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Research Article

Neuroprotection Of *Calotropis Procera* Leaf Extract In Neuropathy Induced Rat Model

Priya Patil*, Dr. Nitin mahurkar, Aatefa harmyn

MTRIPS, HKE's College of Pharmacy, Kalaburagi-585105

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ABSTRACT

Neurons are the fundamental units of the brain and nervous system, the cells responsible for receiving sensory input from external world. Neurological disabilities include a wide range of disorder, such as epilepsy learning disabilities, neuromuscular disorders, autism, attention deficit disorder, brain tumours and cerebral palsy. Neuroprotection is the ability for a therapy to prevent neuronal cell death by intervening in and inhibiting the pathogenetic cascade that results in cell dysfunction and eventual death. Numerous natural products, but primarily plant extracts, have been reported to be used in traditional medicine for neuroprotective, memory enhancing, and antiaging purposes. Calotropis Procrera known as "milkweeds "which comes under the family of Apocyanacece. Ethanolic extract of Calotrpis procera that contains active chemical constituents such as flavonoids, tannins, phenols show neuroprotective activity. In view of this, the present study was aimed to evaluate neuroprotective activity of ethanolic extract of leaves of Calotropis Procrea in neuropathy induced rat model.

INTRODUCTION

Neurological disorders are defined as disorders that affect the brain as well as the nerves found throughout the human body and the spinal cord. Structural, biochemical or electrical abnormalities in the brain, spinal cord or other nerves can result in range of symptoms include paralysis, muscle weakness, poor coordination, loss of sensation, seizures, confusion, pain and altered levels of consciousness. The specific cause of neurological problems vary, but can include genetic disorders, congenital abnormalities or disorders, infections, lifestyle or environmental health problems including malnutrition, and brain injury, spinal cord injury or nerve injury. There are many recognized neurological disorders some relatively common, but many rare. Mental disorders, on the other hand, are "psychiatric illness" or diseases which appear primarily as abnormalities of thought, feeling or behaviour, producing either distress or impairment of function. Neurological disabilities include a wide range of disorders, such

***Corresponding Author:** Priya Patil
Address: *MTRIPS, HKE's College of Pharmacy, Kalaburagi-585105*
Email : piupatil2312@gmail.com
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as epilepsy, learning disabilities, neuromuscular disorders, autism, Attention Deficit Disorder, brain tumors, and cerebral palsy. Some neurological conditions are congenital, emerging before birth. Other conditions may be caused by tumors, degeneration, trauma, infections or structural defects. Regardless of the cause, all neurological disabilities result from damage to the nervous system. Depending on where the damage takes place, determines to what extent communication, vision, gearing, movement and cognition are impacted.

Some of the neurological disorders are:

Neuropathic pain:

• Acute spinal cord injury.	• Head Injury.
• Alzheimer's Disease.	• Hydrocephalus
• Amyotrophic Lateral Sclerosis (ALS	• Lumbar Disk Disease (Herniated Disk)
• Ataxia	• Parkinson's disease.
• Bell's Palsy	• Stroke (Brain Attack).
• Cerebral Aneurysm.	• Migraine Headaches
• Epilepsy and Seizures.	• Encephalitis

Neuropathic pain is caused by a lesion or disease of the somatosensory system, including peripheral fibres (Aβ, Aδ and C fibers) and central neurons, and affects 7–10% of the general population. Multiple causes of neuropathic pain have been described and its incidence is likely to increase owing to the ageing global population, increased incidence of diabetes mellitus and improved survival from cancer after chemotherapy. Indeed, imbalances between excitatory and inhibitory somatosensory signalling, alterations in ion channels and variability in the way that pain messages are modulated in the central nervous system all have been implicated in neuropathic pain. The burden of chronic neuropathic pain seems to be related to the complexity of neuropathic symptoms, poor outcomes and difficult treatment decisions. Despite challenges, progress in the understanding of the pathophysiology of neuropathic pain is spurring the development of new diagnostic procedures and personalized interventions, which emphasize the need for a multidisciplinary approach to the management of neuropathic pain.

Although distinct definitions of neuropathic pain have been used over the years, its most recent (2011) and widely accepted definition is pain caused by a lesion or disease of the somatosensory system. The somatosensory system allows for the perception of touch, pressure, pain, temperature, position, movement and vibration. The somatosensory nerves arise in the skin, muscles, joints and fascia and include thermo receptors, mechanoreceptors, chemoreceptor, pruriceptors and nociceptors that send signals to the spinal cord and eventually to the brain for further processing; most sensory processes involve a thalamic nucleus receiving a sensory signal that is then directed to the cerebral cortex. Lesions or diseases of the somatosensory nervous system can lead to altered and disordered transmission of sensory signals into the spinal cord and the brain; common conditions associated with neuropathic pain include postherpetic neuralgia, trigeminal neuralgia, painful radiculopathy, diabetic neuropathy, HIV infection, leprosy, amputation, peripheral nerve injury pain and stroke (in the form of central post-stroke pain). Not all patients with peripheral neuropathy or central nervous injury develop neuropathic pain; for example, a large cohort study of patients with diabetes mellitus indicated that the overall prevalence of neuropathic pain symptoms was 21% in patients with clinical neuropathy. However, the prevalence of neuropathic pain increased to 60% in those with severe clinical neuropathy[1]. Importantly, neuropathic pain is

mechanistically dissimilar to other chronic pain conditions such as inflammatory pain that occurs, for example, in rheumatoid arthritis, in which the primary cause is inflammation with altered chemical events at the site of inflammation; such pain is diagnosed and treated differently[2].

Common mechanisms include increased levels in oxidative stress, mitochondrial dysfunction, excitotoxicity, inflammatory changes, iron accumulation, and protein aggregation[3,4,5]. Of these mechanisms, neuroprotective treatments often target oxidative stress and excitotoxicity—both of which are highly associated with CNS disorders. Not only can oxidative stress and excitotoxicity trigger neuron cell death but when combined they have synergistic effects that cause even more degradation than on their own[6]. Thus, limiting excitotoxicity and oxidative stress is a very important aspect of neuroprotection. Common neuroprotective treatments are glutamate antagonists and antioxidants, which aim to limit excitotoxicity and oxidative stress respectively.

Neuroprotective herbs:

Identification and characterization of new medicinal plants to cure neurodegenerative diseases and brain injuries resulting from stroke is the major and increasing scientific interest in recent years. There are more than 120 traditional medicines that are being used for the therapy of central nervous system (CNS) disorders in Asian countries.

In the Indian system of medicine, the following medicinal plants have shown promising activity in neuropsychopharmacology:

Bacopa monnierae, *Centella asiatica*, *Allium sativum*, *Celastrus paniculatus*, *Unacaria tomentosa*, *Nicotiana tabaccum*, *Hypericum perforatum*, *Withania somnifera*, *Physiostigma venosum* *Ricinus communis*, *Acorus calmus* *Salvia officinalis*, *Curcuma longa* *Gingkgo biloba*, *Terminalia chebula* *Huperiza serrata*, *Crocus sativus*, *Angelica sinesis*, *Valeriana wallichii*, *Glycyrrhiza glabra, etc.*

Calotropis procera are such plants that contain active chemical groups including, cardenolides, steroids, tannins, glycosides, phenols, terpenoids, sugars, flavonoids, alkaloids and saponins constituents that have neuroprotective activity and have not been critically evaluated so far. In view of this, the present study was aimed to evaluate the potential neuroprotective activity of *Calotropis procera* leaf extract in neuropathy induced rat model.

It exerted many pharmacological effects such as antimicrobial, anthelmintic, anti-inflammatory, analgesic and antipyretic, anticancer, anti-angiogenic, immunological, antidiabetic, cardiovascular, hypolipidemic, gastroprotective, hepatic protective, renal protective, antidiarrheal, antioxidant, anticonvulsant, enhancement of wound healing, antifertility and smooth muscle relaxant effect.

MATERIALS AND METHOD

Collection of plant material:

The leaves of *Calotropis procera* were collected from the local area of kalaburagi, Karnataka, India. The plant leaves were authenticated at Botony department of Smt. Veeramma Gangasiri college for Women, Kalaburagi. The leaves were dried in shade, powdered and stored in air tight containers for the study.

Preparation of extract:

The powdered material was subjected to batch extraction in Soxhlet apparatus. The solvent used was ethanol. The powdered material of *Calotropis*

procera was evenly packed in a Soxhlet extractor for extraction for about 7 days with solvent. The temperature was maintained on an electric heating mantle with thermostat control. Appearance of colourless solvent in the siphon tube was taken to be the termination of extraction. The extracts were then concentrated by distilling the solvent. The concentrated extracts were air dried at room temperature, weighed and percentage yield was calculated. The color and consistency of the extracts were noted.

Acute toxicity studies:

The acute toxicity (LD_{50}) value of the alcoholic extract of *Calotropis procera* was determined using standard conventional procedures as described by [7]. This method has two phases which are phases 1 and 2 respectively. Phase 1 required nine animals divided into three groups of three animals per group. Each group of animals were administered different doses (10, 100 and 1000 mg/kg) of test substance. The animals were observed for 24 hours to monitor their behaviour and possible mortality. In phase 2, animals were grouped into 4 groups of one animal each and then the alcoholic extract are administered at doses to be determined after the phase I and then observed for 24 hours for behaviour as well as mortality. The LD_{50} is calculated by the formula:

$LD50 = \sqrt{D0 \times D100}$ Where:

LD_{50} = lethal dose that kills 50 % of the test population

D_0 = Highest dose that gave no mortality,

D_{100} = Lowest dose that produced mortality.

The LD_{50} was calculated to be 774mg/kg in albino rats.

Animals:

Wister albino rats 8 weeks to 1 year old (180-220g) of either sex was procured from Hyderabad. The animals were acclimatized for seven days and housed under standard well aerated condition of temperature 22^0C (+ 3^0C) and relative humidity (30%) with a 12:12 light dark cycle. The animals were fed with standard pellet and water *ad libitum*. The animals were randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to the start of dosing to allow for acclimatization to the laboratory conditions.

EXPERIMENTATION METHODS

1. Chronic sciatic nerve constriction:

Wistar albino rats were selected and divided into 4 groups each containing 6 animals.

Group 1- Control: 2% w/v gum acacia.

Group 2-Standard: Amitriptyline (10mg/kg) + Chronic Constriction Injury

Group 3- Ethanolic extract of *Calotropis procera* leaves (36.5mg/kg) + Chronic Constriction Injury

Group 4- Ethanolic extract of *Calotropis procera* leaves (77mg/kg) + Chronic Constriction Injury

Procedure[8]:

The animal was anesthetized according to the species, age or weight. Analgesia was administered subcutaneously. The operative site was shaved using electric clippers. The animal was placed on an individual sterile field for aseptic preparation. CCI was performed under anaesthesia, with the sciatic nerve on one side exposed by making a skin incision, and cutting through the connective tissue between the gluteus superficialis and biceps femoris muscles. Four chromic gut ligatures are tied loosely around the sciatic nerve at 1 mm intervals, to just occlude but not arrest epineural blood flow. The wound is closed with sutures in the muscle and staples in the skin. The animal is then allowed to recover from surgery for 24 hrs before pain hypersensitivity testing begins.

Post operative care:

Immediately following surgery, the animals were placed on an absorbent pad inside a heated, filter-topped recovery cage. Animals were regularly observed until they were ambulatory. Once they have established their righting reflex and have recovered from anaesthesia, they were moved to

the holding area for postoperative management until. In the postoperative holding area, the animals were observed daily and observations were recorded.

2.Chemotherapy induced neuropathy:

Wistar albino rats were selected and divided into 4 groups each containing 6 animals.

Group 1- Control: 2% w/v gum acacia.

Group 2-Standard: Amitriptyline (10mg/kg) + Cisplatin.

Group 3- Ethanolic extract of *Calotropis procera* leaves (36.5mg/kg) + Cisplatin.

Group 4- Ethanolic extract of *Calotropis procera* leaves (77mg/kg) + Cisplatin.

The chemotherapeutic drug cisplatin was selected to induce neuropathy.

Cisplatin was dissolved in 0.4% dimethyl sulfoxide (DMSO) in saline just before administration, to a final concentration of 0.2 mg/ml, 0.1 mg/ml or 0.05 mg/ml. The volume for intraperitoneal injection was 1 gm body weight per 0.01 ml. The control group received 0.4% DMSO in saline. Cisplatin was administered once a day for four days. To 21 days from the start of cisplatin administration, tests for mechanical and thermal allodynia and thermal hyperalgesia were performed and general behaviours were observed.

Neurobehavioral studies:

All the neurobehavioral studies were tested in each group of animals at beginning of experiment and 7, 9 11, and 21 days after the lesion.

Assessment of Behaviour Parameters:

Assessment of Hyperalgesia and Allodynia:

The hyperalgesia was assessed in both ipsilateral and contralateral hind paws by immersing paws in the water, maintained at a temperature of 52.5° ± 0.5°C. Withdrawal thresholds for allodynia were measured in both ipsilateral and contralateral hind paws by immersing in the water, maintained at a temperature of 4°± 0.5°C.

Locomotor activity:

The locomotor activity was caried out by open field in a sound attenuated room. The rat was initially placed at centre of the field and observed for 5 minutes in all parameters i.e. latency (sec), rest (sec), fall of time (sec).

Evaluation for peripheral nerve regeneration:

1. **Grasping test[9]:**

The rats were gently lifted by the tail and allowed to grasp a grid connected to an ordinary electronic balance. While grasping the animal continued to be lifted by the tail with increasing firmness until it lost its grip. At this precise moment the value shown by the balance was recorded. In the crushed nerve, recovery of function was clearly demonstrated by the grasping test. The test also indicated the exact day on which recovery began and its improvement with time. This very simple objective behavioural method provides a sensitive quantitative technique for assessing recovery.

2. **Staircase test:**

The staircase test was developed to assess the independent use of forelimbs of rats and was later adopted to assess skilled reaching in mice. The apparatus was designed to encourage the animal to gain access to food by entering a narrow space, a natural rodent behaviour. After the animal climbs on a platform, it reached to either side to retrieve food from a double set of staircases. Pellets were placed on each step on both sides. The animal could not simply scoop the pellet; it made a coordinated reach and grasp to retrieve it. Latency and the number of pellets from each side and location at increasing distances were calculated to determine impairments.

Statistical analysis:

The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett comparison test. The values are expressed as mean ± SEM and $P<0.05$ was considered significant.

RESULT

1. Chronic sciatic nerve constriction:

i. Assessment of Behaviour Parameters:

In the test groups, hyperalgesia and allodynia was enhanced compared to normal rats. Administration of leaf extracts of *Calotropis procera* for 2 weeks at 36.5mg/kg and 77mg/kg doses significantly ($p<0.01$) attenuated the mechanical hyperalgesia, thermal hyperalgesia and tactile allodynia in CSNC induced neuropathy rats as compared to control rats.

ii. Locomotor Activity: There was a decrease in the locomotor activity. The fall off time (motor coordination) was also decreased.

iii. Grasping Test:

After the administration of the drug, the animals were performed the grasping test, a significant restoration in motor functions was observed in terms of grip strength in the test groups as compared to the control. Test groups had shown a prominent difference relevant to control on day 7 post injury, particularly the 77 mg/kg group appeared to be suggestively effective ($p = 0.04$). On subsequent time points (day 9 and 11 post injury), all treatment groups showed meaningfully enhanced gripping strength differences ($p = .0001$) as compared to the control. However, the 77 mg/kg group also showed weighty differences relevant to 36.5 mg/kg group on day 11 post injury ($p = 0.02$). Data indicates the prominent improvement in pattern of walking in animals of treated groups. The 36.5 and 77 mg/kg groups appeared to be highly effective in normalizing the values on day 6 ($p = 0.001$)

day 9 ($p = 0.006$) post injury. These findings indicate the potential of *C. procera* leaves in escalating motor functional recovery.

iv. Staircase Test:

In the staircase test, the number of pellets retrieved were observed. All damaged groups were significantly impaired compared to control and standard animals at post injury (24 hr) and day 7. At day 14, animals from the test group remained impaired and animals from both treated groups improved but without a difference between them. Data are reported as means ± SEM. *P < 0.05 compared to the control and test groups.

Effect of *Calotropis procera* on grip latency and staircase latency using CCI in rats.

Grp no.	Treatment	Dose	Grip latency	Staircase latency
1	Control	-	19±1.265	29.5±1.02
2	Amitriptyline	10mg/kg	23 ±0.0844	24.03±0.09
3	C.P low dose	36.5mg/kg	13±1.005	9.85±0.004
4	C.P high dose	77mg/kg	18.16±1.007	12.67±0.007

Chemotherapy induced neuropathy:

i. Assessment of Behaviour Parameters:

No deterioration in general status was observed; no alterations in the body temperature and no abnormal clinical signs were observed. No rats in the cisplatin control group died during the course of the experiment. At the end of the 4th week, cisplatin group exhibited significant decrease in pain threshold from noxious stimuli as compared with control rats.

ii. Locomotor Activity:

There was a decrease in the locomotor activity. The fall off time (motor coordination) was also decreased.

iii. Grasping Test:

Administration of *Calotropis procera* for 2 weeks significantly improved the latency of grip strength in cisplatin induced neuropathy rats. The % of grip strength was more in highest dose group treated rats as compared to lower dose group but was less than that of the standard.

iv. Staircase Test:

Injury with the chemotherapeutic drug cisplatin resulted in sustained deficits of the affected

forepaw and retrieval of significantly fewer pellets on the staircase test. The test groups showed less activity when compared to the standard.

Effect of *Calotropis procera* on grip latency and staircase latency in chemotherapy induced neuropathy in rats.

Grp no.	Treatment	Dose	Grip latency	Staircase latency
1	Control	0.4% in saline	35.15±1.66	29.20±0.088
2	Toxicant	1g/kg	22.57±0.054	27.87±0.057
3	Standard	10mg/kg	29.80±0.86	28.70±0.065
4	Test drug	77mg/kg	27.50±0.075	26.30±0.078

CONCLUSION

The studies revealed that the higher dose of the drug i.e. 77mg/kg had shown more activity when compared to lower dose of the drug i.e. 36.5mg\kg. But both the groups had shown the activity comparing to that of the standard. Neurobehavioral studies were conducted by assessing the behaviour parameters like assessment of hyperalgesia and allodynia, assessing the locomotor activity and finally by performing the grasping test and staircase test.

In the behaviour parameter study, the hyperalgesia and allodynia were increased and there was a decrease in locomotor activity. In the grasping test and the staircase test, the % of grip strength and recovery in staircase test was calculated to be lesser than that of standard. The extracts were assessed for their neuroprotective activity.

The extracts were screened for neuroprotective activity by two methods - Chronic sciatic nerve constriction, and chemotherapy induced neuropathy in rats. In the study, the changes in behaviour patterns and their grasping strength and results of staircase test in rats were used as markers. In the grasping test and the staircase test, the % of grip strength and recovery in staircase test was calculated to be almost same than that of standard.

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