Tuijin Jishu/Journal of Propulsion Technology
ISSN:1001-4055
Vol. 44 No.3 (2023)

# Neuro Protection of Calotropis Procera Leaf Extract in Neuropathy-Induced Rat Model

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**Abstract**

Objective: To assess and validate the neuroprotective effect of the Calotropis procera leaf extract for safe use in traditional medicine. Method: Ethanolic leaf extract of Calotropis procera was tested in neuropathy-induced rat model with two different doses; high dose (77mg/kg) and low dose (36.5mg/kg). Results: The studies revealed that in group 3 and 4 Calotropis procera leaf extract treated animals have shown neuroprotective effect. Conclusion: The extract significantly mitigated the effects of neuropathy in rats and shown neuroprotective action.

**Keywords:***Neuroprotective, Chronic Constriction Injury (CCI), Spinal Contusion Injury (SCI), herbal medicine, ethanolic extract, Calotropis procera.*

## Introduction

The herbal remedies have its origin from ancient times. The natural products have been used from the beginning of human history. In recent years, Herbal medicines have gained popularity because of their less complexity and few aftereffects than synthetic drugs. With the boosting demand for medicinal plants and related compounds, the use of such therapies for the treatment of painful neuropathy has become more common globally[1]. Various herbal remedies are used in treating the nerve pain. Those compounds mainly impact the peripheral and central nervous systems[2]. The milkweed family's *Calotropis procera* (Aiton) (CP) perennial shrub is a dry, soft-wooded member of the Apocynaceae family and Asclepiadaceae subfamily. It is a xerophytic evergreen plant that often

inhabits dry and semi-dry areas[3]. CP is indigenous to the Arabian Peninsula, West Africa, North and East Africa, Madagascar, Malaysia, Macaronesia, and Indo-China[4]. In folk medicine, various portions of the plant are used to treat many kinds of ailments, and scientific research has proven the effectiveness of such cures[5]. Numerous biological properties, including proteolytic, antibacterial, larvicidal, nematocidal, anticancer, and anti-inflammatory properties, have been recorded with these[6].

**Figure 1: Calotropis procera plant**

CP is a 5.4-meter-tall, upright, massive, heavily-branched shrub with milky latex all across the plant[7]. Triterpenoids, flavonoids, cardiac glycosides, cardenolides, anthocyanins, α, β-amyrins, lupeol, β-sitosterol, flavanols, mudarine, resins, a potent bacteriolytic enzyme called calactin, a nontoxic proteolytic enzyme called calotropin, and wax were isolated from the heartwood of CP[8]. Phytochemicals of similar class have shown neuroprotective action sourced from other plants. Hence, the current study was taken up to assess the possible neuroprotective efficacy of CP leaf extract in a rat model of neuropathy.

**Materials And Methods**

**Plant material:** Fresh leaves of CP were gathered locally in Kalaburagi, Karnataka, India. A voucher specimen of the plant (19PP200) certified by botanist is deposited in the department.

**Extract preparation:** For around 7 days, hot ethanol extraction was performed on the powdered shade-dried leaves of CP. The extract thus obtained was concentrated and dried in-vacco[9].

**Chemicals**: Amitriptyline (HI Media, Mumbai, India), cisplatin (Merck KGaA, Darmstadt, Germany), gum acacia (SD Fine Chemicals, Mumbai, India), ethanol (SD Fine Chemicals, Mumbai, India), ketamine HCl (Ciron Drugs & Pharmaceuticals Pvt Ltd, Mumbai, India), Dimethyl sulphoxide (SD Fine Chemicals, Mumbai, India).

**Animals:** Albino Wistar rats of either sex (180–200 g) were purchased from Mahaveera Enterprises, Hyderabad (146/99 CPCSEA).Acclimatization was progressed in clean polypropylene cages for 1 wk under standard conditions [ambient temperature (22 ± 2 °C); natural 12-h light and dark cycles; unrestricted access to water; and regular laboratory meal]. Rats were arbitrarily chosen for different experimental groups after 1 wk of acclimatization. The Committee for Control and Supervision of Experiments on Animals (CPCSEA), which established the procedures, was strictly followed in the actual conduct of all of the experiments. Prior to the study, approval from the Institutional Animal Ethical Committee was obtained from the H.K.E.S. MTR Institute of Pharmaceutical Sciences in Kalaburagi, Karnataka (1948/PO/Re/S/17/CPCSEA 23-2-2017).

**Phytochemical analysis:**

**Quality chemical test:**

The leaf extract was suspended in water and ethanol to determine the presence of various phytochemicals.

Spectral analysis:

FT-IR spectrum of the ethanolic extract was recorded on infrared (IR)

**High performance liquid chromatographic (HPLC) studies**

The number of components present in the ethanolic extract fraction of CP was determined using analytical HPLC (SYKAM)), with a gradient mobile phase made up of A: methanol: B: 0.1% HCOOH in water using HPLC column of C18 - ODS (25cm * 4.6 mm), and the detected at 254 nm with a flow rate of 1.0 ml/min. A= 70% (0 - 5 min), A= 40% (5 - 8 min), and A= 90% (8 - 15 min).

**Experimental methods**:

**Chronic sciatic nerve constriction (CCI):** All of the chosen rats were split into four groups, each with six animals. All the rats were given oral gavage,

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

Group 2 – Standard group: Amitriptyline (10mg/kg) + CCI.

Group 3 - Ethanolic extract of CP leaves (36.5mg/kg) + CCI.

Group 4 - Ethanolic extract of CP leaves (77mg/kg) + CCI.

Under general anesthesia, CCI was performed to expose the sciatic nerve by making a blunt incision into the skin and slicing through the connective tissue between the gluteus superficialis and biceps femoris muscles (in the center of the thigh). To only occlude but not stop epineural blood flow, four loose chromic gut ligatures are positioned around the sciatic nerve at a distance of one millimeter. Staples in the skin and stitches in the muscle are used to seal the incision. The animal was then given 24 hours to recover from the procedure before being put through a pain hypersensitivity test[10].

**Contusion model (SCI):** All of the chosen rats were split into four groups, each with six animals. All the rats were given oral gavage[9].

Group 1 – Control group: Gum acacia - 2% w/v.

Group 2 – Standard group: Amitriptyline (10mg/kg) + SCI.

Group 3 – Ethanolic extract of CP leaves (36.5mg/kg) + SCI.

Group 4 – Ethanolic extract of CP leaves (77mg/kg) + SCI.

The rats were anesthetized using 90 mg/kg of Ketamine and waited until there is no toe-pinch response. With a 1.5mm impactor, 150 or 200 kdyn of force was applied with a 0's dwell period to cause a contusion of the spinal cord at the midline of T9. Post operative care was taken by placing the rats in warm environment of about 33-35$^{o}$c for 24hrs post-surgery. The examination of locomotor activity on the first, fourth, seventh, tenth, and fourteenth days after the injury, followed by weekly assessments, revealed the behavioral changes.

**Chemotherapy-induced neuropathy:** Selected Wistar albino rats were separated into 4 groups, each with 6 animals.

Group 1 – Control group: Gum acacia - 2% w/v.

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

Group 3 – Ethanolic extract of CP (36.5mg/kg) + Cisplatin.

Group 4 – Ethanolic extract of CP leaves (77mg/kg) + Cisplatin

Cisplatin was selected as chemotherapeutic drug to induce neuropathy. Just prior to injection, 0.4% solution of dimethyl sulfoxide (DMSO) in saline was produced to a final concentration of 0.2mg/ml, 0.1mg/ml, or 0.05mg/ml. A 0.01 ml per 1g body weight was given by intraperitoneal injection. The control group was administered with 0.4% DMSO in saline. Cisplatin was given once daily for the following four days. At the 21st day following the initiation of cisplatin administration, the tests for mechanical and thermal allodynia, thermal hyperalgesia were carried out, and general behaviors were observed.

**Assessment of Hyperalgesia and Allodynia:** In order to measure hyperalgesia, the epsilateral and contralateral hind paws were assessed while the water's temperature was kept at roughly 52.5 ± 0.5°C. The epsilateral and contralateral hind paws were submerged in water that was kept at a temperature of 4 ± 0.5°C to evaluate the withdrawal thresholds for allodynia.

**Locomotor activity**[4]: The locomotor activity was carried out by an open field in a sound-attenuated room. The rats were initially kept at the center of the field and noticed for 5 minutes in all parameters i.e latency (sec), rest (sec), and fall of time (sec).

**Evaluation for peripheral nerve regeneration**:

1. **Grasping test**[11]**:** Rats were raised by the tail with increasing strength and allowed to grab the grid. The grid was linked to a digital balance. The value that the balance displayed at this specific instant was noted. The grasping test showed that the crushed nerve recovered its function. The test also revealed the precise day when recovering started as well as how much better it got over time. This basic objective behavioral approach offers a precise quantitative way for evaluating recovery.
2. **Staircase test**[12]: The staircase test is commonly referred to as skillful forelimb usage, but it also depends on the rat's capacity to acquire new and complex motor patterns. The apparatus was made to encourage the animal to engage in a normal rodent habit of entering a small compartment in order to acquire food. The animal used a platform on a different side of the staircases to access for food. On both sides and on each step, pellets were placed. The animal made a coordinated reach and grab to acquire the pellet because it was unable to simply scoop it up. The number of food pellets from each side and location at increasing distances were calculated together with latency to determine the impairments.

**Statistical analysis:**

A one-way analysis of variance (ANOVA) was performed before the Dunnett comparison test to determine the statistical significance. The values are shown as mean ± SEM, and significance was set at $p<0.05$.

**Results**

**Preliminary Phytochemical tests**

Tannins, saponins, flavonoids, glycosides, and resins were found during the phytochemical evaluation of CP leaf ethanolic extract.

**FTIR analysis**

FTIR analysis of CP leaves extract indicated the presence of different functional groups which are associated with some of the phytochemical indicated by qualitative chemical tests.

The spectral plot showed a significant absorption peak at 3400 $cm^{-1}$ which indicated the existence of a phenolic-OH stretching mode, and a peak at 2994 $cm^{-1}$ showed the presence of an aromatic C-H stretching mode, both confirmed the presence of steroids in the plant extracts.

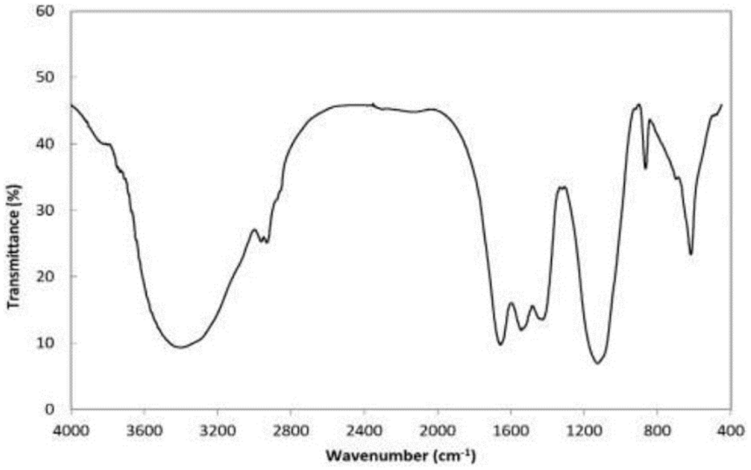


**Figure 2: FTIR spectra of the ethanolic CP leaf extract**

**HPLC analysis**

The HPLC chromatogram indicated the resolution of six different components present in the ethanolic extract at Rt 2.58, 3.10, 5.28, 6.52, 7.32 and 8.13 respectively.

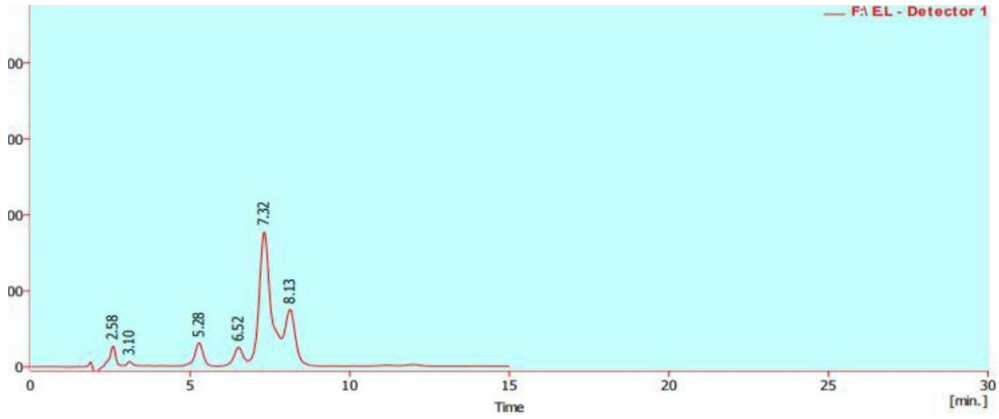


**Figure 3: Chromatogram of ethanolic extract of CP obtained by HPLC**

**Chronic sciatic nerve constriction**:

**i. Assessment of Behavior Parameters**: In the test groups, hyperalgesia and allodynia were enhanced compared to normal rats. Administration of leaf extracts of *Calotropis procera* for 2 weeks at 36.5mg/kg and 77mg/kg doses significantly attenuated the mechanical hyperalgesia, thermal hyperalgesia, and tactile allodynia in CSNC-induced neuropathy rats as compared to control rats.

**ii. Locomotor Activity**: The degree of locomotor activity dropped. Additionally, the motor coordination (fall-off period) was shortened.

**iii. Grasping Test**: After giving the drug, the grasping test was carried out and found to exhibit recovery in motor functioning where the test group's grip strength significantly improved compared to the control group. On day 7 after the injury, test groups had distinguished out significantly from the control group, with the 77 mg/kg group standing forth as potentially effective. Days 9 and 11 after the injury, all treatment groups exhibited markedly improved gripping strength differences from the control group. On day 11 following the injury, the 77mg/kg group exhibited weight differences comparable to the 36.5 mg/kg group. Data indicates that animals in treated groups walk with an improved locomotor pattern. The readings on days 6 and 9 post-injury appeared to

be significantly normalised by both 36.5 mg/kg and 77 mg/kg groups. These results show the *C. procera* leaves capacity to enhance motor functional recovery.

**iv. Staircase Test:** In this test, the number of pellets that are retrieved was observed. At post injury (24hr) on 7$^{th}$ day all test groups were significant impaired compared to control and standard animals. At day 14, animals from the test group remained impaired and animals from both treated groups improved but with no change between them. All the data reported was in terms of mean ± SEM. Statistically, *$p < 0.05$ for both the control and test groups indicated the significance level.

**Contusion Model:**

**i. Assessment of Behavior Parameters**: The behavior patterns were assessed after the rats received the contusion of 150kDyn or 200kDyn SCI. These rats exhibited mild mechanical allodynia and persistent thermal hyperalgesia. The rats treated with *Calotropis procera* showed substantial, momentary mechanical allodynia ($p<0.05$), but no thermal hyperalgesia.

**ii. Locomotor Activity**: The degree of locomotor activity dropped. Also, the motor coordination (fall-off period) was shortened ($p<0.05$).

**iii. Grasping Test**: The contusions in rats displayed chronic forelimb deficits. The test indicated that there were pronounced impairments when determined the success of reaching for food from a tray. The animals showed greater impairments when compared to the standard and control groups. In case of treatment groups, the group with higher dose of the drug was found to be more effective compared to the group administered with the lower dose ($p<0.05$).

**iv. Staircase Test**: Rats suffering from a 75-kilogram dyne contusion injury have a persistent, moderate-to-severe motor dysfunction. When compared to the control and standard, the injuries resulted in prolonged deficits of the injured forepaw and considerably fewer pellet retrievals on the staircase test. The data are presented as mean ± standard error of the mean (SEM).

**Chemotherapy-induced neuropathy:**

**i. Assessment of behavior Parameters**: No changes in body temperature or clinical changes were noticed in the test group, nor was there a change in general behavior. Death rate in the cisplatin experimental group was zero. At the end of the fourth week, the rats in the cisplatin group exhibited a significant lower pain threshold from noxious stimuli than the control group rats ($p<0.05$).

**ii. Locomotor Activity**: Locomotor activity in test group was found to be decreasing with fall-off time (motor coordination) ($p<0.05$).

**iii. Grasping Test:** The latency of grip strength was dramatically improved in the test group with cisplatin-induced neuropathy after receiving *Caltropis procera* treatment for two weeks. The percentage grip strength was more in C.P high dose group rats as compared to the C.P low dose group rats, but the percentage grip strength of both doses was comparatively lower than that of the standard ($p<0.05$).

**iv. Staircase Test**: The chemotherapeutic agent cisplatin was utilized to inflict the damage on wistar rats, which resulted in long-term impairments of the injured forepaw and significantly less food pellets retrieval on the staircase test. The test groups showed low activity when compared to the standard ($p<0.05$).

**Discussion**

*Acute toxicity study:* Even there are various drugs available, there is no standard cure/ treatment for neuropathic pain. The natural products have been used from the beginning of human history [1]. Nerve injury has been treated using a variety of natural medications. They were able to show a recovery impact on both the CNS and peripheral nerves[2].

A illness that affects peripheral nerves, or nerves outside the brain and spinal cord, is referred to as neuropathy[13]. Peripheral neuropathy is frequent, often upsetting, and occasionally crippling or even fatal. The

prevalence rate is approximately 2400 per 100,000 people (2.4%) and increases with age to 8000 per 100,000 people (8%)[9]. Some peripheral neuropathies may develop more quickly and are progressive in nature, but they often take months to years to manifest[14]. Although there are several causes of peripheral neuropathy, the nerve has only a limited number of ways to react to injury[15]. The most prevalent illness in society that causes generalized peripheral neuropathy is diabetes[16]. A lesion or illness of somatosensory system, which includes central neurons and peripheral fibers (Aβ, Aδ, and C fibers) results in neuropathic pain[17]. Numbness and paresthesia are the most typical signs of peripheral neuropathy but may also be accompanied by pain, weakness, and a loss of deep tendon reflexes [14].

The ethanolic extract of *Calotropis procera* was employed in the current study as a neuroprotective medicine. In tropical and subtropical areas of Asia and Africa, the genus Calotropis is prevalent. *Calotropis procera* is an evergreen perennial shrub that is an erect, soft-wooded member of the Asclepiadaceae family. Common names for *C. procera* include "Akra" and "milkweed." This plant is well-known as it generates an enormous amount of latex. The entire plant, including the roots, stem, leaves, and flowers of *C. procera*, are frequently used as traditional medicine[7]. The leaves and bark of the plant have been shown to have anti-cancer, analgesic, antipyretic, neuromuscular blocking, anti-hyperglycemic, and wound healing properties[6].

The following models were utilized to carry out the neuroprotective activity:

1. Chronic sciatic nerve constriction.

2. Contusion Model.

3. Chemotherapy-induced neuropathy.

The studies revealed that the standard 10mg\kg of Amitriptyline had shown more neuroprotective activity when compared to the test groups of *Calotropis procera*. Group administered with the highest dose of the drug i.e., 77mg/kg had shown more activity when compared to the group given with lowest dose i.e., 36.5mg/kg. However, both groups had shown an activity less than that of the standard. Neurobehavioral studies were conducted by assessing the behavior parameters like an assessment of hyperalgesia and allodynia, assessing the locomotor activity, and finally by performing the grasping test and staircase test. In the behavior parameter study, the hyperalgesia and allodynia were increased and the degree of locomotor activity dropped. In the grasping test and the staircase test, the percentage of grip strength and recovery in the staircase test was calculated to be lesser than that of the standard.

LD50 studies were conducted in albino rats using OECD guidelines No- 423 for *Calotropis procera* leaves extract. It was found that the extract even at 500mg/kg dose had not shown any mortality confirming it falls in GHS category 5.

This research study helped to investigate the neuroprotective activity of extracts of *Calotropis procera* leaves in rats. The acute toxicity studies were conducted as per OECD guidelines 423 for Calotropis procera leaf extract. It was found that the extract even at 500mg/kg dose had not shown any mortality confirming it falls in GHS category 5.

**Conclusion:** The leaf extract of CP exhibits statistically significant ($p<0.05$) neuroprotective action in three neuropathy induced rat models, thereby validating its safe use in traditional medicine.

**Table Legends:**

**Table 1: Effect of CP on grip latency and staircase latency using CCI in rats**

| Grp no. | Treatment | Dose | Grip latency(sec) | Staircase latency(sec) |
|---|---|---|---|---|
| 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 |

All values represent mean SEM ‘p<0.05 versus control.

Values are means ± SEM; control+CCI (A); Standard10mg/kg+CCI (B); ethanolic extract 36.5mg/kg+CCI (C); ethanolic extract 77mg/kg + CCI (D).

p<0.05 Compared with Control rats.

p<0.0 Compared with ethanolic extract rats.

**Table 2: Effect of Calotropis procera on grip latency and staircase latency in contusion-induced neuropathy in rats.**

| Grp no. | Treatment | Dose | Grip latency(sec) | Staircase latency(sec) |
|---|---|---|---|---|
| 1 | Control | - | 20±0.09 | 18.65±1.04 |
| 2 | Amitriptyline | 10mg/kg | 19±0.04 | 20.3±0.04 |
| 3 | C.P low dose | 36.5mg/kg | 11±1.08 | 8.95±0.08 |
| 4 | C.P high dose | 77mg/kg | 19.66±0.05 | 12.67±0.05 |

All values represent mean SEM (p<0.05) versus control.

Values are means ± SEM; control+SCI (A); Standard10mg/kg+SCI (B); ethanolic extract 36.5mg/kg+SCI (C); ethanolic extract 77mg/kg + SCI (D).

p<0.05 Compared with Control rats.

p<0.0 Compared with ethanolic extract rats.

**Table 3: Effect of Calotropis procera on grip latency and staircase latency in chemotherapy-induced neuropathy in rats.**

| Grp no. | Treatment | Dose | Grip latency(sec) | Staircase latency(sec) |
|---|---|---|---|---|
| 1 | Control | - | 35.15±1.66 | 29.20±0.088 |
| 2 | Amitriptyline | 10mg/kg | 22.57±0.05 | 27.87±0.05 |
| 3 | C.P low dose | 36.5mg/kg | 29.80±0.86 | 28.70±0.65 |
| 4 | C.P high dose | 77mg/kg | 27.50±0.75 | 26.30±0.78 |

All values represent mean SEM (p<0.05) versus control.

Values are means ± SEM; control (A); Standard10mg/kg+Cisplatin(B); ethanolic extract 36.5mg/kg+Cisplatin (C); ethanolic extract 77mg/kg + Cisplatin(D).

p<0.05 Compared with Control rats.

p<0.0 Compared with ethanolic extract rats.

**Acknowledgment:** The authors are thankful to the Department of Pharmacognosy, HKES’s College of Pharmacy Kalaburagi and Department of Botany, Smt. Veeramma Gangasiri College for Women, Kalaburagi for their valuable help during our research work.

**Conflict Of Interest:** The authors declare that there have no competing financial interests.

**Funding Source:** This study has not received funding from any of the government or private agencies.

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