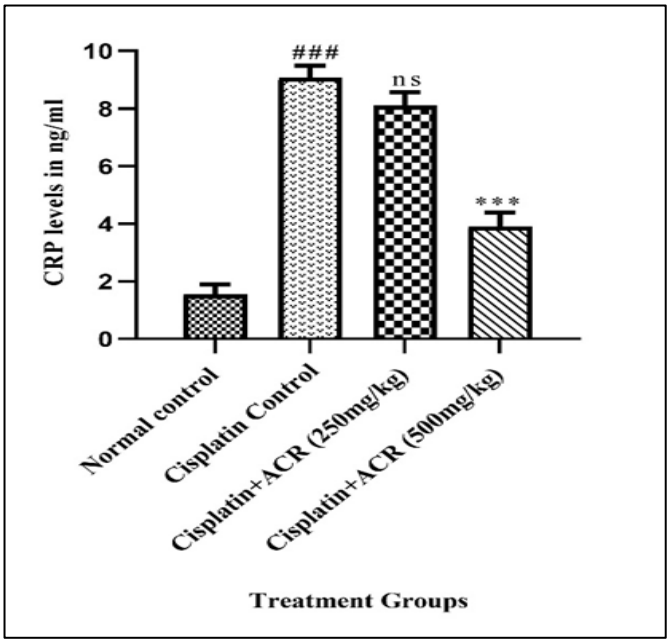


Figure 25: The effect of administration of ACR and Cisplatin on CRP level. (All numbers represented in mean± SEM, n=6). ### indicates P<0.001 Vs. control; ns P>0.05, *** P<0.001 against Cisplatin.

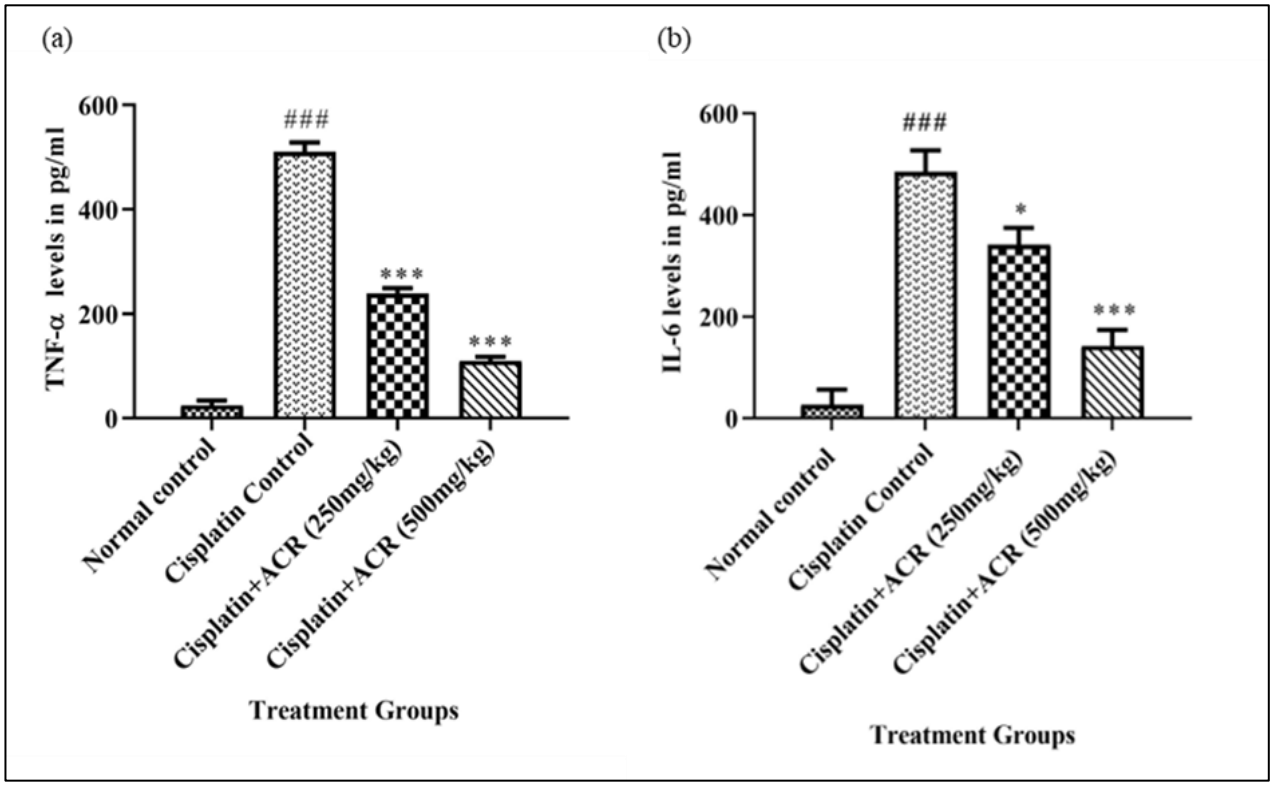


Figure 26: Effect of ACR and Cisplatin **a)** TNF-α and **b)** IL-6. Values represented in mean± SEM, n=6. ###: P<0.001 against control; **P<0.01, and *** P<0.001 against Cisplatin.

3.4.4.2. **Oxidative stress markers:**

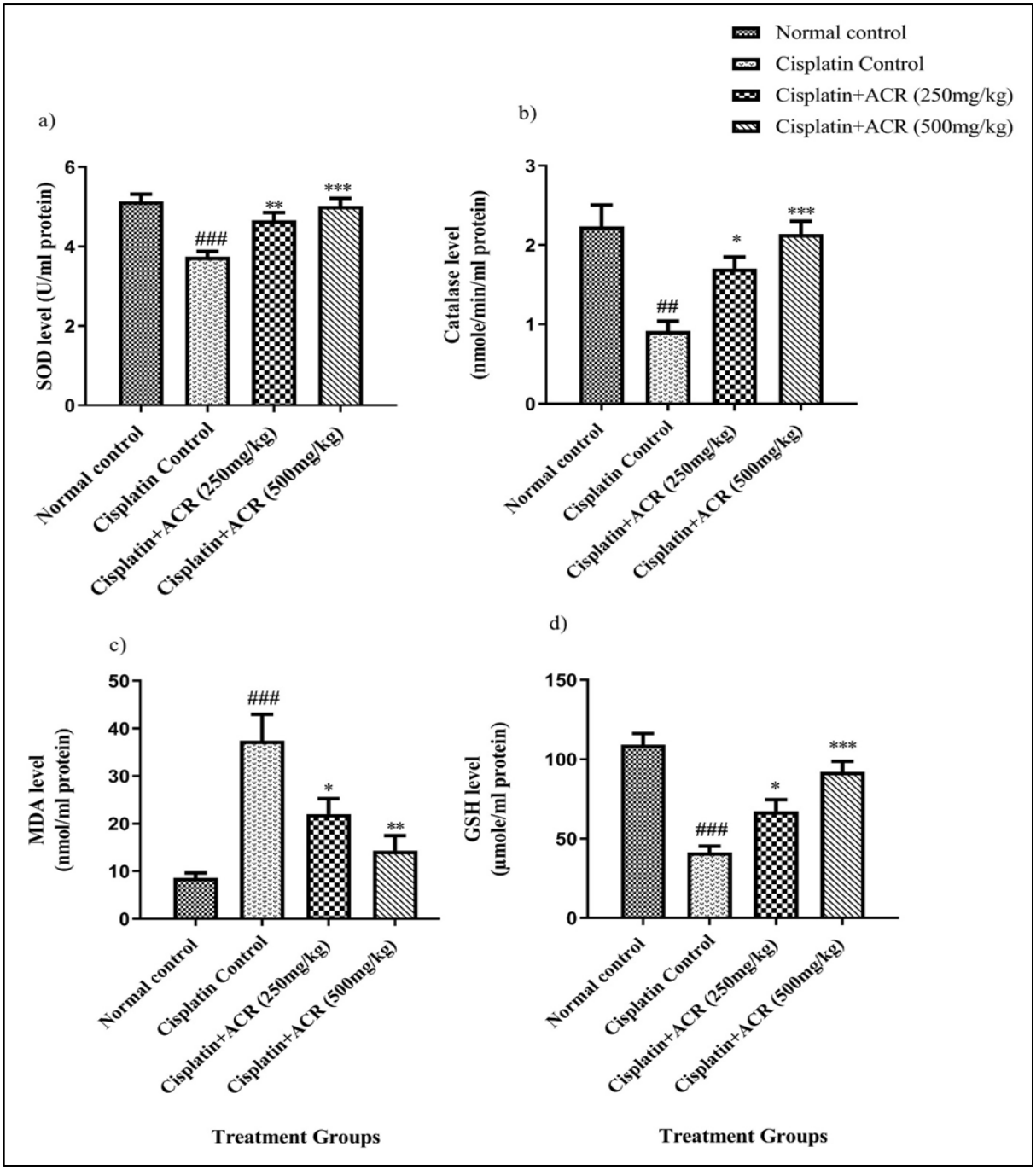


Figure 27: Effect of administration of ACR and Cisplatin **a)** SOD, **b)** CAT **c)** MDA, **d)** GSH. (values represented in mean± SEM, n= 6). ###: P<0.001 against control; *P<0.05, **P<0.01, and *** P<0.001 against Cisplatin.

3.4.5. **Histopathological Parameters:**

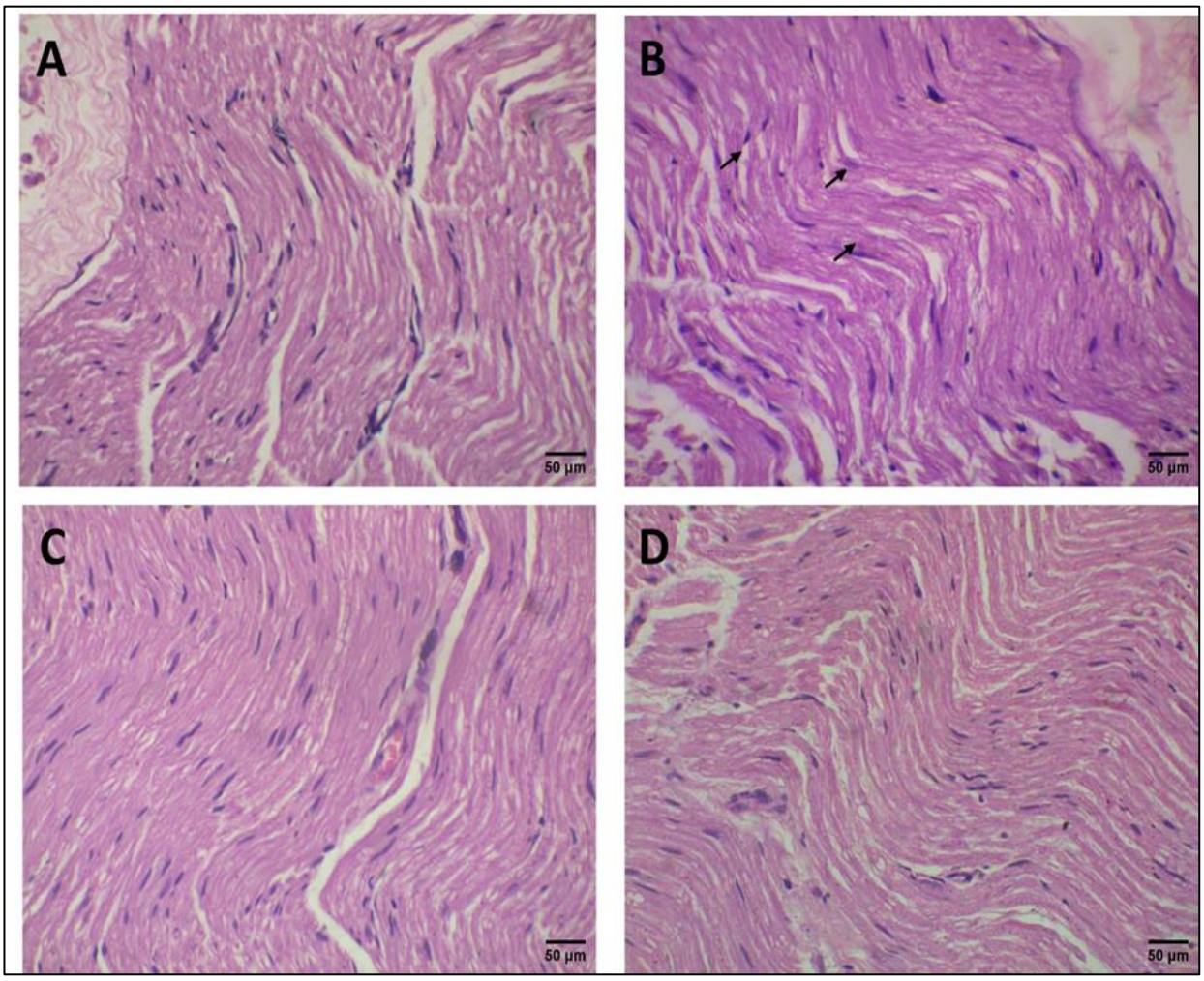


Figure 28: Effect of ACR treatment on the histopathological sections of rat sciatic nerve-(Haematoxylin-eosin (H & E) staining with zoom 40×): **a)** Control-group, **(b)** Cisplatin-group **(c)** & **(d)** Cisplatin+ ACR 250mg/kg and 500mg/kg.

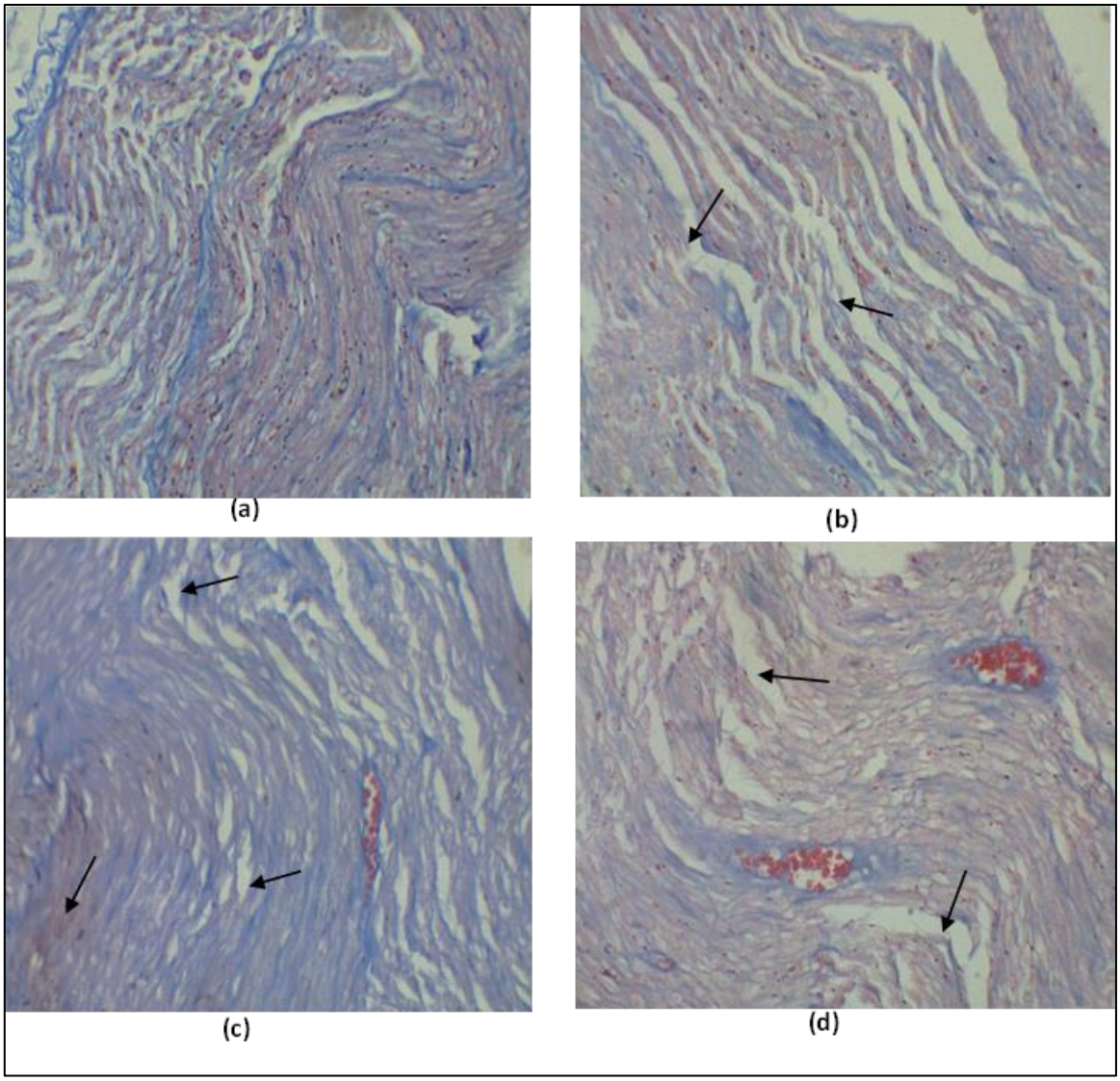


Figure 29: ACR treatment effect on histopathological sections of rat sciatic nerve- Masson Trichome staining with zoom 40x: **a)** Control-group **(b)** Cisplatin-group, **(c)** & **(d)** Cisplatin+ ACR 250mg/kg and 500mg/kg.

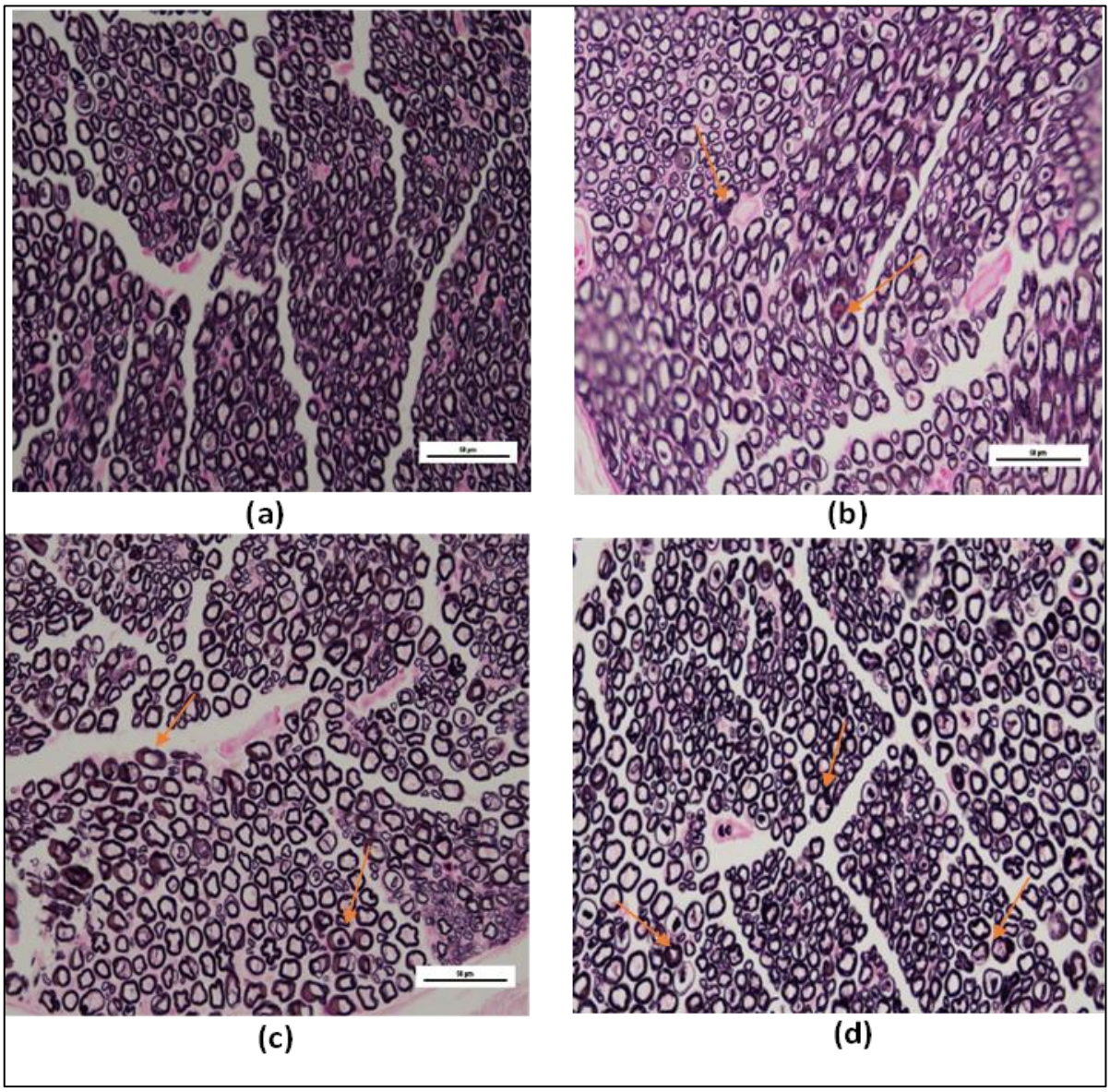


Figure 30: ACR treatment effectiveness on histopathology of rat sciatic nerve-KulchitskyPal (Kpal) stain using zoom 40x: **a)** Control-group **(b)** Cisplatin-group, **(c)** & **(d)** Cisplatin+ ACR (250mg/kg and 500mg/kg).

4. DISCUSSION

4.1. DISCUSSION ON QUALITATIVE PHYTOCHEMICAL SCREENING:

A systematic qualitative phytochemical analysis was conducted to evaluate prevalence of bioactive elements in EEAC. The phytoconstituents in *Alpinia calcarata* are presented in (table 2).

4.2. DISCUSSION ON HPLC STUDIES:

4.2.1. HPLC method development and optimization:

The proposed HPLC method (Figure 8) with UV detection was meticulously developed and optimised to determine stigmasterol, briefly summarised in (Table 3). Subsequently, the developed method underwent a comprehensive and rigorous validation process in strict adherence to the guidelines set forth by the ICH and USP. This process ensures the method's accuracy. The analytical method was validated rigorously following ICH (Q2) R1 guidelines (Table 8). Several mobile phases were attempted to elute the analyte. The main focus was to achieve a sharp peak. The chromatogram (Figure 8) obtained with an Acetonitrile: Orthophosphoric acid (90:10v/v) was observed to exhibit good symmetry and the smallest Rt (3.64) and sharp peak. Therefore, the Acetonitrile: Orthophosphoric acid (90:10v/v) was used as the optimised mobile phase.

4.2.2. Method validation:

Method validation in hplc ensures reliability by systematically assessing key variables like linearity, range, limit-of-detection (lod), quantification(loq), precision, specificity, and robustness. Regression analysis data for the calibration curve indicated a strong linear relationship within the specified concentration range of 10–50 µg/ml for stigmasterol, as presented in (table 4 and figure 9). The linear regression equation was y=138.68× + 2442.7, with a regression coefficient (r2) 0.998. Lod and loq values were observed as 2.08 and 6.33µg/ml, respectively, indicating that the method is highly sensitive and stigmasterol was detected at a very low concentration. Precision, assessed through intra-day and inter-day variations, showed %rsd values below 2% and is presented in supplementary (table 5). Specificity tests confirmed the method's selectivity. A typical hplc peak of stigmasterol is shown in supplementary (figure 10). Ruggedness reproducibility results are presented in (table 6). Robustness is assessed through deliberate variations in the retention time of stigmasterol by changing flow rate and wavelength, yielding %rsd values consistently below 2%, indicating that the developed method was robust, as shown in supplementary (table 7). The system's suitability was found to be within acceptable limits. Maximum retention duration was 3.6min. Theoretical plate count was found ($n > 2000$), and the tailing factor was recorded as 0.99; the relative standard deviation of the peak area (% rsd $\leq$ 2%) is presented in table 8. In summary, the method proved reliable, sensitive, and specific, and it meets the highest analytical standards.

4.2.3. Stigmasterol quantification in AC extract

Developed procedure used for quantifying stigmasterol in an *Alpinia calcarata* extract. Stigmasterol shows retention at 3.58, and the area under the peak is 4088.86. The amount of stigmasterol detected in the extract was 12.61 µg (figure 11). Eeac phytochemical assessment showed stigmasterol existence, novel labdane-type diterpenoids, and other bioactive compounds, underscoring its therapeutic potential. Hplc, a robust and reliable method for quantifying these compounds, supports the standardisation of eeac and further demonstrates its potential therapeutic benefits.

4.3. DISCUSSION ON DIABETES-INDUCED PERIPHERAL NEUROPATHY

DIPN rat models exhibited attenuation in rapid body weight loss, impaired muscle coordination, thermal hyperalgesia, cold hyperalgesia, reduced grip strength, elevated % glycosylated haemoglobin levels, and Electrophysiological dysfunctions, such as decreased nerve conduction velocity, compared to normal rats. This suggests that our DIPN models are suitable for studying EEAC treatment in DIPN in rats. The signs of diabetes induced peripheral neuropathy in our study were similar to those of previous reports, further validating our findings.

4.3.1. Effect of EEAC on rat weight and intake of food and water

Reduced food consumption while increased water intake of group II indicates loss of appetite (diabetic rats) unlike normal rats. As a result, major weight loss was seen in the diseased group ($P < 0.001$), which was improved in EEAC treatment groups of 250mg/kg and 500mg/kg (III: $P<0.01$, IV: $P<0.001$) (Figure 12). Also, as mentioned in Table 10, the treatment groups showed balanced intake with maintained weight. Unattended diabetes can cause neuronal damage, leading to PN-related pain. Neural dysfunction affects stimuli balance, resulting in aches even if the nociceptor is not activated.[90]

4.3.2. Effect of EEAC on Plasma glucose level and % Glycosylated haemoglobin (Hba1c)

Elevated blood glucose concentrations and Hba1c were observed in group II (317.4±9.28 mg/dl and 11.02±0.42 ($P<0.001$) vs. normal control). The four-week EEAC treatment regimen resulted in a controlled diabetes state by medication of both 250mg/kg and 500mg/kg ACR (278.7mg/dl, $P<0.05$) and (254.8 mg/dl, $P<0.001$) unlike the diabetic test group levels. However, ACR-treated groups of 250mg/kg and 500mg/kg recorded much ($P<0.05$, $P<0.001$) lesser HbA1c values at the end of experiments. ACR has shown insulin-release enhancement, thereby aiding glucose transportation and lowering diabetes.[91] The reduction of diabetes by ACR treatment is particularly intriguing and may be linked to its insulin-stimulating effects. Higher diabetic conditions may lead to overexpressing kappa-

light chain catalyst behind live B-cells (NF-kB) along with protein-kinase triggered by Mitogen (MAPK), leading to the initiation of an inflammatory response. Haemoglobin is among many proteins that undergo non-enzymatic glycation to form glycosylated haemoglobin. Glycosylated haemoglobin is a general term for non-enzymatically glycated by glucose and was first described in 1968 as diabetic haemoglobin. The glycosylated haemoglobin levels are a marker of a diabetic state for days.[92] Previous studies on diabetic rats have reported a marked increase in the percentage of glycosylated haemoglobin. Similar findings were observed in diabetes control animals in our experiment. Treatment effects on plasma glucose level and Hba1c were Significantly and positively correlated.

4.3.3. Effect of EEAC on Behavioural Parameters

According to earliest studies, rats often concentrate on their responses to nociceptive stimuli as they do not exhibit clear symptoms of sudden ache. Nociceptive testing carried out in these experiments includes assessments for hot and cold hyperalgesia. These tests assess tail response to hot/cold stimuli. Hyperalgesia is the most common symptom associated with diabetic neuropathy. A four-week EEAC treatment considerably counteracted this reduction in latency, demonstrating its effectiveness at all evaluated dose levels 250mg/kg ($P< 0.05$, $P< 0.001$) and 500mg/kg ($P<0.001$) against the diseased group (Table-10, Figures 14-a and 14-b). According to the underlying mechanism, tissues get invaded, leading to signalling and expression changes of non-specific ionic and specific potassium and sodium channels.[93,94]

In terms of Rota rod time reduction by (28.5 ± 4.41, $P<0.001$) and limb strength by grip-strength (485.0 ± 35.47, $P<0.001$), indicating muscle weakness confirmed peripheral neuropathy in the diabetic control, which was significantly improved in treatment groups rota-rod performance and grid grip strength in (Figure 14-c, $P<0.001$ & 14-d, $P<0.05$, 0.001) concerning group-II.

Further observations from the wire hang and beam walking tests (Figures 14-e and 14-f) reveal that the diseased group experienced heavily reduced suspension time ($P<0.01$) against the group I. Conversely, EEAC treatment 250mg/kg ($P < 0.05$) and 500mg/kg ($P<0.01$) improved suspension time vs. the diabetic group (Table 10). Our findings are consistent with

another study on peripheral neuropathy, highlighting the correlation between STZ administration, sensory-motor dysfunction, and reduced grip strength. This supports the validity of our experimental approach and outcomes.[76,95]

4.3.4. Effect of EEAC on Electrophysiological Parameters

NCV was greatly degraded in the diseased compared to the normal group (P< 0.001). However, post-treatment with 250mg/kg and 500mg/kg (P< 0.001) improvement was noticed unlike in the diseased group (Figure 15). ROS accumulation increased aldose-reductase actions in PN.[96] This unwanted level of ROS and sorbitol disturbs NCV by suppressing Na+, and K+ ATP actions.[97] Treatment with EEAC significantly enhanced motor and sensory NCV in diseased animals, likely an indication of oxidation suppression. To support this, It releases inflammatory mediators, leading to the depleting anti-oxidant-enzymes like SOD, CAT, MDA and GSH, which are assessed in the present study.

4.3.5. EEAC effectiveness on Biochemical parameters

4.3.5.1. EEAC effectiveness on inflammatory markers and serum insulin parameters

As shown in the level of hs-CRP (Figure16) and Cytokines TNF-α and IL-6 (Figure 17) showed a great increase in rats following STZ-induced diabetes, with per cent changes of 85% and 80%, respectively, compared to Group I. Data showed severe inflammation with STZ-caused neuropathic damage indication. Treatment of ACR led to a notable inflammation reduction (hs-CRP, TNF-α, IL6) in Diabetic rats exhibited significant improvements when administered doses of 250mg/kg and 500mg/kg, concerning diseased (P > 0.05, P < 0.01, P < 0.001), documented in (Table-11). Hyper-expression of NFkB leads to neural damage due to the promotion of (TGF-ß) tumor-growth beta factor, TNF-α, IL-6. Furthermore, the activation of NF-kB triggers arachidonic acid in PNs, leading to an increase in cyclooxygenase-2 (COX-2) levels. Moreover, the overexpression of NF-kB, driven by increased (PKC) Protein-kinase-C, ROS, and AGEs, leads to peripheral neuron degradation via leuko-cytic aggression and decreased expression of NGF, IL-6, TNF-α, IL-1ß.[98,99] Figure 16 shows the serum insulin response of the diabetic rat's significant reduction compared to

their normal control (7.722±0.209, P<0.001). Conversely, EEAC medication at 250 as well as 500mg/kg significantly increased (P<0.05 and P<0.001) against diseases shown in (Table 11).

4.3.5.2. EEAC treatment effectiveness in oxidative stress parameters

In the diseased group, a marked alteration in antioxidant enzyme levels was observed in sciatic nerves. This was verified by significantly increased MDA indicated (P<0.001) and corresponding reduced activities of GSH, SOD and CAT. Concentrations dropped to <40% concerning Group I (P<0.001). All markers mentioned above were improved with EEAC medication 250 as well as 500mg/kg, leading to reduced MDA indicated (P<0.001 and P<0.001, resp.) comparison with diseased animals. GSH was elevated in ACR medicated 250 and 500mg/kg indicated (P<0.05 &P<0.01). CAT and superoxide dismutase exhibited significant elevation following ACR groups against diseased animals (Figure 18). This is particularly relevant considering the oxidative stress and inflammation associated with STZ-induced neuropathy. This restoration of oxidative enzyme levels aids in preventing demyelination and provides neuroprotection.[97]

4.3.6. EEAC effectiveness in Histopathological study

In the ACR treated group, the haematoxylin-eosin- (H&E) stain demonstrated protected neural morphology, characterised by unaltered myelin-sheath and invisible inflammatory cell intrusion, concerning the II group. In contrast, the II group exhibited negative changes like axon swelling, myelin damage, and significant inflammatory cell invasion. These findings indicate the preventive effect of ACR on sciatic-nerves (Figure 19).

Masson trichrome (MT) revealed organised collagen in ACR groups unlike in diseased control. In contrast, diseased control showed disorganised collagen surrounding the nerve, indicative of fibrosis. These findings suggest ACR potentially has a role in preventing fibrotic effects in sciatic-nerves (Figure 20).

KulchitskyPal- (Kpal) stain demonstrated the absence of neurofibrillary tangling in ACR-treated in contrast to disease-control. The diseased group exhibited a higher prevalence of

neurofibrillary tangles, indicative of potential neural damage. Reduction in neural fibrillary tangling observed in ACR animals suggests ACR's neuroprotection role (Figure 21).

Well-maintained nerve structures found in histopathology, and myelin sheaths and minimal signs of degeneration, suggesting that EEAC treatment helps maintain the integrity of the sciatic nerves. The diseased animals showed histological damage, including thinner neural fiber density in endoneurium, with a loss of smaller myelinated fibers while larger diameter fibers were better preserved. Additionally, endoneurial vascular thickening, indicative of diabetic microangiopathy, was observed, further highlighting the extent of nerve damage. Diseased group has ill-effects such as axon swelling, which further emphasized the extent of nerve damage. However, the myelin sheath remained unaffected by STZ in diabetic peripheral neuropathy. The disruption of the sodium cell gradient, caused by impaired Na+/K+ ATP ase activity, results in an altered membrane environment. This disturbance contributes to histological damage and a reduction in myelin protein expression. In summary, the results suggest ACR's role in preserving nerves and their reconstruction, offering strong evidence of its potential as a protective treatment.

4.4. DISCUSSION ON CISPLATIN-INDUCED PERIPHERAL NEUROPATHY

Approximately 10.3 million people died from cancer worldwide in the year 2020, and 19.1 million fresh cases were reported. CIPN remains a significant ill effect impacting patients' quality of life. First-line anticancer drug classes such as vinca alkaloids, taxanes, and pt-based compounds are widely used to treat various cancers but are also associated with neuropathic complications.[100,101] the exploration of EEAC as a neuroprotectant has not been done so far. Still, multiple other allied therapeutic potentials have been developed, such as anti-inflammatory, antioxidant, and antinociceptive of ACR. Cisplatin (CDDP), as a primary member of platinum-containing anticancer agents, induces DNA crosslinking, leading to apoptosis, but is also known for its adverse impact on the neural system, manifesting as PN indicated by motor, sensory, and autonomic malfunction.[102] this study specifically explored ACR efficacy in counteracting these detrimental effects of CDDP, utilising behavioural, biochemical, and histological assessments in rats subjected to cisplatin-induced neurotoxicity. Cisplatin-treated rats exhibited decreased body weight percentage, impaired motor coordination and grip strength, hyperalgesia, electrophysiological deficits such as diminished NCV, histopathological changes including fibre density reduction in the endoneurium, myelin sheath degradation, axonal loss, and decreased antioxidant enzyme levels in sciatic nerve homogenates. The changes in these parameters could be attributed to the toxicity of cisplatin to the peripheral nerves. Our investigation into the preventive effect of the EEAC against CDDP-induced neurotoxicity reveals significant findings that contribute to the understanding and potential mitigation of CIPN. Cisplatin mainly induces peripheral neuropathy by forming cisplatin forms specific DNA adducts, including guanine-guanine intra-strand crosslinks. The gathering and sustaining of DNA lesions intervene with transcription and reproduction, ultimately triggering apoptotic phenomenon.

4.4.1. Effect of EEAC on weight, food and water consumption

Increased water intake with reduced food consumption in the CDDP group indicates a diseased condition. As a result, the normal group animals exhibited an increased b.w. compared with those treated with CDDP, significantly reducing weight indicated ($P<0.001$).

Treatment with ACR 250 and 500mg/kg significantly improved weight comparison with CDDP animals, achieving statistical significance (P<0.001)., as mentioned in (Table 12 & Figure 22).

4.4.2. Effect of EEAC on Behavioural Parameters

As listed in Table 12, reduced tail retention time in hot and cold (by 56% & 42%) water in the CDDP control group indicates an increased sensitivity to pain (Hyperalgesia), a known symptom of peripheral neuropathy compared with normal group(P<0.001); improved by ACR treatment (P<0.001) in both dosage group-III 250 mg/kg (5.45 and 11.3) and group IV 500 mg/kg (8.07 and 14.8), P<0.001, (Figures 23-a and b) with the 500 mg/kg dose restoring latency to levels similar to the normal control group. Hyperalgesia is one of the critical symptoms of neuropathic pain in patients, seen in 15-50% of individuals with peripheral or central neuropathy.[93]

Further observations from motor coordination and grip strength, measured by the grid suspension test, the cisplatin group exhibited significant reductions, decreasing by 25% and 40%, respectively (P <0.001), vs group I. EEAC treatment 250 and 500mg/kg doses for animals receiving cisplatin for five weeks significantly improved neuromuscular coordination and grip suspension duration against cisplatin set (Figures 23-c, P<0.01 and 23-d, P<0.05 and P<0.001resp.). The grip strength reduction aligns with another study about peripheral neuropathy.[103]

The wire hangs and beam walking tests reveal that the CDDP group experienced a significant decrease in suspension time (18.9±2.40, P<0.01) vs the normal group. Conversely, ACR input 250 (P<0.05) and 500mg/kg (P<0.01) greatly improved suspension time. The beam walking test results indicated a decrease in performance of the normal group (p <0.01) vs the cisplatin batch. However, EEAC treatment significantly improved the distance travelled, number of slips, and number of turns as shown in (Table 12).

4.4.3. EEAC Effect on Electrophysiological Parameters

Severe reduction (34.9±1.06, P<0.001) in sciatic NCV was found in the cisplatin batch over the normal one. A five-week ACR treatment, 250mg/kg and 500mg/kg doses showed significantly improved NCV (P<0.001), Figure 24 over the cisplatin group, indicating nerve functionality reversal against cisplatin-induced deficits. NCV reduction could be due to myelin thickness reduction caused by cisplatin toxicity, and the myelin abnormality could be due to Schwann cells apoptosis by CDDP.[104,105] The demyelination observed in these studies also explains the NCV reduction in the cisplatin group and the possibility of myelin regeneration with ACR treatment showing increased NCV. Though the mechanisms underlying cisplatin-induced peripheral neuropathy and NCV reduction are not fully revealed, the available evidence suggests oxidative stress was one of the major culprits. The ROS formation was found elevated in cisplatin-altered neurons. CDDP also causes mitochondrial injuries in DRG neurons.[106] Several reports indicate CIPN is due to lost competency of mitochondrial membrane which is attributed to reduced Na+/K+-ATPase activity. Remarkably, the decreased NCV is due to Na+/K+ ATPase activity degradation.

4.4.4. EEAC treatment effectiveness on biochemical factors

4.4.4.1. EEAC treatment on inflammatory markers and Oxidative stress parameters

C reactive protein (hs-CRP) (Figure 25), TNF-α and IL6 (Figure 26-a&b) data showed severe inflammation with cisplatin-caused neuropathic damage. Indication. Additionally, the overexpression of NF-κB, triggered by increased PKC and AGEs, and reactive oxygen species (ROS) caused by leukocytic infiltration, contributes to peripheral nerve degeneration and reduced TNF-α, IL-6, neuronal growth factor (NGF), and IL-1β.[107] ACR showed anti-inflammatory and analgesic properties via inhibiting NF- kß and TNF-α. A five-week regimen of ACR treatment on CIPN models in this study showed inflammation reduction (hs-CRP, TNF-α, IL6, P<0.001) as detailed in Table 13.

Effects of ACR on antioxidant enzymes: different doses of EEAC administered with CDDP showed significant restoration of antioxidant activity. ACR 250 mg/kg with CDDP regained

activities of SOD, GSH (<40%, P<0.001), and CAT (P<0.01). All markers mentioned above were improved with ACR treatment. On the other hand, the activity of Malondialdehyde (MDA), an indicator of oxidative stress, greatly improved in group II concerning I (P<0.001), highlighting increased oxidative stress due to CDDP. Co-administration with EEAC significantly mitigated this effect. At a 250 mg/kg dose of ACR with CDDP, MDA reduced vs CDDP set (P <0.01). This reduction was even more substantial at the higher 500 mg/kg dose of ACR, where MDA levels nearly approached those of the control group, showing a profound protective effect against CDDP-induced oxidative stress (P<0.001) as detailed in (Table 13, Figure 27). Suggested reactive oxygen species (ROS) generation elevation in cisplatin-exposed neurons. Another research revealed cisplatin-induced oxidative stress due to mitochondrial dysfunction (caused by mitochondrial defects) and increased ROS levels.[108] Thus, the protective role of ACR treatment, is due to attenuation. It improved oxidative stress by improving the expression of anti-oxidant enzymes and exerting anti-apoptotic effects., which is attributed to neuroprotection. Further, in our model, these drugs could prevent neurotoxicity when administered along with CDDP for five weeks.[109]

4.4.5. EEAC treatment effect on Histopathological results

The histopathology revealed surface modification of endoneurial fibrosis. Staining with H&E was used to identify treatment in neuro-morphology, as shown in Figure 28 a; the normal control group had typical myelinated fibres with good myelin sheath. In contrast, cisplatin control batch showed moderate nerve fibre degeneration (arrow) and demyelination (Figure 28-b). ACR-treated groups had restored neuro-morphology with myelin sheath, appearing closer to normal, indicating the neuroprotective role of ACR (Figure 28-c & d). The sciatic nerves of the control and experimental groups were stained with Masson trichrome and Kulchitsky-Pal (Kpal). The results of Masson trichrome staining (Figure 29) demonstrated that the cisplatin group had significant endoneurial fibrosis, with axons surrounded by a trichrome-positive area. However, treatment with EEAC at 250mg/kg and 500mg/kg doses showed progressive improvement in endoneurial fibrosis. The Kulchitsky-Pal (Kpal) staining results (Figure 30) showed that the control group had uniform myelinated fibres, whereas the cisplatin group had demyelination and collagen distribution. The histopathological analysis

revealed that the CDDP group had increased deposition of axonal loss in the form of endoneurial fibrosis. However, treatment with ACR at 250mg/kg and 500mg/kg prevented axonal loss, myelin ovoids, and demyelinating neuropathy. The myelin stain decreased neurofibril entanglement in the transverse section of the sciatic nerve, indicating that it is a promising treatment for neural protection. These morphological observations align with NCV, oxidative stress and inflammatory parameter evaluations. Also, the cisplatin-induced neuro-morphological defects observed align with cisplatin neurotoxicity histopathological results in another study.[110]

5. SUMMARY

Alpinia calcarata Roscoe (ACR), Zingiberaceae family, is a rhizomatous perennial herb that thrives in tropical/subtropical areas abundantly throughout India as well as oriental countries. ACR rhizome has flavonoids, terpenoids, and phenolic compounds with antioxidant, anti-inflammatory, antidiabetic, antifungal, and analgesic, and was found to be an effective antinociceptive in rats. EEAC has been a medication for bronchitis and cough and is an anti-inflammatory agent. Among its bioactive metabolites, stigmasterol is particularly notable for its significant role in managing and treating conditions. Stigmasterol has already been reported in the literature to play an essential role in treating and managing cancer, diabetes, thyroid, osteoarthritis and other inflammatory disorders. This interest has also sparked significant attention from the scientific community, which is increasingly exploring the medicinal properties of plants to validate their effectiveness and discover new therapeutic compounds. Therefore, the current study is to identify and evaluate high-performance liquid chromatography (HPLC) procedures under the ICH Guidelines. An International Conference on Harmonization for Phyto chemical assessment of EEAC proved stigmasterol prevalence, novel labdane-type diterpenoids, and other bioactive compounds, underscoring its therapeutic potential. The use of HPLC to quantify these compounds supports the standardisation of EEAC. Analysing stigmasterol content in EEAC rhizomes and evaluating the neuroprotective potential in peripheral neuropathy in a rat model. The present study evaluates physical, behavioural, and biochemical parameters, along with direct measurements of nerve conduction velocity (NCV) and histopathological analysis of sciatic nerves, for exploring the neuroprotective role of EEAC in DIPN and CIPN.

Diabetes can result in numerous medical complexities of which PN is one major. DPN affects close to 50% population, leading to substantial morbidity and a diminished quality of life. It is characterised by uneasiness, ache and tingling-like condition in limbs. In streptozotocin-induced diabetes, neuropathy was induced with streptozotocin administered intraperitoneally at 55 mg/kg/week, four doses. This was selected as an animal model of

diabetic peripheral neuropathy for further studies. Two intervention groups received different EEAC concentrations (250 & 500mg/kg).

The alteration of body weight, pain sensitivity, grip strength, muscle coordination, and electrophysiological abnormalities like decreased nerve conduction velocity and oxidative stress. It exhibited beneficial effects in delaying neuropathy in diabetic rats. Treatment with EEAC showed a significant attenuation of neurotoxicity, as evidenced by behavioural improvements in mechanical allodynia, thermal hyperalgesia, cold allodynia, wire hang and beam walk. ACR medication corrected increased plasma glucose and % Hba1c in diabetic rats and showed a non-significant impact on lowering the plasma glucose level and % glycosylated haemoglobin. FBG and HbA1c corrections indicate both together can complement and help getting accurate results. Inflammatory markers and serum insulin significantly increased in diabetic rats. It showed severe inflammation with STZ-caused neuropathic damage indication. Treatment of ACR led to a notable inflammation reduction. The treatment of diabetic rats with EEAC delayed histopathological deterioration and improved the functional abilities of the sciatic nerve.

CDDP, commonly known as cisplatin, is a potent antineoplastic medication for cancer. CDDP causes severe peripheral neuropathy as a side effect, impacting patients' quality of life by damaging nerve tissues. However, it forms DNA-platinum complexes, which can result in neural damage through axonal modifications. Cisplatin can react with mitochondrial DNA, generating reactive oxygen (RO) compounds that affect axonal transmission due to mitochondria's axonal localisation, leading to chronic neural weakness. Cisplatin is a platinum-based anticancer drug known for its efficacy but associated with severe side effects, including peripheral neuropathy. It also exhibits molecular closeness with PNS, which doesn’t have adequate protection of vascular barrier. CDDP is caused by DNA crosslinking, triggering apoptosis and resulting in peripheral neural damage and sensory dysfunction. It has also been reported to reduce anti-angiogenesis effects, possibly contributing to nerve damage.

The administration of cisplatin 2mg/kg i.p. weekly once for five weeks induced peripheral neuropathy. This CIPN model was selected for further studies. Physical, behavioural,

biochemical, and electrophysiological parameters were evaluated, and sciatic nerve histopathology was studied. Administration with EEAC significantly attenuated neurotoxicity, as evidenced by body weight, which was improved in ACR treatment groups, and behavioural improvements in pain nociception, motor coordination, grip strength, and distance travelled by beam. CDDP administration is associated with sensory-motor dysfunction, including diminished withdrawal latency and reduced grip strength, thereby supporting the validity of our experimental approach and outcomes. EEAC mitigated these effects and demonstrated the potential to improve muscle strength and coordination. The ACR treatment, 250mg/kg and 500mg/kg doses showed improved NCV over cisplatin control. This is particularly significant given the oxidative stress and inflammation linked to CDDP-induced neuropathy. EEAC's anti -inflammatory and -oxidant traits, demonstrated by normalisation of antioxidation enzyme concentration and reduced oxidation-stress, play a pivotal role in its therapeutic effects. This study confirms CDDP's neurotoxic effects and highlights EEAC as a promising neuroprotective agent against (CIPN) and (DIPN). The identification of EEAC’s bioactive constituents and the elucidation of its protective mechanisms are crucial. These efforts could potentially lead to integrating EEAC into diabetes and cancer treatment regimens, offering relief from diabetic-induced peripheral neuropathy (DIPN) and CIPN, positively impacting diabetic and cancer patients.

6. CONCLUSION

The study conclusively shows the neuroprotective potential of an Ethanolic Extract of *Alpinia calcarata Roscoe*, which mitigates the harmful effects of Streptozotocin (STZ)-induced DIPN and CDDP-induced CIPN in rat models. Through comprehensive observations like behavioural, improved motor coordination, biochemical, and histopathological analyses, EEAC shows counteraction against Streptozotocin and cisplatin-induced neurotoxicity in rats, showing marked improvements in sensory-motor function and oxidative stress markers. Developing and validating the HPLC method for quantifying stigmasterol in EEAC enhances its standardisation and supports its potential clinical application. Our findings highlight EEAC's therapeutic potential as a complementary treatment for CIPN, offering light on elevating living quality while undergoing chemotherapy. This novel finding could improve diabetes and cancer patient survival and life quality. Given the promising results of this study, future work should try to find precise mechanisms underlying EEAC's protective role. This is a pressing need, as exploring its integration into current diabetes and cancer treatment regimens could be a significant step in neutralising the impact of DIPN and CIPN effectively.

7. LIMITATIONS AND FUTURE SCOPE

Limited Scope: Although the results in rat models are promising, extensive clinical trials are needed to transition from lab trials on rats to medicinal use in humans. Other challenges that any medicinal plant needs to address are flawless identification of ACR and type of extract, standardisation, and repeatable and reliable extraction process. Without extensive study, it is challenging to say whether plant extraction method and source or origin affect drug concentration. This study gives hope for effectively managing DIPN and CIPN clinically based on its effectiveness in rat models. However, extensive quantitative analysis would be needed to establish the appropriate human dosage and uncover any safety risks.

Long-Term: The study may not have addressed long-term research. This novel finding could improve Diabetes and cancer patient survival and quality of life. Future work is recommended to identify the underlying mechanism of EEAC's protective role and explore its integration with existing diabetes and cancer treatments to effectively neutralise the impact of diabetes and cisplatin-induced peripheral neuropathy.

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9. ANNEXURES

Authentication letter

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Dr. P.E.RAJASEKHARAN M. Sc;. Ph. D; PGDHRM; PGDE&E;PGDIPRL
Principal Scientist & Nodal officer GAC
Division of Plant Genetic Resources

Certificate

The plant sample (rhizome) brought by **Mrs Apurwa Dhavale,** Ph.D Research Scholar (Pharmacology) student of K.L.E College of Pharmacy, Bangalore was identified and authenticated as "***Alpinia Calcarata Roscoe*** " belongs to the family ***Zingiberaceae.***

P.E.RajaSekharan

Dr. P.E. RAJASEKHARAN
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Ethical Clearance Letter

KLE ACADEMY OF HIGHER EDUCATION & RESEARCH, BELAGAVI
(Formerly known as KLE University's)
KLE College of Pharmacy
(Re-Accredited by NBA, Approved by PCI and AICTE - New Delhi)
[Declared as Deemed-to-be-University under section 3 of the UGC Act, 1956 vide Government of India Notification No. F.9-19/2000U.3(A)]
[A constituent unit of the KAHER, Belagavi]
2nd Block, Rajajinagar, Bengaluru - 560 010, Karnataka, India.
☎ 080-23325611 FAX No. : 080-23425373 Web : http://www.kleblrpharm.org E-mail : princpharmblr@kledeemeduniversity.edu.in

Ref. No. KLE/COPBLR/ Date : .....

INSTITUTIONAL ANIMAL ETHICS COMMITTEE CERTIFICATE

This is to certify that the project proposal no. 03/HP/2021entitled "Exploring ethanolic extract of *Alpinia Calcarata Roscoe* rhizome for its peripheral neuroprotective activity in Wistar rats" submitted by Ms.Apurwa Dhavale has been approved /recommended by the IAEC of KLE College of Pharmacy, Bengaluru in its meeting dated 12/10/2021 and has been sanctioned 66 Albino Wistar Rats under this proposal for a duration of next 24 months

Authorized by	Name	Signature	Date
Chairman:	Dr.Raman Dang		
Member Secretary	Dr.Hariprasad M.G		12/10/21
Main Nominee of CPCSEA	Dr.Giridhar.P		

INSTITUTIONAL ANIMAL ETHICS COMMITTEE ★ KLE College of Pharmacy Bangalore

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Prof. Balram Bhargava
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Mr./Mrs./Ms./Dr. Apurwa Dhavale

for **Oral** Presentation entitled **"Neuroprotective Activity Of Ethanolic Extract Of Alpinia Calcarata Roscoe Rhizome In Cisplatin Induced Peripheral Neuropathy In Rat Models"** in the **International Conference on Biomedical and Clinical Research** held on 21st to 22nd of November 2022 at Shri Dharmasthala Manjunatheshwara University, Dharwad, Karnataka.

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Theme:
"Access to Quality & Affordable Medical Products"

INTAS

72nd Indian Pharmaceutical Congress, Nagpur

Certificate

POSTER

It is our pleasure to certify that,

APURWA DHAVALE presented a Poster in Scientific Session entitled **"NEUROPROTECTIVE EFFECT OF ALPINIA CALCARATA ROSCOE RHIZOME ETHANOLIC EXTRACT IN RATS WITH PAINFUL DIABETIC PERIPHERAL NEUROPATHY"** in the 72nd Indian Pharmaceutical Congress held at Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur during January 20-22, 2023.

Dr. V. G. Somani	Mr. Atul Mandlekar	Prof. Milind Umekar	Prof. Prakash Itankar	Prof. Roop K. Khar	Prof. Dadasaheb M. Kokare
President, IPCA	Chairman,LOC	Organising Secretary,LOC	Organising Secretary,LOC	Convener, IPCA-SSC	Chairman, Scientific Committee, LOC

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Department of Epidemiology and Biostatistics

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Workshop on
Sample Size Estimation, Systematic Review and Meta-Analysis using EXCEL/SPSS
held from 4th to 6th January 2021

This is to certify that **Ms. Apurwa S Dhavale**
has participated as a Delegate in the workshop

Dr. N. K. Tyagi
Professor & Head,
Department of Epidemiology & Biostatistics, KAHER, Belagavi

Dr. V. A. Kothiwale
Registrar,
KAHER, Belagavi

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MANIPAL COLLEGE
OF PHARMACEUTICAL SCIENCES
MANIPAL
(A constituent unit of MAHE, Manipal)

National e-Conference on Drug Discovery for CNS Disorders

CERTIFICATE OF PARTICIPATION

This is to certify that

Ms. Apurwa Dhavale

has participated as a **Delegate**

in the National e-Conference on Drug Discovery for CNS Disorders held between 6 – 10 December 2021.

– Jointly organized by –

Department of Pharmacology, Manipal College of Pharmaceutical Sciences and **Centre for Digital Learning**, MAHE, Manipal, India

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Vision Group on Science and Technology (VGST), **Karnataka Science and Technology Promotion Society** (KSTePS),
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Co-ordinator, Centre for Digital Learning
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Dr. C Mallikarjuna Rao
Convenor, CNSCON-2021
Principal, MCOPS, MAHE, Manipal



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Bengaluru

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This is to certify that APURWA DHAVALE

has participated as a Delegate during the National Conference held at COPS, DSU on **"New Horizons for Drug discovery and Innovation in Health care and Pharmaceutical Research"** on 29th November 2022.

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Organizing secretary

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Dean, SAHS

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has completed the certificate course in medical writing covering the practical aspects of developing manuscripts.

The course covered practical aspects of developing a literature review, systematic review, case reports, and original science articles. The activity-based learning also provided guidance on article submission.

Dr. Betsy Joseph

MDS (Periodontics), Ph D (Dentistry), MFDS RCPS (Glasg)
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28th June, 2023

Date

MedWriters.

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has completed the certificate course in medical writing covering the practical aspects of developing medico-marketing deliverables.

The course covered literature search, content processing, referencing styles, and the development of various medico-marketing inputs including articles, detailing aids, infographics, LBLs, RTEs, EDMs, case reports, slide kits, films, animations, KOL deliverables, rep training material, Advisory Board Meetings, and meeting reports.

Ramesh Mylapilli,

B.Pharm., MBA (Aus)
Program Director, MedWriters
CEO, Crixus Communications Pvt Ltd

28th June, 2023

Date

PUBLICATIONS

Protective effects of Ethanolic extract of *Alpinia calcarata* Roscoe rhizome on streptozotocin-induced diabetic peripheral neuropathy in rats

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Abstract

Background: Diabetic peripheral neuropathy (DPN) is a common and debilitating complication of diabetes, leading to nerve damage and impaired pain sensation. **Objectives:** The present study aimed to investigate the protective effects of ethanolic extract of *Alpinia calcarata* Roscoe rhizome (EEACR) on rats' streptozotocin (STZ)-induced DPN. **Materials and Methods:** Male Wistar rats were divided into control, STZ-induced diabetic, and two treatment groups (250 and 500 mg/kg EEACR). Diabetes was induced by a single intraperitoneal injection of STZ (55 mg/kg), and the treatment groups received EEACR orally for four weeks. **Results:** The results demonstrated a significant reduction in blood glucose levels and increased body weight in the EEACR-treated groups compared to the STZ-induced diabetic group. Moreover, EEACR treatment significantly ameliorated mechanical allodynia, thermal hyperalgesia, and cold allodynia in a dose-dependent manner. Biochemical analysis showed that EEACR treatment significantly reduced oxidative stress and increased antioxidant enzyme activities, such as superoxide dismutase, catalase, and glutathione peroxidase, in the sciatic nerves of treated rats. Histopathological evaluation revealed reduced nerve damage and degeneration in the EEACR-treated groups and improved myelin sheath and axonal integrity. **Conclusion:** This study provides evidence that the ethanolic extract of *Alpinia calcarata* Roscoe rhizome possesses protective effects against streptozotocin-induced diabetic peripheral neuropathy in rats, potentially

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through its antioxidant properties and ability to reduce oxidative stress. The findings suggest that EEACR may be a promising therapeutic agent for managing diabetic peripheral neuropathy.

Key-words: Diabetic, peripheral neuropathy, oxidative stress, *Alpinia calcarata*

Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Olokoba et al., 2012). The global prevalence of diabetes is rapidly increasing, affecting millions of people worldwide (Kharroubi and Darwish, 2015). The International Diabetes Federation (IDF) estimates that by 2045, the number of people with diabetes will reach 784 million, a 51% increase from the 2021 statistics (Christ, 2020). Diabetes can lead to various complications, including retinopathy, nephropathy, and neuropathy, with diabetic peripheral neuropathy (DPN) being the most common and debilitating complication. Diabetic peripheral neuropathy affects approximately 50% of diabetic patients, causing significant morbidity and reduced quality of life (Yang et al., 2022; Selvarajah et al., 2019; Sloan et al., 2021). DPN is characterized by pain, numbness, and tingling in the extremities, particularly the feet (Røikjer and Ejskjaer, 2022). The pathogenesis of DPN is multifactorial, with factors like oxidative stress, inflammation, and advanced glycation end-products playing crucial roles in the development and progression of the condition (Vinik et al., 2013). Current therapeutic options for DPN, including analgesics and anticonvulsants, primarily focus on alleviating symptoms rather than treating the underlying pathophysiological mechanisms. Therefore, there is a pressing need for novel therapeutic interventions to prevent or reverse DPN's progression.

Natural products derived from medicinal plants have been used for centuries to treat various ailments, including diabetes and its complications. *Alpinia calcarata* Roscoe, a plant from the Zingiberaceae family, is widely used in traditional medicine systems in South and Southeast Asia to treat a range of conditions, such as inflammation, pain, and respiratory ailments (Rahman and Islam MS, 2015; Wijayasiriwardena and Premakumara, 2012; Kong et al., 2000). The rhizome of *A. calcarata* is rich in bioactive compounds, including flavonoids, terpenoids, and phenolic compounds, which exhibit potent antioxidant, anti-inflammatory, and analgesic properties (Arawwawala et al., 2012; Arambewela et al., 2010; Arambewela et al., 2004). These properties have

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garnered interest in the potential therapeutic effects of *A. calcarata* for managing diabetes and its complications.

Ethanolic extracts have demonstrated significant biological activities due to their ability to solubilize many phytochemicals. The ethanolic extract of *A. calcarata* rhizome has been shown to possess potent antioxidant and anti-inflammatory activities, which may contribute to its potential protective effects against diabetes and associated complications. Furthermore, previous studies have reported the hypoglycemic and antihyperlipidemic effects of *A. calcarata* rhizome extract in streptozotocin (STZ)-induced diabetic rats, suggesting its potential role in managing diabetes (Rajasekar et al., 2014).

Although the ethanolic extract of *A. calcarata* rhizome has been shown to exhibit hypoglycemic and antihyperlipidemic effects in STZ-induced diabetic rats, its potential protective effects against DPN remain unexplored. This study, therefore, aims to investigate the protective effects of ethanolic extract of *A. calcarata* rhizome on STZ-induced peripheral neuropathy in rats.

Materials and Methods

Chemicals

STZ was acquired from Sigma Aldrich, India. While diagnostic kits for biochemical evaluations were procured from Krishgen Biosystem, India. All other chemicals utilized in the study were of analytical grade.

Plant material and its extraction

Alpinia calcarata Roscoe rhizomes were gathered from IIHR (Indian Institute of Horticulture and Research), Bengaluru. The botanical specimens were verified and confirmed by the same.

Freshly harvested *Alpinia calcarata* Roscoe rhizomes were sectioned into small fragments and subjected to air-drying for 12 to 14 days under shaded conditions. Subsequently, 500 grams of the desiccated rhizome powder were subjected to extraction with 1.5 liters of ethanol via a Soxhlet extraction apparatus for 4 hours. Following filtration of the resultant solution, the filtrate was concentrated under reduced pressure at 55 °C, yielding an 18.5% (w/w, based on dry weight) extract preserved at 4 °C for future utilization. A co-precipitate containing polyvinylpyrrolidone (PVP; molecular weight 44,000) was synthesized by blending the

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crude ethanolic extract (concentration: 1.0 mg/mL in ethanol) with PVP in a 1:1 (w/w) ratio (Arambewela et al., 2004).

Animals

The investigation was conducted using male Wister albino rats weighing 180 to 230 grams; rats were maintained in a controlled environment (temperature: 22 ± 2°C, humidity: 75 ± 5%, 12-hour light/dark cycle) within the research facility for the duration of the study. The animals were given a nutritional diet and water *ad libitum*. Before the commencement of the experiment, the rats were given a one-week acclimation period to adjust to their new surroundings. The study's experimental protocol received approval from the Institutional Animal Ethics Committee, KLE University's College of Pharmacy, Bengaluru (03/HP/2021), which was established in line with the guidelines set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) in India.

Induction of diabetic neuropathy and treatment

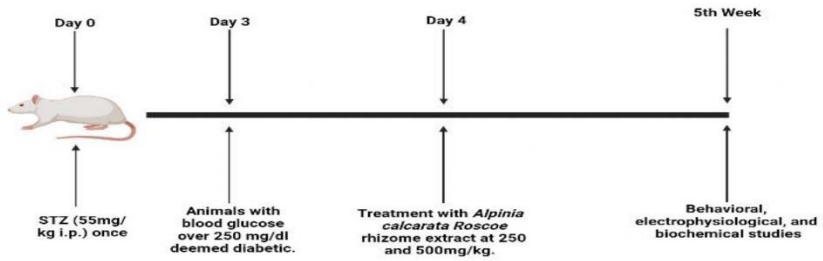
Diabetes was induced through a single intraperitoneal injection of streptozotocin (STZ) at 55 mg/kg, dissolved in ice-cold 0.1 M citrate buffer (pH 4.4), as previously described (Bhatt and Veeranjaneyulu, 2010; Kamdi et al., 2021). Forty-eight hours after STZ administration, plasma glucose levels were assessed to confirm diabetes induction. Animals exhibiting plasma glucose concentrations exceeding 250 mg/dl were identified as diabetic and included in subsequent analyses. Diabetic animals were classified according to their plasma glucose concentrations and body weights.

Group I – Normal Control

Group II – Positive control (STZ; 55mg/kg b.w, i.p)

Group III – STZ + *Alpinia calcarata* Roscoe rhizome extract (250mg/kg) for 4 weeks

Group IV – STZ + *Alpinia calcarata* Roscoe rhizome extract (500mg/kg) for 4 weeks



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Figure 1: Diagram illustrating experiment layout

Evaluation parameters

Body weight and plasma glucose levels

In the Streptozotocin (STZ)-induced diabetes peripheral neuropathy model, body weight was measured at the end of the study. Blood specimens were obtained from the retro-orbital sinus of the rats under light anesthesia upon completion of the experimental procedures. Subsequently, the blood samples underwent centrifugation to isolate serum. Plasma glucose levels were quantified using specialized enzyme-linked immunosorbent assay (ELISA) kits.

Behavioral parameters

Thermal Hyperalgesia

The rat tail was submerged in a heated water bath maintained at 46°C. Observations were made for indications of discomfort or reflexive retraction of the tail, and the latency period was documented using a stopwatch. A maximum duration of 12 seconds was established as the threshold. Decreases in the time taken for tail retraction indicate heightened pain sensitivity. After recording each response, the tail was meticulously dried after each response (Padgaonkar et al., 2017).

Cold Hyperalgesia

The rat tail was dipped in a container filled with cold water (4ºC). A rapid retraction or tail movement in response to the low temperature was observed and documented, with a maximum observation period of 10 seconds. A decrease in the time taken for the tail to retract indicates the presence of cold hyperalgesia (Ostovar et al., 2019).

Rota-Rod Performance

The Rota-rod assessment is a widely employed method for examining motor coordination in rats, specifically by measuring the capacity of rats to maintain their position on a rotating rod with a 75 mm diameter. The rotation speed was adjusted to enable normal rats to remain on the rod for 5 minutes. Rats underwent three practice sessions, each spaced 3 to 4 hours apart. During the evaluation, the rat was positioned on the rod, which rotates at 15 and 25 rpm speeds. The duration each rat successfully stayed on the Rota rod was documented (Vera et al., 2013).

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Grip Strength

A grip strength measurement was used to assess the grip strength of rat models in this study. Prior to the experimentation, the animals underwent an acclimatization process, during which they were placed on the apparatus for a designated period. To perform the measurement, each rat was suspended by its tail, ensuring a nearly horizontal orientation above the grid of the grip strength meter. Subsequently, a gentle force was applied to the base of the tail, aligning with the axis of the sensor until the animal released its hold on the grid. The exerted force by the rat was documented in either kilograms or newtons units (Nayak et al., 2021).

Electrophysiological parameter

Measurement of Nerve conduction velocity

The assessment of nerve conduction velocity was conducted non-invasively utilizing a PowerLab data acquisition system. An anesthetic combination of ketamine and xylazine (ranging from 80-100 mg/kg and 5-10 mg/kg, respectively, administered intraperitoneally) was used during the experimental procedure. The sciatic nerve was stimulated by applying supramaximal electrical stimuli through an electrode operating at 8V and a frequency of 20 Hz. Compound muscle action potential latencies were determined using bipolar electrodes. Motor nerve conduction velocity (MNCV) can be calculated by subtracting the proximal latency from the distal latency and dividing the resulting value by the distance separating the stimulation and recording electrodes (Addepalli and Suryavanshi, 2018).

$$\text{Nerve conduction velocity (m/sec)} = \frac{\text{Distance between stimulation sites}}{\text{Latency of M wave (sciatic notch) - Latency of M wave (Achilles tendon)}}$$

Oxidative stress parameters

At the end of the experiment, sciatic nerves were isolated and homogenized in ice-cold phosphate buffer (0.1 M, pH 7.4) using a probe homogenizer. The homogenate was divided into aliquots to obtain post-mitochondrial and post-nuclear fractions (Garud and Kulkarni, 2018). The protein concentration in the tissue homogenate was determined using a previously described method (Lowry et al., 1951). Lipid peroxidation was evaluated by measuring the malondialdehyde (MDA) (Ohkawa et al.,

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1979). The tissue sulfhydryl groups were assessed by determining the levels of reduced glutathione (GSH) (Ellman, 1959). Catalase activity was determined by measuring the amount of hydrogen peroxide decomposed per minute (Aebi, 1984). Superoxide dismutase (SOD) activity was measured using a previously described method (Paoletti et al., 1986).

Assay of cytokines

The TNF-α and IL-6 were measured using ELISA and according to the method adopted by Whiteside (Meager, 2006) using the kits following the manufacturer's instructions (Endogen Company, USA).

Histopathology

After collecting blood samples, the sciatic nerves were carefully isolated and submerged in a 10% formalin solution for fixation. Subsequently, the fixed nerves were immersed in paraffin wax in preparation for sectioning. Utilizing a microtome, ultra-thin sections of 5 micrometers in thickness were obtained. For histological evaluation, these sections undergo staining with Mayer's hematoxylin and eosin for general morphology, Masson's trichrome to visualize collagen distribution, and the Kulchitsky-Pal to assess myelin integrity.

Statistical analysis

All the data were analyzed by one-way ANOVA following post hoc Dunnett's multiple comparisons using Graph pad Prism software (version 9).

Results

Impact of *Alpinia calcarata* Roscoe Rhizomes Administration on Body Weight, and Blood Glucose

The diabetic group experienced a notable decline in body weight, mitigated by administering *Alpinia calcarata* at 250mg/kg and 500mg/kg compared to the diabetic group (Figure 2). Elevated blood glucose concentrations were observed in diabetic rats relative to healthy controls (317.4mg/dl versus 105.8mg/dl, $P<0.001$). A 4-week *Alpinia calcarata* treatment regimen led to a substantial decrease in blood glucose concentrations at dosages of 250mg/kg (278.7mg/dl, $P<0.05$) and 500mg/kg (254.8 mg/dl, $P<0.001$) compared to the diabetic group.

Impact of *Alpinia calcarata* Roscoe rhizomes treatment on behavior parameters

The observed decrease in response latency and tail withdrawal latency was markedly significant (P<0.001) in the diabetic group compared to the normal group for both the tail immersion test in hot and cold water. This reduction in latency was considerably counteracted by a four-week *Alpinia calcarata* treatment at all evaluated dose levels when compared with the diabetic group. The assessment of the Rota-rod response was carried out using the Rota-rod apparatus. Compared to normal animals, diabetic rats substantially declined motor coordination. Their capacity to sustain themselves on the rotarod apparatus was significantly diminished (P<0.001) compared to non-diabetic control animals. Administration of 250mg/kg and 500mg/kg *Alpinia calcarata* in diabetic animals led to an increase in retention time. Nerve strength was assessed by measuring grip strength in diabetic rats. Diabetic animals significantly reduced grip strength compared to normal rats, indicating muscle weakness. Administration of *Alpinia calcarata* for 4 weeks significantly improved grip strength latency compared to STZ-treated rats (Figure 3).

Impact of *Alpinia calcarata* treatment on NCV

Diabetic rats exhibited a substantial decline in nerve conduction velocity (NCV) compared to their healthy counterparts (P<0.001). A 4-week regimen of *Alpinia calcarata* treatment led to a notable enhancement in NCV among diabetic rats when administered at doses of 250mg/kg (P<0.001) and 500 mg/kg (P< 0.001) in comparison to the diabetic control group (Figure 4).

The impact of *Alpinia calcarata* therapy on oxidative stress markers

In diabetic animals, a marked elevation in oxidative stress within the sciatic nerves was observed, as evidenced by a considerable increase in malondialdehyde (MDA) levels and a decrease in glutathione (GSH), superoxide dismutase (SOD), and catalase concentrations when compared to non-diabetic animals. *Alpinia calcarata* treatment at 250mg/kg and 500mg/kg led to a substantial decrease in MDA levels (P< 0.001 and P<0.001, respectively) compared to the diabetic group. Additionally, GSH levels experienced a significant rise following *Alpinia calcarata* administration at doses of 250mg/kg (P<0.05) and 500mg/kg (P<0.01). Furthermore, catalase and SOD concentrations showed a notable increase after *Alpinia calcarata* treatment compared to the diabetic control group (Figure 5).

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IL-6 and TNF-α Levels

As shown in Figure 6, Levels of cytokines TNF- α and IL-6 in rats after induction of diabetic with STZ revealed high significant increase with percent change (85% and 80%), respectively, compared with the control group. A 4-week regimen of *Alpinia calcarata* treatment led to a notable enhancement in both TNF- α and IL-6 among diabetic rats when administered at doses of 250mg/kg ($P<0.001$) and 500 mg/kg ($P< 0.001$) in comparison to the diabetic control group.

Effect of *Alpinia calcarata* treatment on histopathology of sciatic nerves

In the *Alpinia calcarata*-treated group, H&E staining revealed a well-preserved nerve morphology with intact myelin sheaths and minimal infiltration of inflammatory cells compared to the disease group. The disease group displayed evidence of degenerative changes, including axonal swelling, loss of myelin, and infiltration of inflammatory cells. These findings suggest a protective effect of *Alpinia calcarata* treatment on the sciatic nerves (Figure 7).

Masson's trichrome staining demonstrated a more uniform and well-organized collagen distribution in the *Alpinia calcarata*-treated group compared to the disease group. The disease group exhibited increased deposition of disorganized collagen fibers around the nerve, indicating fibrosis. This result suggests that *Alpinia calcarata* treatment may help prevent fibrotic changes in the sciatic nerves (Figure 8).

Kulchitsky-Pal staining revealed a lower incidence of neurofibrillary tangles in the *Alpinia calcarata*-treated group compared to the disease group. The disease group displayed more neurofibrillary tangles, suggesting a possible neurodegenerative process. The reduced neurofibrillary tangles in the treated group indicate that *Alpinia calcarata* may have a neuroprotective effect on the sciatic nerves (Figure 9).

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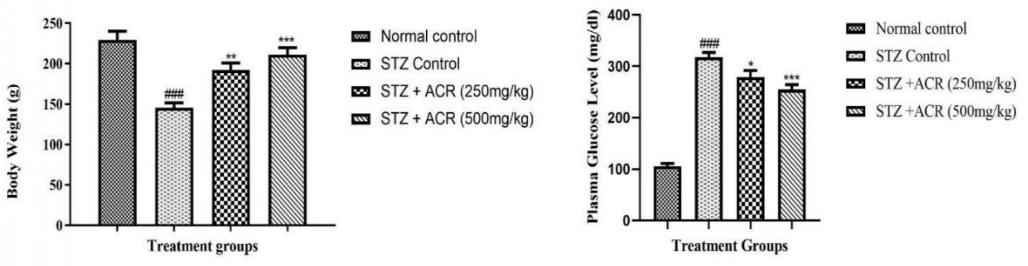


Fig. 2. Effect of treatment *Alpinia calcarata* on body weight and plasma glucose levels. All data are expressed as Mean ± SEM (n=6). ### P<0.001 when compared with normal control, ***P<0.001, **P<0.01, *P<0.05 when compared with diabetic control.

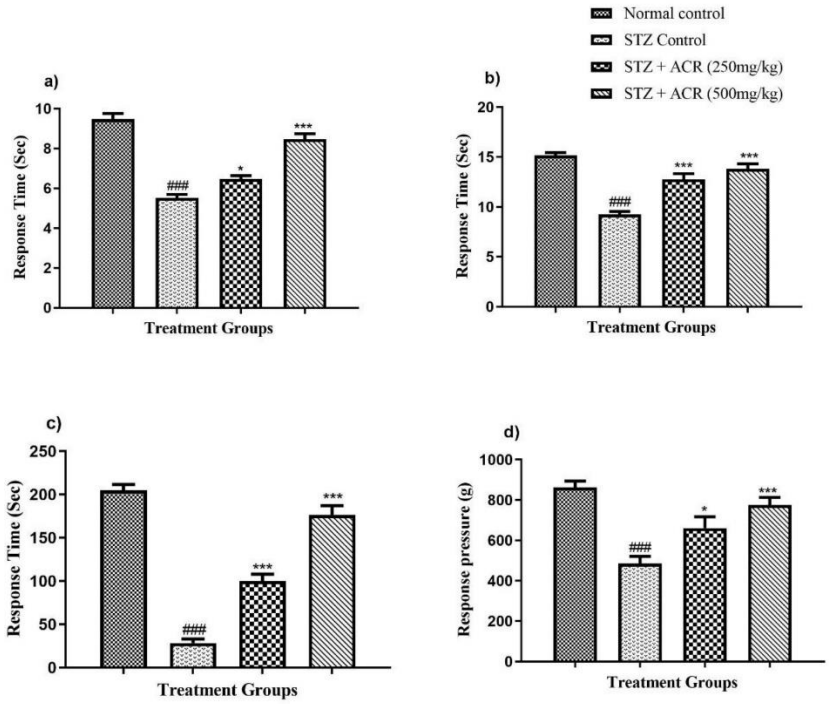


Fig. 3. Effect of *Alpinia calcarata* treatment on a) Tail immersion in hot water, b) Tail immersion in cold water, c) Rota-rod test, and d) Grip strength test. All data are expressed as Mean ± SEM (n = 6). ### P<0.001 when compared with normal control, *P<0.05, **P<0.01, ***P<0.001 when compared with diabetic control.

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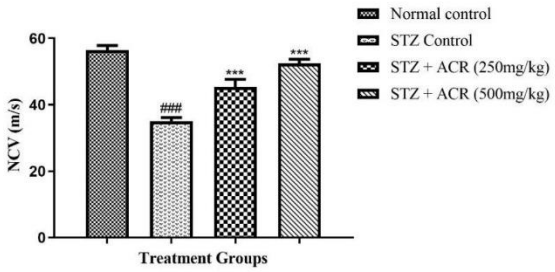


Fig. 4. Effect of *Alpinia calcarata* treatment on NCV. All data are expressed as Mean ± SEM (n = 6). ### P<0.001 when compared with normal control, ***P<0.001 when compared with diabetic control.

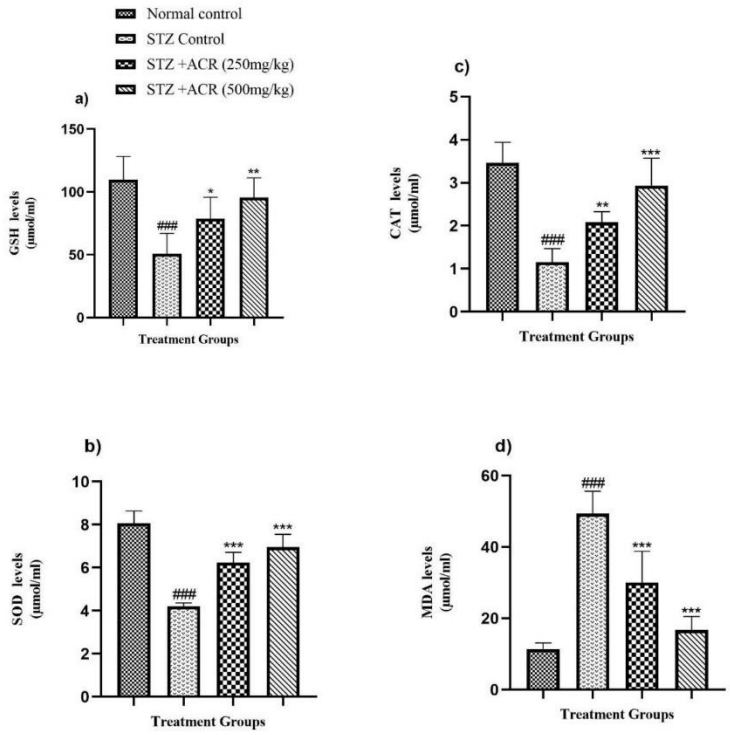


Fig. 5. Effect of *Alpinia calcarata* treatment on a) GSH, b) CAT, c) SOD, and d) MDA. All data are expressed as Mean ± SEM (n = 6). ### P<0.001 when compared with normal control, *P<0.05, **P<0.01, ***P<0.001 when compared with diabetic control.

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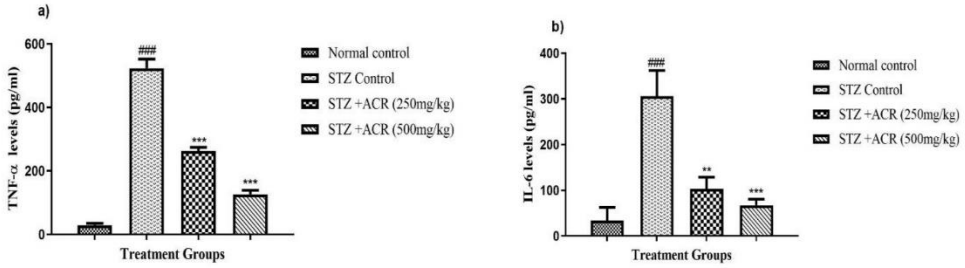


Fig. 6. Effect of *Alpinia calcarata* treatment on a) TNF-α, b) IL-6. All data are expressed as Mean ± SEM (n = 6). ### P<0.001 when compared with normal control, **P<0.01, ***P<0.001 when compared with diabetic control.

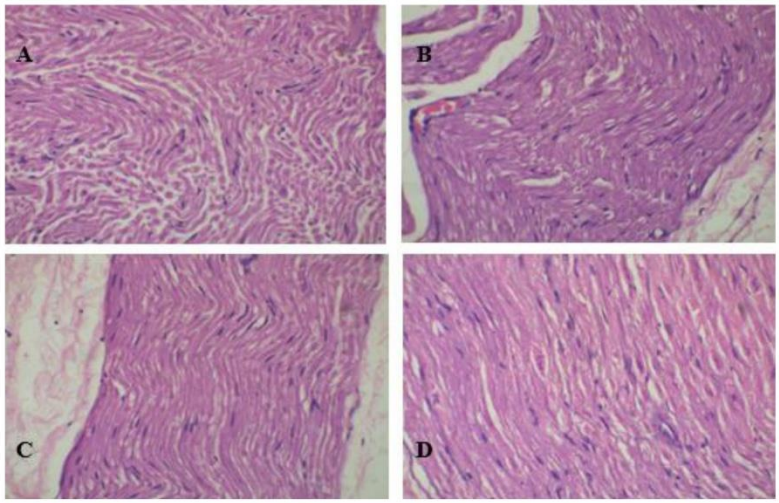


Fig. 7. Effect of *Alpinia calcarata* treatment on sciatic nerves- H&E staining (40×) a) Normal Control: showing a well-preserved nerve morphology with intact myelin sheaths and minimal infiltration of inflammatory cells b) Diabetic Control: showing degenerative changes, including axonal swelling and loss of myelin c,d) *Alpinia calcarata* (250 mg/kg and 500 mg/kg): showing normal histology, myelin sheath minimal infiltration of inflammatory cell.

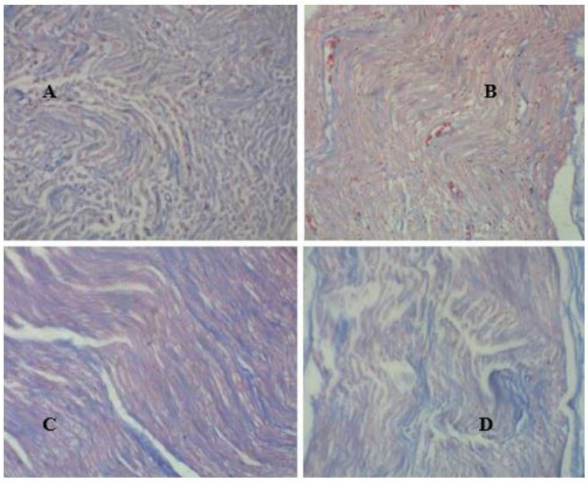


Fig. 8. Effect of *Alpinia calcarata* treatment on sciatic nerves- Masson's trichrome staining (40×) a) Normal Control: showing lower incidence of neurofibrillary tangles b) Diabetic Control: displayed more neurofibrillary tangles, suggesting a possible neurodegenerative process. c,d) *Alpinia calcarata* (250 mg/kg and 500 mg/kg): revealed a lower incidence of neurofibrillary tangles.

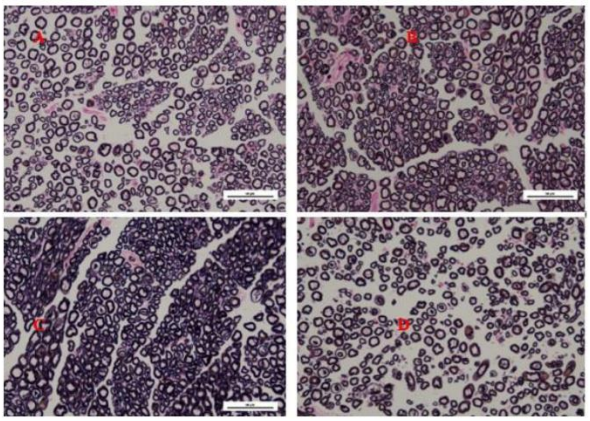


Fig. 9. Effect of *Alpinia calcarata* treatment on sciatic nerves- Kulchitsky-Pal staining (40×) a) Normal Control: showing uniform and well-organized collagen distribution b) Diabetic Control: showing increased deposition of disorganized collagen fibers around the nerve, indicating fibrosis. c,d) *Alpinia calcarata* (250 mg/kg and 500 mg/kg): showing normal histology and uniform and well-organized collagen distribution.

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Discussion

Diabetic complications, including neuropathy, nephropathy, retinopathy, and cardiomyopathy, are primarily attributed to uncontrolled blood sugar levels, elevated advanced glycation end products (AGEs), and increased receptors for AGEs (RAGE), leading to stress-induced inflammatory cascades. Diabetic peripheral neuropathy (DPN) is a persistent microvascular complication characterized by decreased vibration perception and nerve conduction velocity (Yagihashi et al., 2007). Although the precise mechanism underlying DPN remains elusive, it is predominantly associated with heightened oxidative stress and increased polyol pathway activity (Ahmad and Hoda, 2020). Various risk factors contribute to peripheral nerve damage, such as glycated hemoglobin (HbA1c) and collagen accumulation in peripheral nerves (Sugimoto et al., 1997). This accumulation provokes neuronal injury by activating apoptotic and inflammatory cascades by stimulating inflammatory and apoptotic mediators, including nuclear factor-kappa B (NF-kB) and tumor necrosis factor-alpha (TNF-α). Concurrently, an elevated polyol flux leads to sorbitol and reactive oxygen species (ROS) buildup, impairing nerve conduction velocities (Suryavanshi and Kulkarni, 2017; Padgaonkar et al., 2017).

The overexpression of NF-kB is central to neuronal injury, as it activates inflammatory mediators like tumor growth factor-beta (TGF-ß), TNF-α, and interleukin-6 (IL-6). Additionally, the arachidonic acid pathway is triggered in peripheral nerves, and cyclooxygenase-2 (COX-2) levels rise in response to NF-kB activation (Khan et al., 2013). This complex interplay of factors contributes to the development and progression of diabetic peripheral neuropathy, emphasizing the need for further research to elucidate the underlying mechanisms and develop effective treatments.

In individuals with diabetes, either inadequate insulin production or heightened insulin resistance disrupts the movement of glucose across cellular membranes, resulting in elevated blood glucose levels. Because of this impaired glucose transport, stored fats are metabolized for energy, which can lead to significant weight loss. This phenomenon is particularly evident in patients with type 1 diabetes. *Alpinia calcarata* has been demonstrated to promote insulin secretion, facilitating glucose transport across cell membranes and decreasing plasma glucose levels (Wang et al., 2009). The observed decrease in plasma glucose levels following *Alpinia calcarata* administration may be attributed to its insulin-stimulating properties. Furthermore, *Alpinia calcarata* treatment has been linked to a notable increase in overall body weight. Uncontrolled

diabetes can lead to neuronal damage, resulting in neuropathic pain. Neuronal dysfunction disrupts the balance between non-painful and painful stimuli by impairing the inhibitory or excessively stimulating the nociceptive pathway. This dysfunction induces pain in the absence of nociceptor involvement (Yagihashi et al., 2007).

Elevated glucose levels can lead to the overexpression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kß) and the mitogen-activated protein kinase (MAPK) pathway, initiating an inflammatory response. In addition, this hyperglycemic state triggers the release of inflammatory cytokines, such as transforming growth factor-beta (TGF-ß), cyclooxygenase-2 (COX-2), and tumor necrosis factor-alpha (TNF-α) in the sciatic nerves, resulting in mechanical allodynia and hyperalgesia (Ahmad and Hoda, 2020). *Alpinia calcarata*, a natural compound, demonstrates significant anti-inflammatory properties without impacting the immune system (Suryavanshi and Kulkarni, 2017). It has been shown to mitigate nociceptive pain by inhibiting the inflammatory response (Padgaonkar et al., 2017). Treatment with *Alpinia calcarata* has been observed to significantly reduce thermal hyperalgesia, mechanical hyperalgesia, and mechanical allodynia in diabetic animals. This attenuation of neuropathic pain may be attributed to inhibiting the NF-kß pathway, subsequently disrupting the inflammatory cascade.

The accumulation of reactive oxygen species (ROS) and dysregulated polyol flux elevate the activity of aldose reductase and sorbitol dehydrogenase in peripheral nerves (Yagihashi et al., 2007). This increase in ROS and sorbitol in the sciatic nerves disrupts nerve conduction velocity by inactivating Na+/K+ ATPase activity (Ahmad and Hoda, 2020). Moreover, the overexpression of NF-kß, resulting from elevated protein kinase C (PKC), ROS, and advanced glycation end products (AGEs), contributes to the degeneration of peripheral neurons through leukocytic infiltration and the reduction of neuronal growth factor (NGF), TNF-α, IL6, and IL1ß (Sugimoto et al., 1997).

Alpinia calcarata has demonstrated anti-inflammatory and analgesic effects by inhibiting the TNF-α and NF-kß pathways (Suryavanshi and Kulkarni, 2017). Treatment with *Alpinia calcarata* significantly improved motor and sensory nerve conduction velocities in diabetic rats, potentially due to its neuroprotective effect through the inhibition of oxidative stress (Padgaonkar et al., 2017). The generation of ROS in neuronal cells initiates a pro-apoptotic cascade. It releases inflammatory mediators, depleting antioxidant enzymes, such as malondialdehyde, glutathione, catalase, and superoxide dismutase. The disruption of these antioxidant enzymes results

in mitochondrial dysfunction, demyelination of neurons, and extracellular matrix accumulation (Ahmad and Hoda, 2020). *Alpinia calcarata* treatment has been observed to normalize antioxidant enzyme levels and decrease oxidative stress in the sciatic nerves. This normalization of oxidative enzymes helps prevent demyelination and confers neuroprotection.

Alpinia calcarata treatment demonstrated a significant protective effect on the sciatic nerves. Histological examination using H&E staining showed preserved nerve morphology and intact myelin sheaths. It reduced inflammatory cell infiltration in the *Alpinia calcarata*-treated group compared to the disease group, which displayed degenerative changes like axonal swelling and myelin loss. Masson's trichrome staining exhibited a more organized collagen distribution in the treated group, suggesting a possible preventive effect against fibrotic changes. Furthermore, Kulchitsky-Pal staining revealed decreased neurofibrillary tangles in the *Alpinia calcarata*-treated group, implying a potential neuroprotective role. Overall, these findings indicate that *Alpinia calcarata* may be beneficial in preventing nerve damage and neurodegeneration.

Conclusion

The present study provides compelling evidence that the ethanolic extract of *Alpinia calcarata* Roscoe rhizome exerts significant protective effects against streptozotocin-induced diabetic peripheral neuropathy in rats. Our findings demonstrate that the extract ameliorates hyperglycemia, alleviates oxidative stress, and promotes nerve regeneration, thereby mitigating the structural and functional damage caused by diabetic peripheral neuropathy. These results suggest that bioactive compounds in *Alpinia calcarata* Roscoe rhizome may serve as potential therapeutic agents for treating and managing diabetic peripheral neuropathy. However, further research is warranted to elucidate the precise molecular mechanisms underlying the protective effects of the extract.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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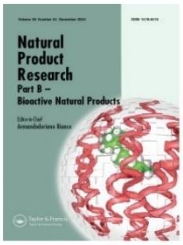
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Neuroprotective potential of *Alpinia calcarata* and HPLC analysis of stigmasterol against cisplatin-induced neuropathy in rat model

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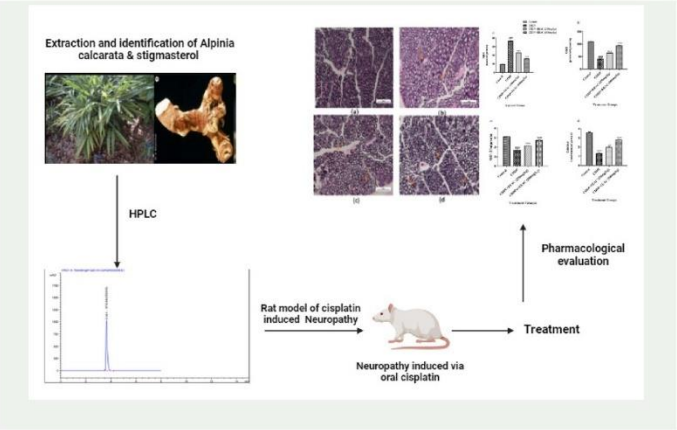
ABSTRACT

Cisplatin is a chemotherapeutic agent known for causing severe peripheral neuropathy as a side effect, impacting patients' quality of life by damaging nerve tissues. This study aims to explore the neuroprotective effects of the ethanolic extract of *Alpinia calcarata* Roscoe rhizome (EEACR) and stigmasterol identified by high-performance liquid chromatography (HPLC) in a rat model of cisplatin-induced neuropathy. Male Wistar rats were divided into control, cisplatin-induced neuropathic, and two intervention groups receiving different concentrations of EEACR (250 and 500 mg/kg). Neuropathy was induced with cisplatin administered intraperitoneally at a dose of 2 mg/kg per week for five weeks. The intervention groups were treated orally with EEACR daily during the induction period. Treatment with EEACR showed a significant attenuation of neurotoxicity as evidenced by behavioural improvements in mechanical allodynia, thermal hyperalgesia, and cold allodynia. HPLC analysis confirmed the presence of stigmasterol in EEACR, which may contribute to its therapeutic effects. Biochemical assessments revealed a significant decrease in oxidative stress markers and an increase in antioxidant enzyme activities, including superoxide dismutase, catalase, and glutathione peroxidase, in the nervous tissues of EEACR-treated rats. Histopathological examination indicated a reduction in nerve damage and enhanced preservation of myelin and axonal structures in the treatment groups. The findings from this study suggest that EEACR, enriched with stigmasterol, offers promising neuroprotective effects against cisplatin-induced neuropathy in rats.

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1. Introduction

Peripheral neuropathy is a complex condition characterised by damage to the peripheral nervous system, with diverse aetiologies including genetic factors, autoimmune diseases, infectious agents, nutritional deficiencies, and metabolic disorders (Stubblefield et al. 2009; Hsiao et al. 2010; Lehmann et al. 2020). Among its various subtypes, chemotherapy-induced peripheral neuropathy (CIPN) stands out, often resulting from exposure to anticancer chemotherapeutic agents. Cisplatin (cis-diamminedichloroplatinum II), a widely used platinum-based compound in cancer treatment, is a known cause of sensory peripheral neuropathy, typically presenting in a 'sock and glove' distribution pattern (Stubblefield et al. 2009; Colvin 2019). While cisplatin is highly effective as a chemotherapeutic agent, its neurotoxic effects—often dose-limiting and potentially long-lasting-are primarily attributed to mitochondrial dysfunction, apoptosis, and damage to the dorsal root ganglia (DRG) neurons (Staff et al. 2019). Research highlights the role of elevated inflammatory cytokines and chemokines in CIPN pathogenesis, positioning inflammation as a key contributor to neuropathy development. (Alberti 2019). As a result, therapeutic strategies involving anti-inflammatory agents and immunomodulators are being actively investigated for their potential to alleviate CIPN. (Kim et al. 2015; Brandolini et al. 2019).

In traditional medicine, *Alpinia calcarata* Roscoe, a member of the Zingiberaceae family, is highly regarded across several Asian countries, including Bangladesh, India, Indonesia, Vietnam, Thailand, Malaysia, Sri Lanka, Taiwan, and Cambodia. Native to India and thriving in dense forests at high altitudes, this rhizomatous perennial plant is celebrated for its extensive medicinal properties (Ghosh and Rangan 2013; Philomindoss 2017). The rhizome of *Alpinia calcarata* (AC) is commonly used as a food additive, spice, and in traditional medicine, boasting a rich phytochemical composition that includes flavonoids, glycosides, phenols, phytosterols, and tetracyclic terpenoids (Mathew and Britto 2016). These constituents confer a wide range of pharmacological activities, such as antinociceptive, antioxidant, antidiabetic,

antibacterial, antifungal, anthelmintic, aphrodisiac, and anticancer properties (Raj 1975; Perveen et al. 2012; Ghosh and Rangan 2013; Rahman and Islam 2015; Akbarzadeh et al. 2023). Among its bioactive metabolites, stigmasterol is particularly n for its significant role in managing and treating conditions like cancer, diabetes, thyroid disorders, osteoarthritis, and other inflammatory diseases (Panda et al. 2009; Gabay et al. 2010; Kangsamaksin et al. 2017; Wang et al. 2017). Despite its importance, there is a clear gap in the literature concerning dedicated methods for quantifying stigmasterol in the ethanolic extract of *Alpinia calcarata* rhizomes (EEAC).

To address this, the current study aims to develop and validate a high-performance liquid chromatography (HPLC) method, following International Conference on Harmonisation (ICH) guidelines, for the quantification of stigmasterol in EEAC rhizomes. Furthermore, the study will evaluate the neuroprotective potential of EEAC in a cisplatin-induced neuropathy rat model. This research extends our ongoing exploration of bioactive metabolites in AC, utilising HPLC for its simplicity, precision, and reliability in analyte quantification.

2. Result

2.1. Method development and optimization

The proposed HPLC method with UV detection was developed for the determination of stigmasterol. Several mobile phases were attempted to elute the analyte. The main focus was to achieve a sharp peak. The mobile phases tried for this purpose were water: methanol (30:80 v/v), acetonitrile: water (95:5v/v), acetonitrile: orthophosphoric acid (90:10v/v). The chromatogram (Supplementary Figure 1) obtained with an Acetonitrile: Orthophosphoric acid (90:10v/v) was found to have good symmetry and the lowest Rt (3.64) and sharp peak. Therefore, the Acetonitrile: Orthophosphoric acid (90:10v/v) was used as the optimised mobile phase.

2.2. Method validation

Validating the method in HPLC is crucial to ensuring the reliability, reproducibility, and accuracy of sample analysis. The method validation process was carried out in accordance with ICH guidelines.

2.2.1. Linearity

The linearity range of stigmasterol solution was obtained as 10-50 µg/ml as presented in Supplementary Table 2 & Figure 2. The linear regression equation was found to be $y = 138.68x + 2442.7$ with a regression coefficient of 0.998.

2.2.2. LOD LOQ

LOD and LOQ of the method were determined by the standard deviation method as described in the experimental section. LOD and LOQ were found to be 2.08 µg/ml and 6.33 µg/ml which indicates the method is sensitive and stigmasterol was detected at a very low concentration.

2.2.3. Precision

The precision was determined by injecting 20 µl/ml of a standard solution stigmasterol in the six replicates and respective areas were calculated. The precision results are expressed as the percentage of relative standard deviation (% RSD). Both repeatability and intermediate precision are also reported as % RSD and presented in Supplementary Table 3.

2.2.4. Specificity

The absence of interference at the retention of the analyte was demonstrated by injecting the blank sample which consisted of diluents. The developed method for stigmasterol demonstrated no interference at the analyte's retention, as depicted in Supplementary Figure 3. A typical HPLC peak of stigmasterol is shown in Supplementary Figure 3.

2.2.5. Ruggedness

Additionally, an intra- and inter-day precision reproducibility study was conducted. Precision was assessed using the same instrument, but measurements were taken by different analysts. The reproducibility results are presented in Supplementary Table 4.

2.2.6. Robustness

There was no significant change in the retention time of stigmasterol by changing flow rate and wavelength. The % RSD is less than 2 indicating the developed method was robust as shown in Supplementary Table 5.

2.2.7. System suitability

The system suitability test of the chromatographic system was performed. Six replicate injections for the system suitability test were injected into the HPLC system. The peak retention time was found to be 3.6 min, and the number of theoretical plates in all standard injections for stigmasterol was not less than 2000. The tailing factor was < 2. Relative standard deviation and column efficiency for 6 injections were determined for all sample analyses %RSD was found to be ≤ 2, and the theoretical plate was found to be ≥ 2000. The tailing factor was recorded as 0.99 (Supplementary Table 6).

2.3. Quantification of stigmasterol in Alpinia calcarata extract

The developed method was used to quantify stigmasterol in *Alpinia calcarata* extract. It was found that stigmasterol shows retention at 3.58 and the area under peak was found to be 4088.86. The amount of stigmasterol detected in the extract was 12.61 µg (Supplementary Figure 4).

2.4. Effect of EEAC on body weight

The control group animals exhibited a higher body weight compared to those treated with CDDP, which showed a significant reduction in body weight ($p < 0.001$). However, treatment with EEAC at doses of 250 mg/kg and 500 mg/kg significantly improved

body weight in comparison to the CDDP group, achieving statistical significance ($p < 0.001$) (Supplementary Figure 5).

2.5. Effect of EEAC on behavioural parameters

In the CDDP-treated group, there was a significant reduction in tail withdrawal latency in both cold and hot flick tests compared to the control group ($p < 0.001$). However, this reduction was significantly mitigated by EEAC treatment at doses of 250 mg/kg and 500 mg/kg ($p < 0.001$), with the 500 mg/kg dose restoring latency to levels similar to the control (Supplementary Figures 6-A and 6-B). In terms of motor coordination and grip strength, measured by the grid suspension test, the CDDP group exhibited significant reductions, decreasing by 27% and 42%, respectively ($p < 0.001$), compared with control animals. EEAC treatment at both 250 mg/kg and 500 mg/kg doses significantly improved neuromuscular coordination and grip suspension time compared to the CDDP group (Supplementary Figures 6-C and 6-D).

Further observations from the wire hang and beam walking tests (Supplementary Figures 6-E and 6-F) reveal that the CDDP group experienced a significant decrease in suspension time ($p < 0.01$) compared to the control group. Conversely, EEAC treatment at both 250 mg/kg and 500 mg/kg significantly increased suspension time ($p < 0.05$, $p < 0.01$) compared to the CDDP group. The beam walking test results indicated a general decrease in performance in the CDDP group ($p < 0.01$) compared to the control. However, EEAC treatment significantly improved the distance travelled, number of slips, and number of turns by 20% and 30% for the doses of 250 mg/kg and 500 mg/kg, respectively, compared to the CDDP group as shown in Supplementary Table 7.

2.6. Effect of EEAC on biochemical parameters

To study the effects of EEAC on antioxidant enzymes, different doses of EEAC administered with CDDP showed significant restoration of antioxidant activity. At a dose of 250 mg/kg, EEAC in combination with CDDP restored the activities of SOD, GSH ($p < 0.001$), and CAT ($p < 0.01$) (Supplementary Figure 7). At a higher dose of 500 mg/kg, the restoration effects on SOD, GSH, and CAT were even more pronounced, with all showing significant improvements ($p < 0.001$). On the other hand, the activity of MDA, an indicator of oxidative stress, was significantly increased in the CDDP-only group when compared to the control group ($p < 0.001$), highlighting increased oxidative stress due to CDDP. co-administration with EEAC significantly mitigated this effect. At a dose of 250 mg/kg of EEAC with CDDP, MDA levels were significantly reduced compared to the CDDP-only group ($p < 0.01$). This reduction was even more substantial at the higher 500 mg/kg dose of EEAC, where MDA levels nearly approached those of the control group, showing a profound protective effect against CDDP-induced oxidative stress ($p < 0.001$).

2.7. Histopathology study

A histopathology study was conducted to evaluate the effect of surface modification on endoneurial fibrosis. The sciatic nerves of the control and experimental groups were stained with Masson trichrome and Kulchitsky-Pal (Kpal). The results of Masson trichrome

staining (Supplementary Figure 8) demonstrated that the CDDP group had significant endoneurial fibrosis, with axons surrounded by a trichrome-positive area. However, treatment with EEAC at 250 mg/kg and 500 mg/kg doses showed progressive improvement in endoneurial fibrosis. The Kulchitsky-Pal (Kpal) staining results (Supplementary Figure 9) showed that the control group had uniform myelinated fibres, whereas the CDDP group had demyelination and collagen distribution. The histopathological analysis revealed that the CDDP group had increased deposition of axonal loss in the form of endoneurial fibrosis. However, treatment with EEAC at 250 mg/kg and 500 mg/kg prevented axonal loss, myelin ovoids, and demyelinating neuropathy. The myelin stain decreased neurofibril entanglement in the transverse section of the sciatic nerve, indicating that it is a promising treatment for neural protection.

3. Discussion

Cancer remains one of the leading causes of mortality worldwide. In 2020 alone, an estimated 10.3 million people succumbed to cancer, with 19.1 million new cases reported globally (Sung et al. 2021). Despite the alarming statistics, advances in medical science have led to the development of several anticancer therapies, including chemotherapy, which has significantly extended the life expectancy of cancer patients (Schirrmacher 2019). However, chemotherapy-induced peripheral neuropathy (CIPN) is a major dose-limiting side effect, impacting patients' quality of life. First-line anticancer drug classes such as vinca alkaloids, taxanes, and platinum-based compounds are widely used to treat various cancers, but they are also associated with neuropathic complications (Marmiroli et al. 2012). Moreover, motor, sensory, and autonomic impairment, as well as numbness, tingling, and searing pain, are signs of CIPN. Our investigation into the protective effects of the Ethanolic Extract of *Alpinia calcarata* against CDDP-induced neurotoxicity reveals significant findings that contribute to the understanding and potential mitigation of CIPN.

CDDP, as a primary member of platinum-containing anticancer agents, induces DNA crosslinking leading to apoptosis but is also known for its adverse impact on the nervous system, manifesting as peripheral neuropathy characterised by motor, sensory, and autonomic dysfunction (Wolf et al. 2008). This study specifically explored EEAC's efficacy in counteracting these detrimental effects of CDDP, utilising behavioural, biochemical, and histological assessments in rats subjected to CDDP-induced neurotoxicity. Administering EEAC at doses of 250 mg/kg and 500 mg/kg body weight demonstrated mitigation of rapid body weight loss, improvement in tail withdrawal latency, motor coordination, and grip strength, effectively identifying and addressing neuropathic disorders induced by CDDP. Our findings are in line with existing literature, underscoring the correlation between CDDP administration and sensory-motor dysfunction, including diminished withdrawal latency and reduced grip strength, thereby validating our experimental approach and outcomes (Khan et al. 2003; Ali et al. 2014; Sharawy et al. 2015). Remarkably, EEAC not only ameliorated these effects but also exhibited potential in enhancing muscle strength and coordination at the higher dose of 500 mg/kg, suggesting a dose-dependent neuroprotective effect. This is particularly relevant considering the oxidative stress and inflammation associated with CDDP-induced neuropathy, where EEAC's antioxidant and anti-inflammatory properties, evidenced through the normalisation of antioxidant enzyme levels and reduction of oxidative stress in sciatic nerves, play a crucial role.

The phytochemical analysis of EEAC has revealed the presence of stigmasterol, novel labdane-type diterpenoids, and other bioactive compounds, underscoring its therapeutic potential. The use of HPLC for quantifying these compounds supports the standardisation of EEAC and further demonstrates its potential therapeutic benefits. This study not only confirms the neurotoxic effects of CDDP but also introduces EEAC as a promising neuroprotective agent against CIPN. The identification of EEAC's bioactive constituents, along with the elucidation of its protective mechanisms, lays the groundwork for future research and clinical trials. Such efforts could potentially integrate EEAC into cancer treatment regimens to alleviate CIPN, thereby improving the quality of life for cancer patients.

4. Conclusion

This study conclusively demonstrates the neuroprotective potential of the Ethanolic Extract of *Alpinia calcarata* in mitigating the harmful effects of CDDP-induced peripheral neuropathy. Through detailed behavioural, biochemical, and histopathological analyses, EEAC significantly reduced CDDP-induced neurotoxicity in rats, showing marked improvements in sensory-motor function and oxidative stress markers. Additionally, the development and validation of HPLC method for quantifying stigmasterol in EEAC enhances its standardisation and supports its potential clinical application. Our findings highlight EEAC's therapeutic potential as a complementary treatment for CIPN, offering promise for improving the quality of life in cancer patients undergoing chemotherapy. Future research should focus on uncovering the precise mechanisms underlying EEAC's protective effects and exploring its integration into current cancer treatment regimens to effectively reduce the impact of CIPN.

Ethics approval

The study's experimental protocol received approval from the Institutional Animal Ethics Committee, KLE University's College of Pharmacy, Bengaluru (03/HP/2021).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Neuroprotective Potential of *Alpinia calcarata* and HPLC Analysis of Stigmasterol Against Cisplatin-Induced Neuropathy in rat model

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Abstract

Cisplatin is a chemotherapeutic agent known for causing severe peripheral neuropathy as a side effect, impacting patients' quality of life by damaging nerve tissues. This study aims to explore the neuroprotective effects of the ethanolic extract of *Alpinia calcarata* Roscoe rhizome (EEACR) and stigmasterol identified by high-performance liquid chromatography (HPLC) in a rat model of cisplatin-induced neuropathy. Male Wistar rats were divided into control, cisplatin-induced neuropathic, and two intervention groups receiving different concentrations of EEACR (250 and 500 mg/kg). Neuropathy was induced with cisplatin administered intraperitoneally at a dose of 2mg/kg per week for five weeks. The intervention groups were treated orally with EEACR daily during the induction period. Treatment with EEACR showed a significant attenuation of neurotoxicity as evidenced by behavioral improvements in mechanical allodynia, thermal hyperalgesia, and cold allodynia. HPLC analysis confirmed the presence of stigmasterol in EEACR, which may contribute to its

therapeutic effects. Biochemical assessments revealed a significant decrease in oxidative stress markers and an increase in antioxidant enzyme activities, including superoxide dismutase, catalase, and glutathione peroxidase, in the nervous tissues of EEACR-treated rats. Histopathological examination indicated a reduction in nerve damage and enhanced preservation of myelin and axonal structures in the treatment groups. The findings from this study suggest that EEACR, enriched with stigmasterol, offers promising neuroprotective effects against cisplatin-induced neuropathy in rats, likely through its potent antioxidant properties. These results support the potential use of EEACR as a therapeutic agent in managing chemotherapy-induced neuropathy.

Key-words

Chemotherapy, peripheral Neuropathy, *Alpinia calcarata,* stigmasterol

Experimental

Materials

Acetonitrile and OPA (HPLC grade) were purchased from Merck Ltd Mumbai, India. Stigmasterol (99%) from Sigma□Aldrich, Germany. Cisplatin was procured from Hetero Healthcare Ltd. The chemicals used were of analytical grade.

Collection and Authentication of plant material

Fresh and fully grown rhizomes of *Alpinia calcarata* were collected at the Indian Institute of Horticultural Research, (Bengaluru) Karnataka, India, in January. The authentication was confirmed by Dr. P.E. Rajasekharan, Nodal Officer and Principal Scientist (GAC Division), with a voucher specimen (Voucher No. 375657) has been deposited at the Department of Plant Genetic Resources, (Bengaluru) Karnataka, India. Verified the plant name *Alpinia calcarata* with http://www.theplantlist.org/, accessed on February 2023.

Preparation of ethanol extracts

The fresh rhizome of *Alpinia calcarata* was cut into small pieces and shade-dried for 12 to 14 days at 35 to 40□. Then it was ground to a uniform powder. 500 gm of powder rhizome were extracted with 1.5 L of ethanol using a Soxhlet extractor for 4 hrs. The extraction was filtered to remove all unextractable matter, including cellular materials and other insoluble constituents in the extraction solvent. Moreover, using a rotary evaporator, the filtrate was evaporated to dryness. under reduced pressure at 55□ (yield 18.5%, w/w, dry weight basis) and stored at 4□ until use. For subsequent HPLC analysis, the filtrate obtained was a sample solution (Ghosh and Rangan 2013; Philomindoss 2017).

Preparation of standard sample

10 mg of stigmasterol dissolved in 10 ml diluent (1000 µg/ml). further dilutions were prepared

from these stock solutions. Acetonitrile: Orthophosphoric acid (90: 10) to get the desired concentration. The standard stock was prepared in calibrated flasks.

Preparation of extract solution

10mg of Alpinia calcarata extract was dissolved in 10 ml of diluent Acetonitrile: Orthophosphoric acid (90: 10). The solution was sonicated for 30 min and then filtered.

Equipment

An Agilent (1100) gradient HPLC equipped with a UV detector, RP C-18 Agilent column with G1310A pump, and Chemstation (10.01) software were used.

Chromatographic Condition

The chromatographic column used was a reverse phase 4.6 × 250 MM Agilent C-18 with a particle size packing of 5µm. The column and HPLC system were kept at ambient conditions. The mobile phase was Acetonitrile: 0.1% Orthophosphoric acid (90: 10) with a 0.7 mL/min flow rate. The injection volume was 20µL, and the elute was analyzed at a wavelength of 220nm (Table S1).

Method Development and Optimization

Various solvent systems were tried to develop a suitable HPLC method for analyzing stigmasterol in herbal extract. The suitability of the solvent system was determined by its cost, sensitivity, and the time required for a single analysis. The mobile phase tried for this purpose was water: methanol, acetonitrile: water, and acetonitrile: orthophosphoric acid. The condition that gave the best resolution and symmetry was selected.

Method Validation

Linearity

The concentration of stigmasterol from 10-50 µg/ml was prepared from stock solution (1000 µg/ml). The graph between the concentration and the area under peak was plotted for linearity.

Sensitivity

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ were determined by the standard deviation method. The LOD and LOQ were determined using the slope of the calibration curve.

$$LOD = 3.3 \times \frac{SD}{S}$$

$$LOQ = 10 \times \frac{SD}{S}$$

Where SD is the standard deviation and S is the slope of the calibration curve.

Precision

In the validation of an HPLC method, precision is a key parameter for evaluating the consistency and reliability of analytical results. It refers to the degree of agreement between a series of measurements taken from multiple samples of the same homogeneous substance under specified conditions. The method's precision was assessed through inter-day and intra-

day variation studies, confirming its reproducibility and reliability. **Repeatability (Intraday Precision)**

Repeatability assesses the precision under the same operating conditions over a short interval of time, such as within the same day. It involves analyzing multiple replicates (6) of the same sample solution prepared independently. The relative standard deviation (RSD) is used to express the degree of precision.

Intermediate Precision (Interday Precision)

Intermediate precision assesses the variability of results within the same laboratory under different conditions, such as varying days, analysts, instruments, and other factors. It provides an estimate of the method's robustness. Like repeatability, it is evaluated by analyzing multiple replicates of the same sample solution across different days.

Specificity

The method's specificity was assessed by injecting a blank sample without the analyte and examining any potential interference at the analyte’s retention time. To determine if any interfering substances eluted at the same retention time, the blank solution was injected three times.

Ruggedness (Reproducibility)

In HPLC validation, ruggedness is a measure of an analytical method's ability to maintain performance when small changes occur in experimental conditions. Ruggedness is defined in the current USP guideline as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions. Ruggedness was checked by determining precision on the same instrument but by different analysts.

Robustness

Robustness is the capacity of a method to remain unaffected by small, deliberate variations in method parameters; it measures a method's reliability. In robustness studies, small and

deliberate changes in flow rate, wavelength, or temperature are made. The method's robustness was studied by changing flow rate and wavelength. The standard solution was injected in triplicate, and the changes in RSD were observed.

System suitability

System suitability (SST) testing is a test that verifies that a chromatographic system is working properly before any sample analysis. SST is an integral part of many analytical procedures, according to the United States Pharmacopeia (USP) and the International Conference on Harmonization (ICH). System suitability was determined through six consecutive analyses of a standard solution of stigmasterol. Key parameters such as retention time, column efficiency, peak symmetry, and theoretical plates were determined for standard solutions.

Solution stability

The solution stability was demonstrated by periodic analysis of the same sample solution.

Animals

Wistar albino rats of either sex, average weight of about 200-250 gm, Rats were kept in clean polypropylene cages, housed in an air-conditioned atmosphere at a temperature of 25 ± 2ºC with alternatively 12 hrs light and dark cycles, and kept on a standard diet and water was given *ad libitum*. Standard diet pellets were commercially purchased from VRK Nutritional Solutions, Pune, Maharashtra. Experimentation studies were done and maintained as per the CPCSEA guidelines in the animal facility at KLE College of Pharmacy, Bengaluru, India.

Experimental design

After seven days of acclimatization. The rats were grouped randomly into four groups (N=6) and treated as follows: The first group was (Normal Control; NC): No treatment was given. The second group was (Diseased Control; DC): Animals received the 2 ml Saline oral, CDDP 2 mg/kg b.w., i.p. weekly once for five weeks (8, 15, 22, 28 and 36 days). The third group

was (Low Dose; LD): Animals received the 2 ml Saline oral, CDDP 2 mg/kg b.w., i.p. weekly once and EEAC 250mg/kg, b.w., orally for five weeks (CDDP + EEAC). The fourth group was (High Dose; HD): Animals received the 2 ml Saline oral, CDDP 2 mg/kg b.w., i.p. weekly once and EEAC 500mg/kg, b.w., orally for five weeks (CDDP + EEAC) (Vera et al. 2013; Ghosh and Rangan 2013).

Evaluation parameters

Tail hot hyperalgesia (tail-flick test)

The tail immersion test has been performed to assess thermal sensitivity (Necker and Hellon 1977). When the terminal part of the tail was immersed 1 cm in water at a temperature of 46ºC, as a response to the thermal sensation, the duration of the tail withdrawal reflex was recorded, and the cut-off time limit of 12 seconds was maintained.

Tail cold Hyperalgesia (tail-flick test)

The tail immersion test has been performed to assess cold sensitivity (Ostovar et al. 2020). In this test, the tip of the tail was submerged 1 cm into water maintained at 4°C to induce tail cold hyperalgesia. The time taken for the tail withdrawal reflex in response to the cold stimulus was recorded, with a cut-off time set at 10 seconds to prevent prolonged exposure.

Rota-rod Performance (motor coordination)

The retention time was measured using a rotarod apparatus (Singh et al. 2020). The rats were put on the rotating rods, and the time each rat spent on the rod was measured. The apparatus comprises an 80 cm wide, rough metal rod measuring 8 cm in diameter, which prevents the rats from escaping. In the wood chamber, the rod was set to a height of 50 cm using black painted acrylic glass, the rod was attached to the rotating drum and divided into five segments of 10 cm each. For three days, Rats were trained to walk on a rotating rod at increasing

speeds, from 1 to 50 rpm, for 5 minutes daily. On the test day, in the 5-minute test sessions, the time it takes rats to fall was measured at the speed of 50 rpm.

Grip Strength

The rats were trained to familiarize themselves with the grip strength meter over a specified period (ALI et al. 2014). To measure grip strength, each rat was suspended with its tail, ensuring a roughly horizontal orientation above the apparatus grid. The animals were hung by their forelimbs. The time it took for the animal to fall from the wire was recorded using a stopwatch.

Wire hang test

Rats were suspended from a metal wire secured above a cage lined with bedding (Putten et al. 2013). They were released a few seconds after instinctively grasping the wire with their forelimbs. Depending on the rat's hanging ability, all limbs and tails were used in a five-minute hanging session. The prolonged hanging time was used for the analysis.

Beam walk

A beam walking test was performed on a wood strip of 20 mm width (Gonzalez-Usano et al. 2014). Concisely, the rats had to cross a 1 m long wooden stick located approximately 1m above the surface of the ground, and observers counted the number of slips committed by rats, the total distance of the journey, and the number of turns in the animal. The number of foot errors, or slips, was assessed to measure incoordination.

Biochemical (Oxidative stress parameters)

Sciatic nerves were isolated and homogenized using a probe homogenizer in an ice-cold 0.1 M phosphate buffer with a pH of 7.4. The homogenate was then divided into various aliquots to separate the post-nuclear and post-mitochondrial fractions (Addepalli and Suryavanshi 2018). The level of lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels (Ohkawa et al. 1979). Additionally, the reduction in glutathione (GSH) levels was

evaluated to identify tissue sulfhydryl groups (Norwood et al. 2016). The activity of superoxide dismutase (SOD) was assessed (Paoletti et al. 1986), and catalase (CAT) activity was determined by measuring the decomposition rate of hydrogen peroxide per minute (Suryavanshi et al. 2022).

Histopathology

The sciatic nerves were carefully isolated and immersed in a 10% neutral formalin buffer for fixation. Following fixation, the nerves were embedded in paraffin wax in preparation for sectioning. Fine 5µm sections were obtained using a microtome. These sections were then stained using Masson's trichrome and Kulchitsky-Pal methods. Photographs of the stained sections were taken under a microscope for detailed examination. A pathologist, who was blinded to the study groupings, conducted a histopathological assessment of the slides to identify any pathological changes.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA). Following the ANOVA, post hoc comparisons were conducted using Dunnett's multiple comparison tests. A probability value (p-value) of 0.05 was set as the threshold for statistical significance. Graphs were prepared using Graph Pad Prism software, version 9, and the data were presented as mean ± standard error of the mean (SEM).

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Table S1 Method parameters of the HPLC system.

Column	**4.6 × 250 MM Agilent C-18, 5 µm particle size**
Flow rate	0.7 mL/min
Mobile phase	Acetonitrile: 0.1% Orthophosphoric acid (90: 10)
Run time	8 minutes
Wavelength	220 nm
Temperature of column	Ambient
Injection volume	20 µl
Sample solvent	Acetonitrile:0.1% Orthophosphoric acid (90: 10)
HPLC: Reverse phase high-performance liquid chromatography	

Table S2 Linearity range of stigmasterol

CONC	**AREA**
10	3801.614
20	5060.931
30	6275.39
40	7623.19
50	9054.5

Table S3 Repeatability and intermediate precision

	Inter-day		Intraday 1day	2day	3 days
Sr No	**Area**	**RT**	**Area**		
1	5060.93	3.5	5083.56	5053.51	5023.12
2	5085.21	3.5	5106.27	5107.23	5116.95
3	5103.93	3.5	5112.34	5122.32	5122.12
4	5094.29	3.5	5078.38	5098.48	5068.38
5	5069.38	3.6	5094.59	5084.34	5094.12
6	5073.16	3.6	5073.12	5043.23	5043.12
Avg	5081.15		5091.377	5084.852	5077.968
%RSD	**0.319682**		**0.281779**	**0.556554**	**0.721081**

Table S4 Ruggedness result

	Area by analyst 1	**Area by analyst 2**
	5060.9311	5013.32
	5085.211	5116.78
	5103.93	5102.67
	5094.29	5088.12
	5069.38	5034.43
	5073.16	5048.54
Avg	5081.15035	5067.31

SD	16.24350108	37.5921
%RSD	0.319681567	0.741855

Table S5 Robustness

Condition	**Modification**	**Area**	**Mean**	**SD**	**%RSD**	**Rt**	**SD**
Mobile Phase flow rate	6	5216.23	5260.667	52.67299	1.001261	3.81	0.077675
ml/min	6	5318.85				3.96	
	6	5246.92				3.92	
	7	5060.93	5074.953	16.16929	0.31861	3.51	0.05
	7	5071.29				3.56	
	7	5092.64				3.61	
	8	4822.16	4893.71	70.08881	1.432222	3.17	0.017321
	8	4896.73				3.2	
	8	4962.24				3.2	
Wavelength	219	5016.73	5019.493	12.93825	0.25776	3.56	0.020817
nm	219	5008.16				3.59	
	219	5033.59				3.6	
	220	5060.93	5074.907	16.05984	0.316456	3.65	0.081445
	220	5071.34				3.52	
	220	5092.45				3.5	
	221	5087.93	5062.66	26.73398	0.528062	3.6	0
	221	5034.67				3.6	
	221	5065.38				3.6	

Table S6 System suitability test for stigmasterol

drug	area	Theoretical plate	Tailing factor	Rt
stigamasterol	5060.93SD	8123	0.99	3.6

Table S7 Effect of EEAC treatment on Beam Walking Test (Distance traveled, number of foot slips, and number of turns).

		Parameters (Beam walk)		
Sr no	**Groups**	**Distance traveled (cm)**	**Number of slips**	**Number of turns**
1.	Control	110±10.2	4.50±0.563	4.83±0.792
2.	CDDP	42.0±4.81$^{\#\#}$	1.17±0.477$^{\#\#}$	1.16±0.477$^{\#}$
3.	CDDP+EEAC 250 mg/kg	59.8±3.90**	3.83±0.601*	3.83±0.654*
4.	CDDP+EEAC 500 mg/kg	77.0±3.31**	4.17±0.477*	4.16±0.477**

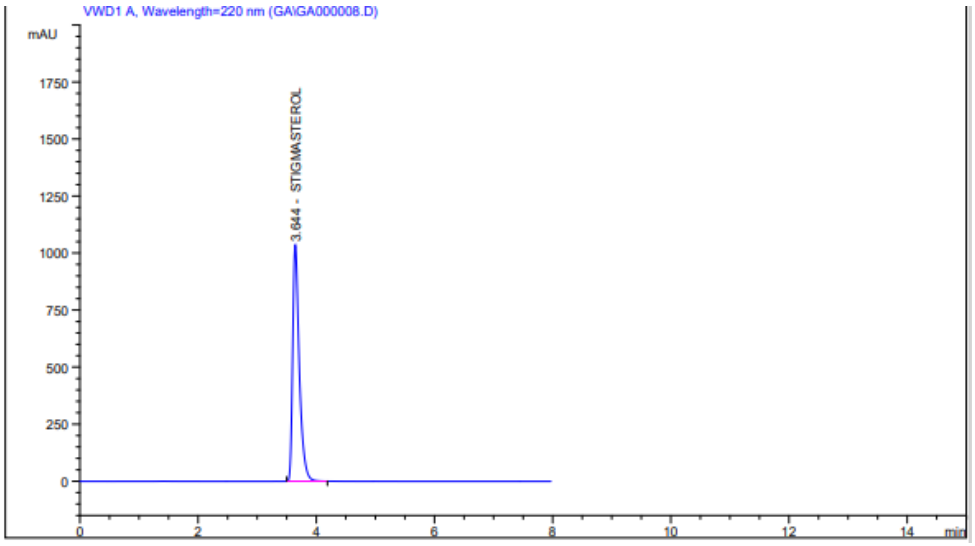


Figure S1 HPLC chromatogram of Stigmasterol

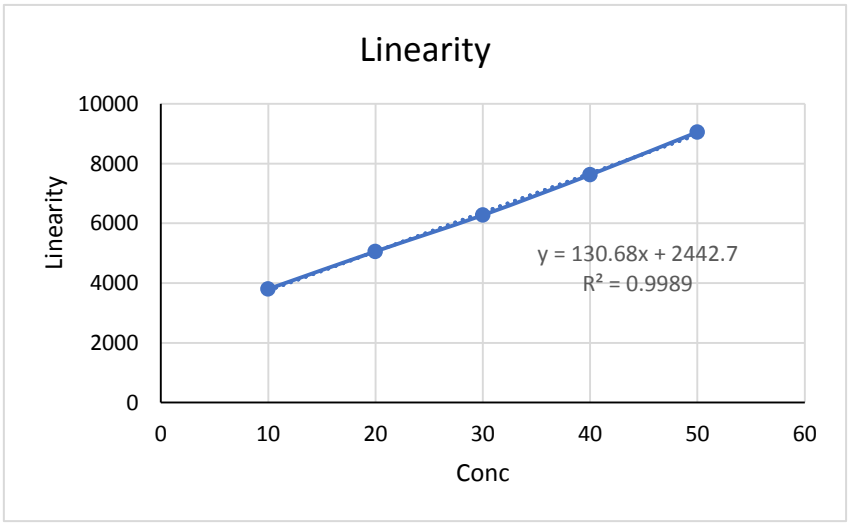


Figure S2 linearity of stigmasterol

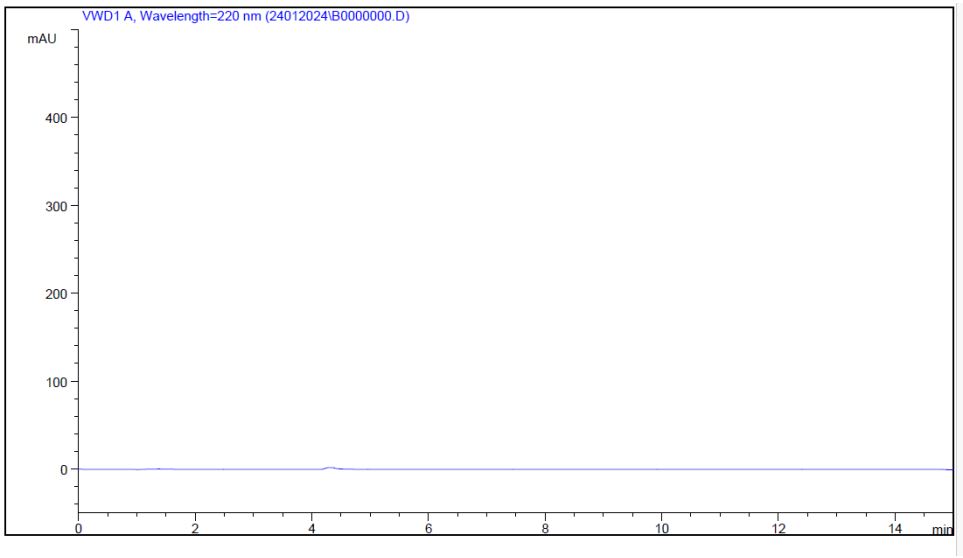


Figure S3 HPLC peak of stigmasterol

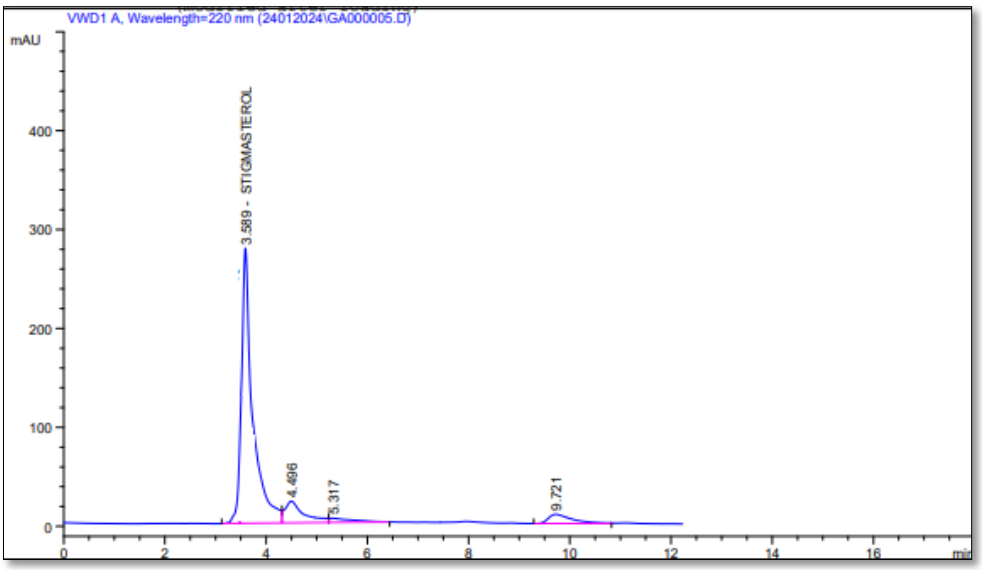


Figure S4 Chromatogram of stigmasterol in *Alpinia calcarata* extract

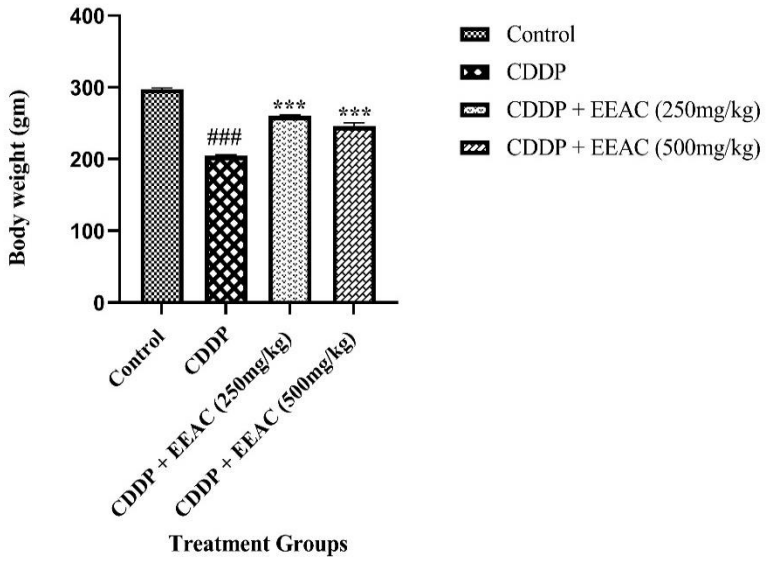


Figure S5 Effect of EEAC and CDDP on Body weight in rats. Mean ± SEM, n=6 rats per group. $^{###}P<0.001$ vs. control; $^{***}P<0.001$ vs. CDDP group.

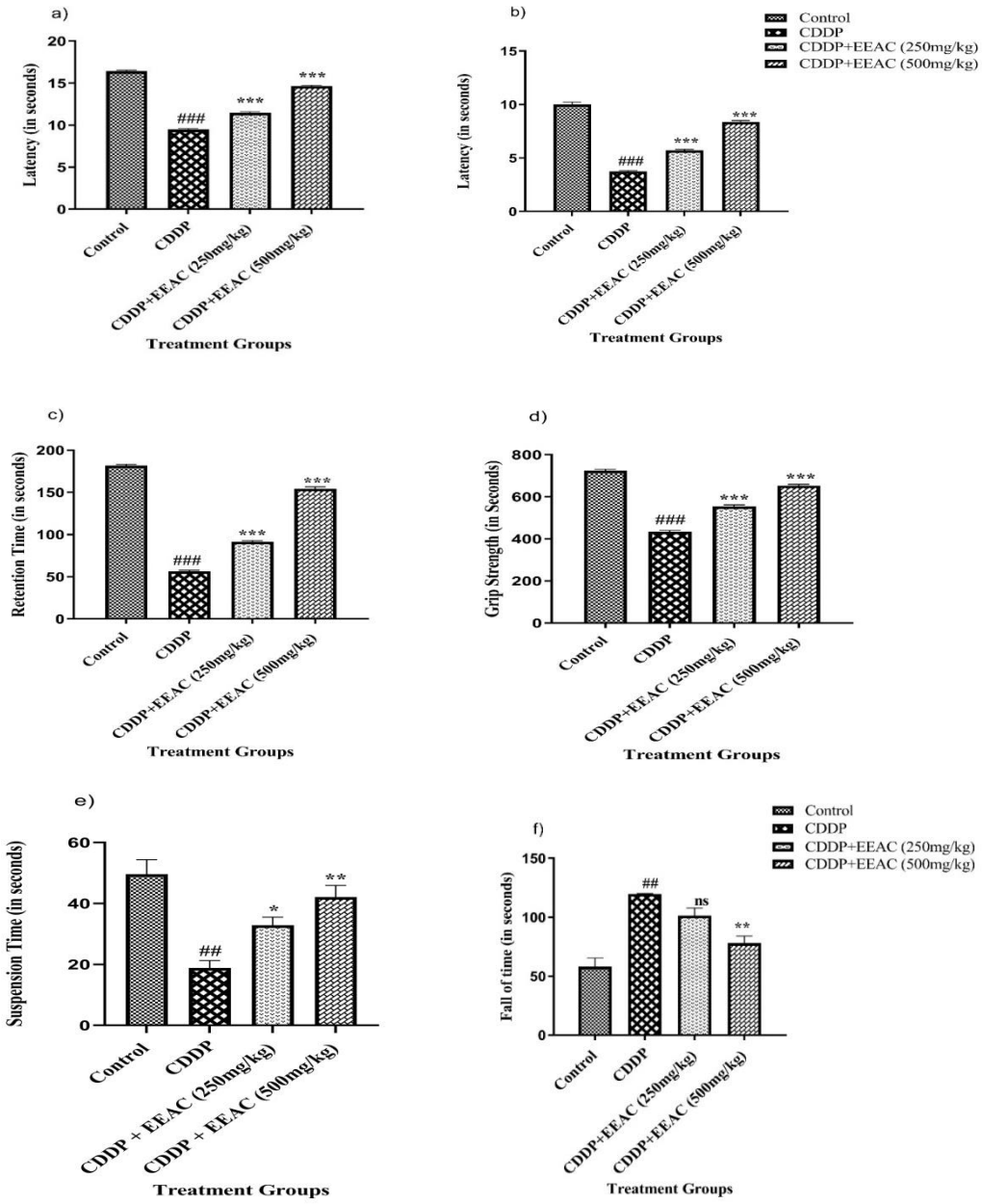


Figure S6 Effect of EEAC treatment on CDDP-induced in rats: **a)** Cold water, **b)** Hot water, **c)** Motor coordination, **d)** Grip strength, **e)** Wire hang, **f)** Beam walk. Mean ± standard error of means (SEM), n=6 rats per group. $^{###}P<0.001$, $^{##}P<0.01$ vs. control; $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ and ns $P>0.05$ vs. CDDP group.

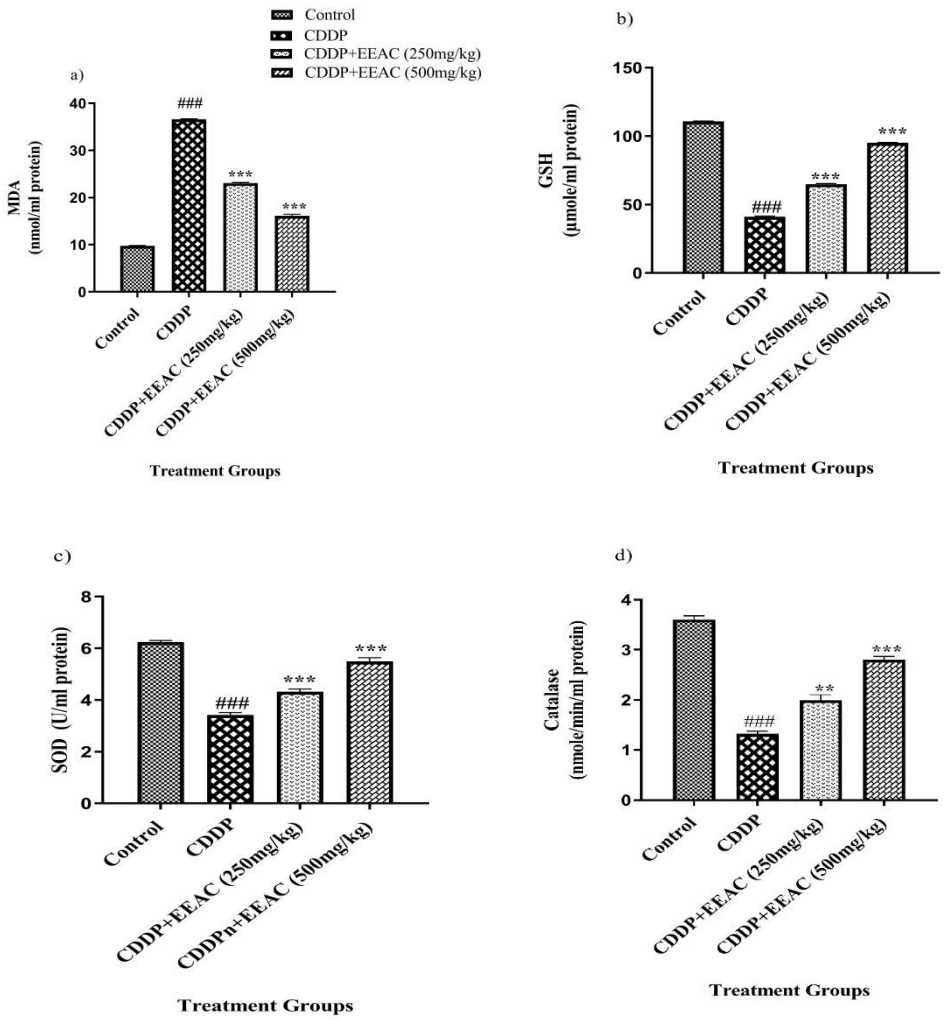


Figure S7 Effect of EEAC treatment on CDDP-induced in rats: **a)** MDA, **b)** GSH, **c)** SOD, **d)** CAT. Mean ± standard error of means (SEM), n=6 rats per group. $^{###}$P<0.001 vs. control; ***P<0.001, **P<0.01 vs. CDDP group.

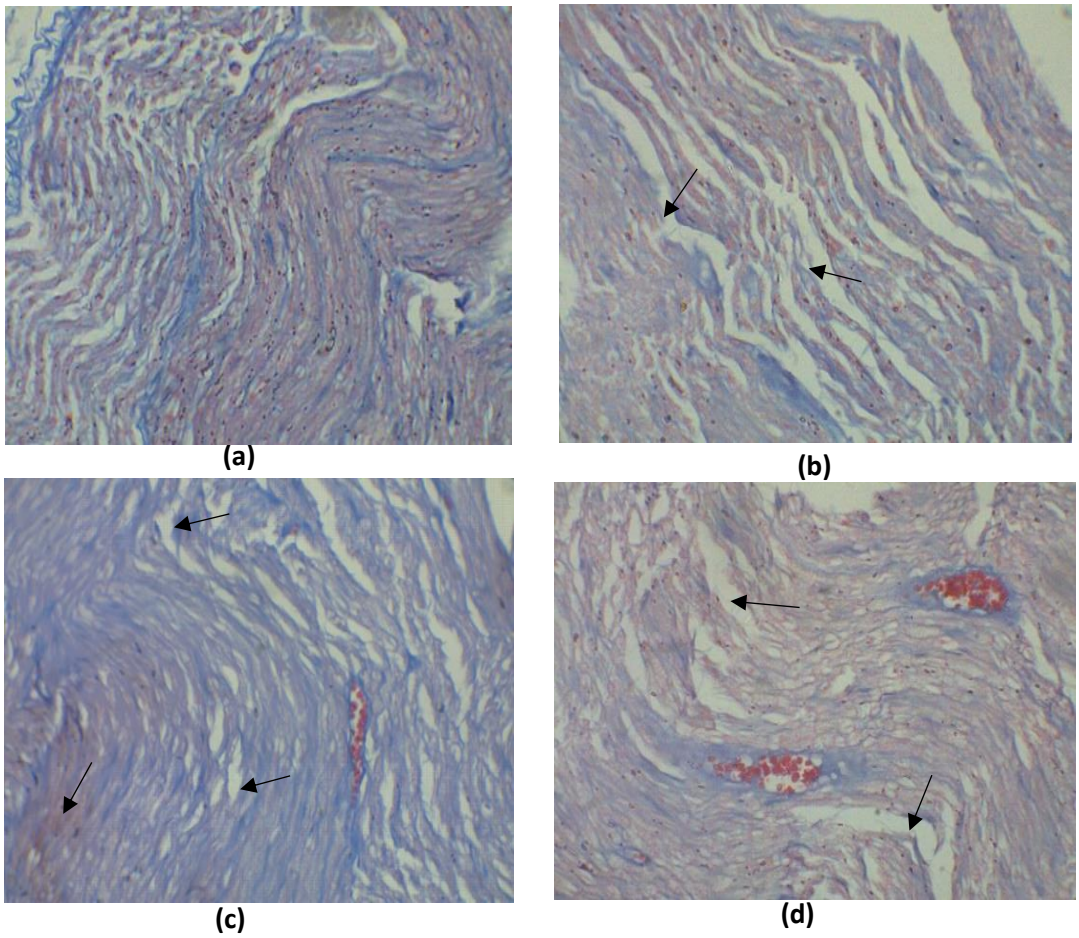


Figure S8 Effect of EEAC treatment on the sciatic nerve- Masson Trichome staining (40×): **a)** Control group **(b)** CDDP group, **(c)** & **(d)** EEAC (250 mg/kg and 500 mg/kg).

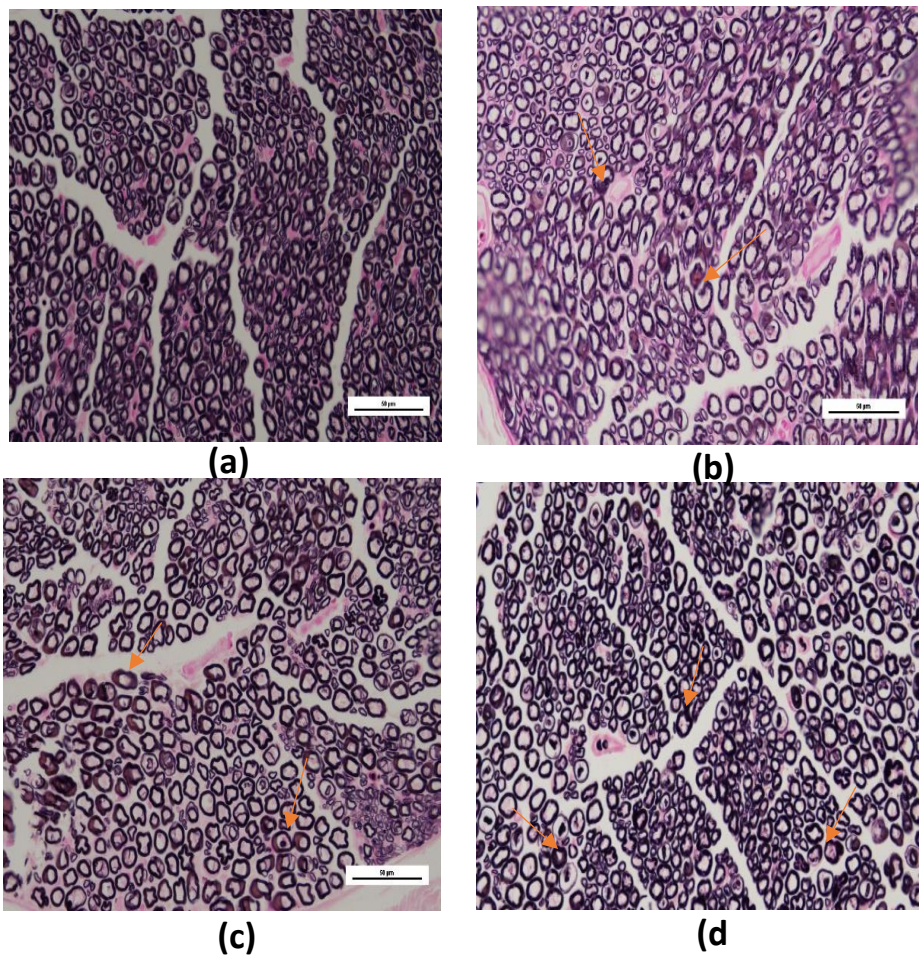


Figure S9 Effect of EEAC treatment on the sciatic nerve- Kulchitsky-Pal (Kpal) staining (40×): **a)** Control group **(b)** CDDP group, **(c)** & **(d)** EEAC (250 mg/kg and 500 mg/kg).

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Exploring neuroprotective activity of ethanolic rhizome extract of *Alpinia calcarata* in cisplatin-induced peripheral neuropathy in rats

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Alpinia calcarata, cisplatin, peripheral neuropathy, cis-diamminedichloroplatinum II, neuroprotection.

ABSTRACT

This study aims to determine the neuroprotective activity of *Alpinia calcarata* (Haw.) Roscoe rhizome ethanolic extract (ACREE) using cis-diamminedichloroplatinum II (CDDP, cisplatin)-induced peripheral neuropathy (CIPN) rat (Wistar albino) models. The rats were maintained in suitable laboratory conditions (12 hours light/dark cycle, 25ºC ± 2ºC temp) with a nutritional diet and water ad libitum, divided into four groups ($n = 6$) for the experiment. After 7 days of acclimation, groups II, III, and IV were injected with 2 mg/kg cisplatin intraperitoneal, with 2 ml saline (oral) on days 8, 15, 22, 28, and 36 along with ACREE oral doses of 250 mg/kg (to group III), and 500 mg/kg (to group IV) daily from day 8 to 42. Physical, behavioral, biochemical, and electrophysiological parameters were evaluated, and sciatic nerve histopathology was studied. Statistical analysis using one-way variance analysis (ANOVA) showed improvement in CIPN condition due to ACREE treatment in nerve conduction velocity ($p < 0.001$), grip strength ($p < 0.001$), rota rod performance ($p < 0.01$), hot and cold hyperalgesia ($p < 0.001$), inflammatory markers high sensitivity C reactive protein, tumor necrosis factor-α, and interleukin ($p < 0.001$), and oxidative stress parameters catalase, glutathione, superoxide dismutase, and malondialdehyde ($p < 0.001$). Histopathology study revealed preservation of neuro-morphology and myelin sheath and minimal inflammatory effect in treatment groups over diseased. This novel finding could be crucial in improving cancer patient survival and life quality. Further research is recommended to elucidate the precise mechanisms of ACREE's protective effects and explore its integration with existing cancer treatments to effectively neutralize the impact of cisplatin-induced peripheral neuropathy.

INTRODUCTION

Alpinia calcarata (Haw.) Roscoe, the Zingiberaceae family herb, has traditionally been used as medicine in Sri Lanka and is produced mainly in tropical regions of Asia [1]. *Alpinia calcarata* rhizome ethanolic extract *Alpinia calcarata* (Haw.) Roscoe rhizome ethanolic extract (ACREE) has been used to treat respiratory diseases such as asthma, bronchitis, and cough and as an anti-inflammatory agent [2]. ACR rhizome contains bioactive compounds such as flavonoids and terpenoids, phenolic compounds with antioxidant, anti-inflammatory, antidiabetic, antifungal, antibacterial, analgesic, and anticancer properties and was found to be an effective antinociceptive in rats [3–7]. Previous studies have also reported ACREE's hypoglycaemic and antihyperlipidemic effects [8]. ACREE was also a possible source of antitumor agents [9]. Cancer is one of the most common causes of death worldwide. Cis-diamminedichloroplatinum II, also known as cisplatin, is an antineoplastic agent effective in cancer treatment [10]. However, it forms a DNA-platinum product, leading to neural damage by axonal modification [11] and significant myelinated neural fiber injury [12]. Cisplatin can react with mitochondrial DNA, generating reactive oxygen compounds affecting axonal transmission due to the axonal localization of mitochondria, leading to chronic neural weakness [13]. Other independent

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studies reported that mitochondrial irregularities are linked to neuronal and axonal degeneration [14]. Cisplatin is a platinum anticancer drug with severe side effects, including peripheral neuropathy [15] and a molecular affinity for the peripheral nervous system not protected by a vascular barrier [16]. It causes DNA crosslinking, leading to apoptosis, causing peripheral neural damage, and sensory dysfunction [17]. They have also been reported to reduce anti-angiogenesis effects, which may cause nerve damage [18]. Other toxicities of cisplatin, including nephron and cardio, were reported [19], while peripheral neurotoxicity is a disabling side effect affecting the living quality of the patient. The therapeutic findings of ACREE and the worldwide spread of cisplatin-induced peripheral neuropathy (CIPN) have garnered interest in exploring ACREE's potential in treating CIPN, which has not been done so far. Plant-derived medicines represent a crucial aspect of proactive medicine, which focuses on preventing diseases and enhancing overall well-being rather than merely treating symptoms. If ACREE is proven to be an effective remedy for CIPN, it is likely to be seen as a gentler alternative to synthetic drugs and produce fewer side effects. Herbal medicines have gained popularity among patients seeking to improve their quality of life. This interest has also sparked significant attention from the scientific community, which is increasingly exploring the medicinal properties of plants to validate their effectiveness and discover new therapeutic compounds. This study evaluates physical, behavioral, and biochemical parameters, the direct measurement of nerve conduction velocity (NCV), and the histopathology study of sciatic nerves to explore the neuroprotective activity of ACREE in CIPN.

MATERIALS AND METHODS

Materials

Alpinia calcarata rhizomes were freshly collected in January 2021 from the Indian Institute of Horticultural Research, Bengaluru (Karnataka state, India), authenticated by Dr. P.E. Rajasekharan, Nodal Officer and Principal Scientist, GAC Division, Department of Plant Genetic Resources, Bengaluru (Karnataka state, India). Verified the plant name *Alpinia calcarata* with http://www.theplantlist.org/, accessed on Sep 20, 2023.

Cisplatin was procured from Hetero Healthcare Ltd. Diagnostic for the high sensitivity C reactive protein (hs-CRP) ELISA (enzyme-linked immunosorbent assay) kit and biochemical evaluations from Krishgen Biosystem, India. Cytokines assay Tumor necrosis factor-α (TNF-α) and IL-6 assessment commercially available ELISA kits, following manufacturer instructions from Pierce Endogen (Rockford, IL, USA). All the chemicals used were of analytical grade. **Ethanolic extraction of *A. calcarata* rhizome**The freshly collected *Alpinia calcarata* (Haw.) Roscoe (ACR) rhizomes were fragmented and air-dried under shade for 2 weeks. Five hundred grams of dried powder was extracted with 1.5 l of ethanol using the Soxhlet process for 4 hours. The extraction then was filtered, followed by concentration under diminished pressure at 55°C temp. The percentage yield was 18.5 w/w (dry weight). The extract was preserved at 4°C for further use. Then, a co-precipitate with Polyvinylpyrrolidone (PVP, 44,000 MW) in a 1:1 (w/w) ratio was used for the administration [3].

CIPN in rats

Wistar albino rats of either sex, weighing 180–200 g, were kept in clean polypropylene cages under suitable laboratory conditions (12 hours light/dark cycle, 25ºC ± 2ºC temp) provided with a nutritional diet and water ad libitum. Before commencing the experiment, an animal ethical clearance and approval were availed from KLE's College of Pharmacy Bengaluru, Institutional Animal Ethics Committee (IAEC no. 03/HP/2021). Cisplatin [(2 mg/kg), intraperitoneal (i.p.)] was administered (followed by 2 ml of normal saline water as nephrotoxicity prevention) to form CIPN rat models [20,21]. The cisplatin dosage routine is mentioned in Table 1.

Experimental design

After a week's acclimatation, a 5-week experiment followed by analysis was performed on rats (4 groups with $N = 6$), as detailed in Table 1. Groups I and II were (normal and diseased groups, respectively), and III and IV were treatment groups with daily ACREE dosages of 250 mg/kg and 500 mg/kg, respectively [3,5].

Parameters evaluation

To explore the neuroprotective activity of ACREE, physical (body weight), food and water intake, behavioral, electrophysiological, and biochemical parameters were analyzed using a one-way ANOVA technique and a histopathological study haematoxylin-eosin (H & E) was performed.

Physical

Changes in body weight and food and water intake profiles were recorded across animals used in the experiment.

Hot and cold hyperalgesia

To test hot and cold hyperalgesia, the tail was immersed in hot (46ºC, cut-off time 12 seconds) and cold (4ºC, cut-off time 10 seconds) water. The reaction time required to remove the tail was recorded as discomfort or reflexive action [22]. Tails were thoroughly dried in each rat sample post experiment [23].

Rota-rod performance (motor coordination)

The ability of the rat to maintain its position on a rotating rod (75 mm diameter) was measured using a rota-rod setup calibrated to a speed at which normal rats (Group I) could withstand >5 min on it. All the rats had three trial runs with a minimum 3-hour gap. The time spent by the rats on rotating rod at 15 and 25 rpm was recorded for each rat [20].

Grip strength

The rats were familiarised with the grip strength meter for a designated period. Each rat was suspended using its tail to measure grip strength, ensuring a roughly horizontal orientation above the apparatus grid. Then, an external force was applied to

Table 1. Experimental design to explore peripheral neuroprotection potential of ACREE.

		Laboratory condition throughout the experiment: 12 hours light/dark cycle, 25°C ± 2°C temp, nutritional diet, and water ad libitum			
	Day	**I (Normal control)**	**II (Cisplatin control)**	**III (Cisplatin + ACREE 250 mg/kg)**	**IV (Cisplatin + ACREE 500 mg/kg)**
Week 1	1–7	Acclimation to laboratory condition			
Week 2	8	-	2 ml Saline oral, 2 mg/kg cisplatin i.p. injection	2 ml Saline oral, 2 mg/kg cisplatin i.p. injection; 250 mg/kg ACREE oral	2 ml Saline oral, 2 mg/kg cisplatin i.p. injection; 500 mg/kg ACREE oral
	9–14	-	-	250 mg/kg ACREE oral	500 mg/kg ACREE oral
Week 3	15	Same as day 8			
	16–21	Same as days 9–14			
Week 4	22	Same as day 8			
	23–27	Same as days 9–14			
Week 5	28	Same as day 8			
	29–35	Same as days 9–14			
Week 6	36	Same as day 8			
	37–42	Same as days 9–14			
	After 43	Parameters evaluation			

the tail base in alignment with the sensor axis, and the response force by the rat was recorded before leaving its grip [24].

Nerve conduction study

Non-invasively NCV was measured using a Power Lab data acquisition system. An anesthetic combination of Xylazine (5–10 mg/kg, i.p.) and Ketamine (80–100 mg/kg, i.p.) was administered during the measurement. The sciatic nerve was stimulated using a bipolar electrode with an 8V, 20Hz signal, and calculated the NCV by dividing the spacing between the stimulating and recording electrodes by proximal and distal latencies difference [25,26]. The stimulating electrode probed the sciatic notch and the recording to the second interosseous muscle of the hindfoot and the ground electrode to the lateral part of the hindfoot. Stimulus latency was measured from the stimulus artefact to the M wave peak. Then, the recording electrode was moved to the tendon, and the procedure was repeated to find the recording latency.

Biochemical (Inflammatory markers and oxidative stress parameters)

hs-CRP level in blood serum was done using hs-CRP ELISA kit [27], procured from Krishgen Biosystems Ltd. Cytokines assay IL-6 and TNF-α assessment was done using commercially available ELISA kits (Spectramax i3x, Molecular devices), following manufacturer instructions from Pierce Endogen (Rockford, IL, USA) and the method used by Whiteside [28]. To measure oxidative stress markers [Superoxide dismutase (SOD), Catalase (CAT), Malondialdehyde (MDA) and Glutathione (GSH)], the sciatic nerve was segregated, followed by homogenization in phosphate-buffered saline (pH 7.4, 0.1M) at ice-cold temp (IKA 3720000 T 18 Digital Ultra Turrax), further segregated into aliquots, getting post-nuclear and mitochondrial fractions [29]. The method elaborated by Paoletti *et al.* [30] was used for SOD assessment. CAT was assessed by measuring the hydrogen peroxide decomposition rate (mole/minute) [31]. Lipid peroxidation was determined by MDA assessment [32]. The tissue sulfhydryl group was assessed by GSH levels [33].

Histopathology

The separated sciatic nerve was immersed in pH-balanced formalin buffer (10%) and planted on paraffin wax for sectioning. Ultra-fine 5-µm transverse segments were taken with a microtome for staining with haematoxylin-eosin (H & E) for general morphology [34,35].

Statistical analysis

The comparison across groups (normal control, disease control, and treatments) was done using ANOVA. Post hoc Dunnett's multiple comparison was done using Graph pad Prism software (v9), and data were represented in mean ± standard mean error (SEM).

RESULTS AND DISCUSSION

Cisplatin or ACREE doses during the study did not cause any mortality. The exploration of ACREE as a neuroprotectant has not been done so far, but multiple other allied therapeutic potentials, like anti-inflammatory, anti-oxidant, and antinociceptive of *A. calcarata* have been shown by studies [3,4,5]. The physical, behavioral, and biochemical parameters, sciatic NCV measurement results, and histopathology analysis are detailed in the following subsections.

Measurement of body weight, food intake, and water intake

Reduced food consumption and increased water intake in group II indicate loss of appetite (diseased condition) compared to group I. As a result, weight loss was observed in

group II ($p < 0.001$), which was improved in ACREE treatment groups (III: $p < 0.001$ and IV: $p < 0.05$) (Fig. 1). Also, as mentioned in Table 2, the treatment groups showed balanced food and water intake with maintained body weight.

Motor coordination and grip strength

Rota rod time reduction by 25% and grip strength by 40% confirmed peripheral neuropathy in group II, which was significantly improved in treatment groups (rota-rod performance, Fig. 2c, $p < 0.01$ and grip strength, Fig. 2d, $p < 0.05$ and $p < 0.001$ for III and IV resp.) compared to group II. The grip strength reduction aligns with another study about peripheral neuropathy [36].

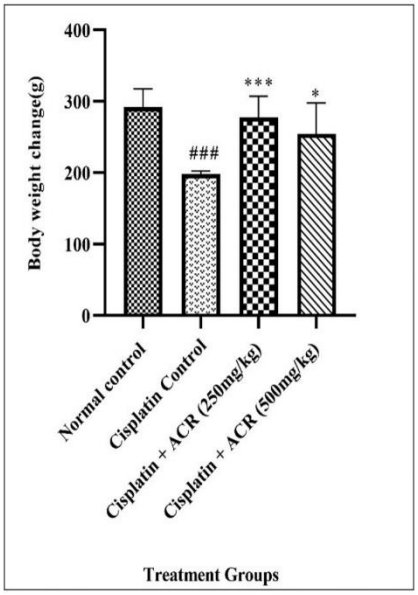


Figure 1. Body weight across test groups. Values are in Mean ± SEM, $n = 6$. ###: $p < 0.001$ with respect to normal group, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$ with respect to cisplatin group.

Hot and cold hyperalgesia

As listed in Table 2, reduced tail immersion time in hot (by 56%, $p < 0.001$) and cold (by 42%, $p < 0.001$) water in the cisplatin control group indicates an increased sensitivity to pain (Hyperalgesia), a known symptom of peripheral neuropathy [37]; improved with treatment ($p < 0.001$) in both the dosage groups III and IV (Fig. 2a and b). Hyperalgesia is one of the critical symptoms of neuropathic pain in patients, seen in 15%–50% of individuals with peripheral or central neuropathy [38]. According to the underlying mechanism, tissues get invaded, leading to signaling and expression changes of non-specific ionic and specific potassium and sodium channels [39–42].

Nerve conduction study

Severe reduction ($p < 0.001$) in sciatic NCV was found in the cisplatin control group over the normal group. The ACREE treatment, both 250 mg/kg and 500 mg/kg doses, showed improved NCV ($p < 0.001$) (Fig. 3) over cisplatin control, indicating nerve functionality reversal against cisplatin-induced deficits. NCV reduction could be due to myelin thickness reduction caused by cisplatin toxicity [43–45], and the myelin abnormality could be due to Schwann cells apoptosis by cisplatin [46]. The demyelination observed in these studies also explains the NCV reduction in the cisplatin group and the possibility of myelin regeneration with ACREE treatment showing increased NCV. Though the mechanisms underlying CIPN and NCV reduction are not fully revealed, the available evidence suggests oxidative stress was one of the major culprits. The reactive oxygen species (ROS) formation was elevated in cisplatin-exposed neurons [47,48]. Cisplatin also causes mitochondrial injuries in DRG neurons [49]. Higher ROS levels, along with mitochondrial dysfunction, cause oxidative stress. Therefore, it is also possible that the favorable effects of ACREE on these impairments in CIPN models are due to oxidative stress reduction. To support this, the oxidative stress parameters (SOD, CAT, MDA, and GSH) were evaluated in the current study.

Inflammatory markers and oxidative stress parameters

hs-CRP (Fig. 4), TNF-α, and Interleukin (IL6) (Fig. 5a and b) data showed severe inflammation with cisplatin-

Table 2. Effect of ACREE on food and water consumption, body weight, and behavioral parameters.

Evaluation parameter	I (Normal control)	II (Cisplatin control)	III (Cisplatin + ACREE 250 mg/kg)	IV (Cisplatin + ACREE 500 mg/kg)
Food consumption (gm/day/animal)	27.7 ± 0.570	21.0 ± 0.370###	25.4 ± 0.557***	23.3 ± 0.640ns
Water Intake (ml/day/animal)	28.0 ± 0.742	34.5 ± 1.14###	29.7 ± 0.595**	32.2 ± 0.926ns
Weight (gm)	292 ± 10.5	198 ± 1.82###	278 ± 11.9***	254 ± 17.7*
Hot Hyperalgesia (second)	8.85 ± 0.44	3.8 ± 0.1###	5.45 ± 0.191***	8.07 ± 0.09***
Cold Hyperalgesia (second)	15.1 ± 0.8	8.87 ± 0.3###	11.3 ± 0.2***	14.8 ± 0.2***
Motor coordination (second)	185 ± 18.1	39.5 ± 6.04##	92.8 ± 7.66**	191 ± 23.1**
Grip strength (newton)	702 ± 26.1	433 ± 24.4###	535 ± 18.6***	677 ± 30.2***

Values are in Mean ± SEM, $n = 6$. ##: $p < 0.001$, ##: $p < 0.01$ with respect to normal group, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ns: $p > 0.05$ with respect to cisplatin group.

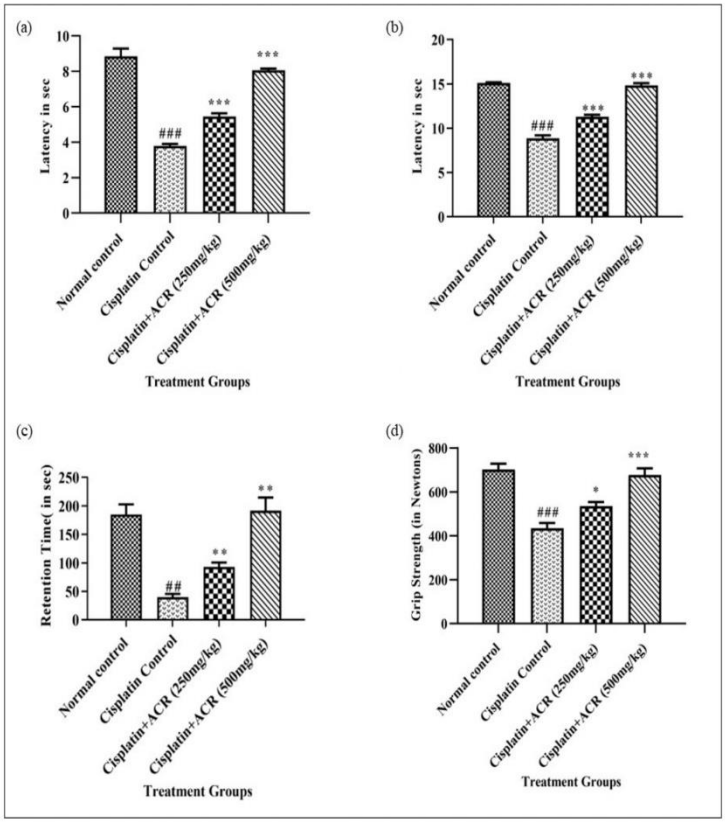


Figure 2. Behavioural evaluation of ACREE treatment: a) Hot hyperalgesia, b) cold hyperalgesia, c) rota-rod performance, and d) grip strength. Values are in Mean ± SEM, $n = 6$. ###: $p < 0.001$ with respect to normal group, ***: $p < 0.001$, **: $p < 0.01$, *:$p < 0.05$ with respect to cisplatin group.

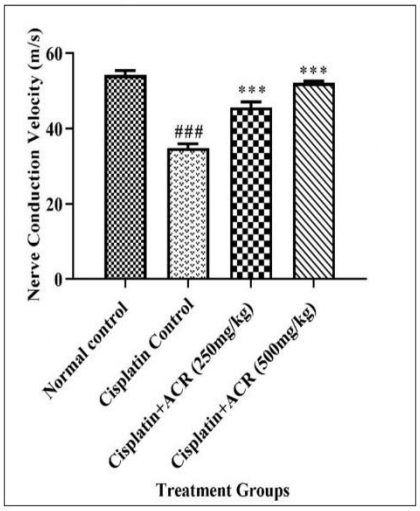


Figure 3. Effect of ACREE treatment on NCV. Values are in Mean ± SEM, $n = 6$. ###: $p < 0.001$ with respect to the normal group, ***: $p < 0.001$ with respect to the cisplatin group.

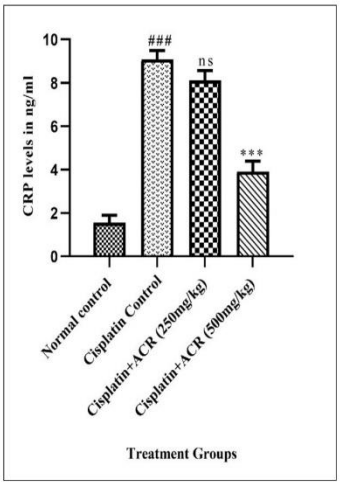


Figure 4. ACREE treatment effect on hs-CRP level. Values are in Mean ± SEM, $n = 6$. ###: $p < 0.001$ with respect to the normal group, ns: $p > 0.05$, ***: $p < 0.001$, ns $p > 0.05$ with respect to the cisplatin group.

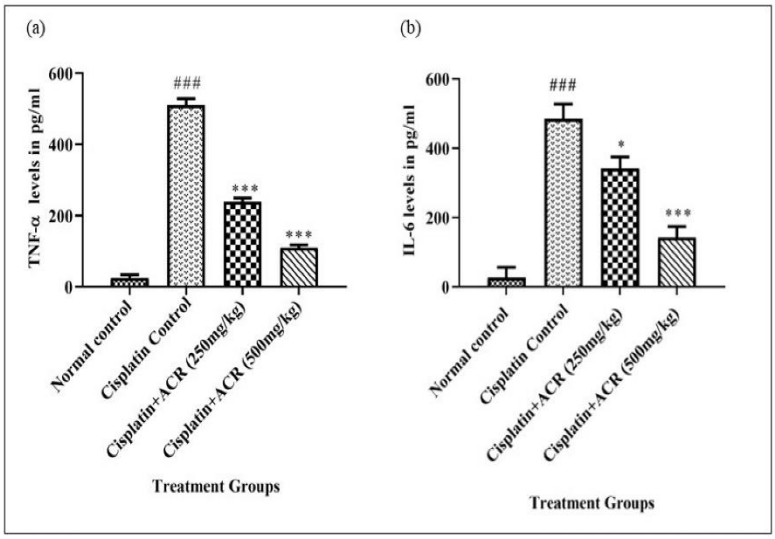


Figure 5. ACREE effect on Inflammatory markers: a) TNF-α and b) IL-6. Values in Mean ± SEM, $n = 6$. ###: $p < 0.001$ with respect to normal group, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, with respect to cisplatin group.

Table 3. Effect of ACREE treatment on inflammatory markers and oxidative stress parameters.

	Evaluation parameter	I (Normal control)	II (Cisplatin control)	III (Cisplatin + ACREE 250 mg/kg)	IV (Cisplatin + ACREE 500 mg/kg)
Inflammatory markers	hs-CRP	1.55 ± 0.34	9.08 ± 0.41$^{\#\#\#}$	8.1 ± 0.47^{ns}	3.91 ± 0.48***
	TNF-α	24.4 ± 9.52	510 ± 17.8$^{\#\#\#}$	238 ± 11**	110 ± 7.82***
	IL6	27.1 ± 29.7	485 ± 42.4$^{\#\#}$	342 ± 33.2*	142 ± 31***
Oxidative stress parameters	SOD	5.14 ± 0.17	3.74 ± 0.13###	4.65 ± 0.19**	5.02 ± 0.19***
	CAT	2.24 ± 0.27	0.91 ± 0.13##	1.7 ± 0.14*	2.14 ± 0.16***
	MDA	8.6 ± 1.05	37.4 ± 5.56###	22 ± 3.27*	14.4 ± 3.11**
	GSH	109 ± 7.11	41.5 ± 3.83###	67.3 ± 7.35*	92.2 ± 6.56***

Values are in Mean±SEM, $n = 6$. ###: $p < 0.001$, ##: $p < 0.01$ with respect to normal group, ***: $p < 0.001$, **: $p < 0.01$, *:$p < 0.05$, ns: $p > 0.05$ with respect to cisplatin group.

caused neuropathic damage indication [15]. Additionally, the NF-kß overexpression, resulting from increased protein kinase C, ROS, and advanced glycation end products caused by leukocytic infiltration, contributes to the peripheral neuron degeneration and reduction in TNF-α, IL6, neuronal growth factor, and IL1ß levels [50]. ACREE showed anti-inflammatory and analgesic properties via inhibiting NF-kß and TNF-α pathways [51]. ACREE treatment on CIPN models in this study showed inflammation reduction (hs-CRP, TNF-α, IL6) ($p < 0.001$), as detailed in Table 3.

CAT, an antioxidant enzyme, and reduced GSH, an antioxidant level, dropped to <40% from normal ($p < 0.001$); the same was confirmed by lowered oxidative stress markers SOD ($p < 0.001$) and elevated MDA ($p < 0.001$). All the markers mentioned above were improved with ACREE treatment (Table 3, Fig. 6). A study by Jiang *et al.* [47], suggested ROS generation elevation in cisplatin-exposed neurons. Another research [48,52] revealed cisplatin-induced oxidative stress due to mitochondrial dysfunction (caused by mitochondrial defects in dorsal root ganglion neurons) and increased ROS levels. Also, an inhibition of oxidative stress was associated with NCV improvement [53].

SOD, CAT, GSH, and MDA level alterations from groups I to II and then II to III and IV in this study showed cisplatin-induced oxidative stress and its reduction with ACREE treatments.

Histopathology study

Sciatic nerve histopathology (staining with H&E) was performed to confirm the effect of ACREE treatment on neuro-morphology. As shown in Figure 7A, the normal control group had typical myelinated nerve fibers with intact myelin sheath. In contrast, the cisplatin control group showed moderate nerve fiber degeneration (arrow) and demyelination (Fig. 7B). ACREE-treated groups had restored neuro-morphology with myelin sheath, appearing closer to normal, indicating

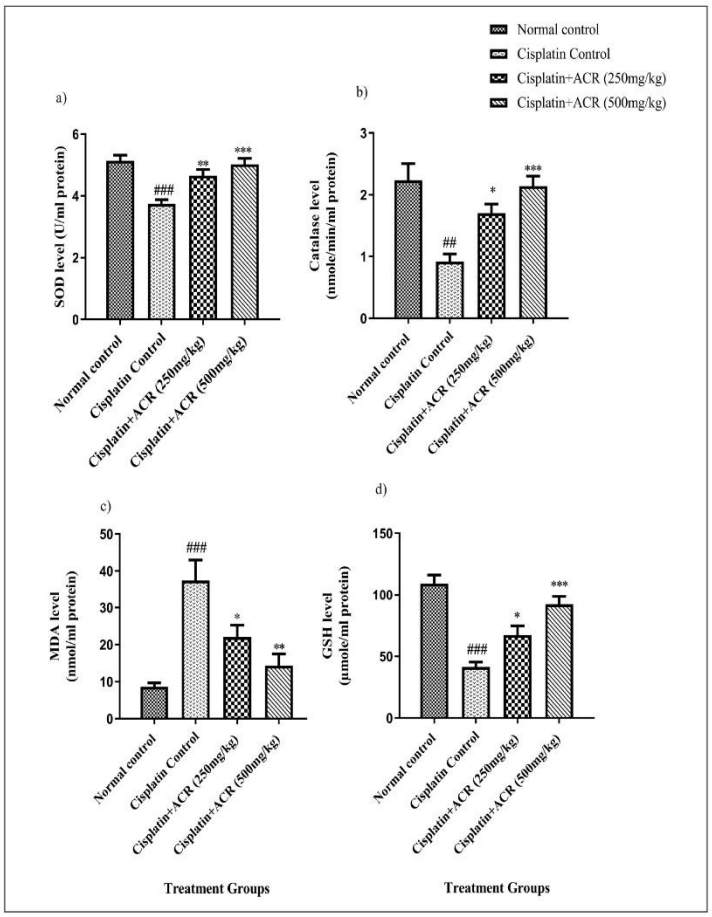


Figure 6. ACREE treatment effect: a) SOD, b) CAT, c) MDA, and d) GSH. Values in Mean ± SEM, $n = 6$. ##: $p < 0.01$, ###: $p < 0.001$ with respect to normal group, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, with respect to cisplatin group.

the neuroprotective role of ACREE (Fig. 7C and D). These morphological observations align with NCV, oxidative stress, and inflammatory parameter evaluations. Also, the cisplatin-induced neuro-morphological defects observed are in line with cisplatin neurotoxicity histopathological results in another study by [54].

Although the results in rat models are promising, extensive clinical trials are needed to transition from lab trials on rats to medicinal use in humans. Other challenges that any medicinal plant needs to address are flawless identification of ACR and type of extract, standardization, and repeatable and reliable extraction process. Without extensive study, it is challenging to say whether plant extraction method and source or origin affect drug concentration. This study gives hope for effectively managing CIPN clinically based on its effectiveness in rat models. However, extensive quantitative analysis would be needed to establish the appropriate human dosage and uncover any safety risks.

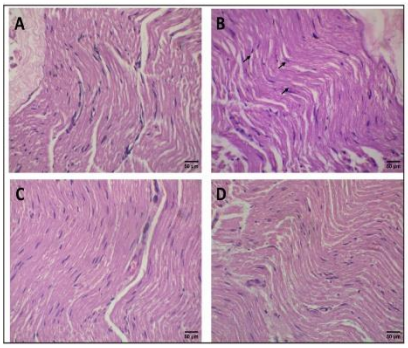


Figure 7. Representative photos of sciatic nerve sections (Haematoxylin-eosin (H & E) stain with magnification 40x): (A) Normal control group showing normal myelinated nerve fibers; (B) Cisplatin control group showing focal areas of demyelination and moderate degeneration of nerve fibers (arrows); (C and D) *A. calcarata (Haw.) Roscoe* extract treated (250 mg/kg and 500 mg/kg) groups showing normal appearance of nerve fibers with a myelin sheath.

CONCLUSION

This study demonstrates ACREE's neuroprotective effect in ameliorating CIPN's detrimental impact in rat models. Through comprehensive observations like hot and cold hyperalgesia amelioration, oxidative stress and inflammation alleviation, improved motor coordination, and promoted nerve revival by limiting and reverting functional and structural (sciatic nerve morphology) damage, ACREE shows counteraction against cisplatin-induced neurotoxicity in rat models. detrimental impact in rat models. Through comprehensive observations like hot and cold hyperalgesia amelioration, oxidative stress and inflammation alleviation, improved motor coordination, and promoted nerve revival by limiting and reverting functional and structural (sciatic nerve morphology) damage, ACREE shows counteraction against cisplatin-induced neurotoxicity in rat models. This novel finding could improve cancer patient survival and life quality. Further research is recommended to elucidate the precise mechanisms of ACREE's protective effects and explore its integration with existing cancer treatments to effectively neutralize the impact of CIPN.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

ACREE, *Alpinia calcarata* (Haw.) Roscoe rhizome ethanolic extract; ACR, *Alpinia calcarata* (Haw.) Roscoe; CAT, Catalase; CDDP, cis-diamminedichloroplatinum (II); CIPN, cisplatin-induced peripheral neuropathy; GSH, Glutathione; hs-CRP, high sensitivity C reactive protein; IL6, Interleukin 6; i.p., intraperitoneal; MDA, Malondialdehyde; NCV, nerve conduction velocity; SOD, Superoxide dismutase; TNF-α, Tumor necrosis factor-α.

AUTHOR CONTRIBUTIONS

Apurwa Shyam Dhavale designed and conducted the experimental work and contributed to the writing and revision of this manuscript. Hariprasad M.G. supervised the entire experimental work and reviewed the written manuscript. Rajendran Ramaswamy guided on drug identification and the ethanolic extract. All authors have read and approved the final manuscript for publication and agree to be accountable for all aspects of the work, ensuring integrity and accuracy.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of the KLE College of Pharmacy, Bengaluru, India with approval number 03/HP/2021.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

PUBLISHER'S NOTE

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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