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Calotropis procera (root) escalates functions rehabilitation and attenuates oxidative stress in a mouse model of peripheral nerve injury

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Abstract: Peripheral nerve injuries result in sensorimotor functional loss, leading to permanent disability and physical dependency with immense cost and reduced quality of life. These injuries are among those complicated medical situations which still are waiting for their first-line treatment. This study was designed to investigate the role of *Calotropis procera* (crude roots) in accelerating functional retrieval following mechanically induced sciatic nerve injury in healthy albino male mice. Following acclimatization, mice were grouped equally as "Control" fed on normal chow and "Root" fed on *C. procera* root (100mg/kg/day) mixed chow. A mechanical crush was induced in right sciatic nerve of animals. Behavioral analyses (grip strength, SFI, pinprick and hot plate tests) were conducted for assessing sensorimotor function reclamation and blood was collected for oxidative stress assessment. Significantly earlier retrieval of sensorimotor activities ($p<0.05$), reduced total oxidant status, increased total antioxidant capacity with prominently enhanced arylesterase and paraoxonase activities ($p<0.001$) in treatment group suggested positive impact of *C. procera* roots on quickening functional recovery and combating oxidative stress following nerve injury. Thus *C. procera* root can be considered as potential candidate drug for further investigation to seek bioactive compound/s that may actually responsible for ameliorative functional recovery following nerve injury.

Keywords: Peripheral nerve injury, total oxidant status, total antioxidant capacity, arylesterase and paraoxonase-1

INTRODUCTION

Peripheral nerve injuries (PNIs) are among those quite prevalent health issues in our society which are still incurable (Bota & Fodor, 2019). Nerves of the peripheral nervous system are delicate long cords made up of bundles of axons that are more susceptible to be injured as a result of any kind of physical trauma such as roadside accidents, gunshots, industrial or household accidents leading to mechanical stress and stretch. These result in the loss of sensory and motor functions (Hussain *et al*., 2020; Li *et al.*, 2020). Although the injured peripheral nerve owns an ability to regenerate yet this phenomenon of regeneration is very much slow and difficult to accomplish without adjuvant therapy (Hussain *et al*., 2020). Unfortunately, owing to the sluggish recovery process, the exacerbated muscular atrophy occurs even before the onset of complete functional restoration accompanied by nerve rejuvenation (Hussain *et al*., 2020; Imran *et al*., 2019; Rasul *et al*., 2019). Even though extensive work in the field of nerve injury and nerve regeneration is going on, reliable treatments ensuring the complete functional recovery remains a challenge (Bota & Fodor, 2019). The natural flora possesses a variety of phytochemicals that have been achieving more attention as health-promoting agents because of their historical use in traditional systems of medicines (Hussain *et al*., 2018a; Hussain *et al*., 2018b; Hussain *et al*., 2019; Hameed *et al*., 2020).

Calotropis procera (*C. procera*), belonging to the family Apocynaceae, is a commonly available tropical plant with renowned medicinal importance. In local languages, it is known as Madar, Apple of Sodom, or Aak (Yogi *et al*., 2016). This plant exhibits enormous health-related properties such as anthelmintic, hepatoprotective, antitumor, antimicrobial, antioxidant, and anticonvulsant (Parihar & Balekar, 2016). It has been reported to have neuroprotective effects in neurodegenerative diseases such as Alzheimer's disease (Paul *et al*., 2018). Based on the reported pieces of evidence regarding the pharmacological importance of *C. procera* against various diseases, this plant may possess a positive role in healing the nerve injuries, however not even a single study is available till now, showing the role of this plant in it. Therefore, the current study was planned to investigate its effects in accelerating the nerve regeneration and associated functional recovery in the scenario of PNI. For this, we took advantage of our previously established model of sciatic nerve injury to evaluate the nerve regenerative potential of this plant using a mouse model.

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MATERIALS AND METHODS

Experimental animals

Healthy albino mice of an average age of 8 to 10 weeks and body weight 30-32 g were bought from the animal house of the Department of Physiology, Government College University Faisalabad, Pakistan. All mice were housed in separate polycarbonate plastic rodent cages at ambient room temperature (25±1℃), 12 hours light and dark cycle, and *ad libitum* supply of food and water. The mice were grouped into two: Control (n=8), fed on normal chow and Root (n=8), fed on *C. procera* root containing chow. The guidelines adopted for the use of animals and their care, in this study, were established on the rules given by the National Institute of Health for the use and care of experimental animals in its guide (Guide for the Care and Use of Laboratory Animals, 1978). The design of the study was approved by the Institutional Review Board, Government College University Faisalabad (IRB no. 586).

Collection and processing of plant material

The roots of *C. procera* were collected from the area of Faisalabad, Pakistan, and were identified and confirmed by the taxonomist from the Department of Botany, Government College University Faisalabad. The roots were dried in shade, ground, and mixed with the rodents' diet at the dose rate of 100mg/kg of body weight/day. The treatment dose was mixed in the mice' chow in such a way that the daily average consumed diet by animals (5-6 grams) must contain the given dose of *C. procera* crude roots. This was calculated using the following formula: Daily dose = Bodyweight (dose of treatment per kg/1000) (Hussain *et al*., 2013). The treatment group was given root containing diet from the day of sciatic nerve mechanical crush till the end of the experiment, while the control group was administered the normal rodent chow throughout the experiment.

Method to induce Sciatic nerve crush

Following the one week acclimatization period, a mechanical crush to the sciatic nerve on the right leg was induced in all mice as previously described (Aziz *et al*., 2019; Imran *et al*., 2019; Rasul *et al*., 2019). Briefly, the mice were subjected to anesthesia by injecting a mixture of ketamine (70mg/Kg body weight) and xylazine (5mg/kg body weight) through the intraperitoneal route. A 2cm long fine cut was made following the smooth shaving of the desired part of the skin. The sciatic nerve was exposed and mechanically crushed by applying constant pressure with forceps for 15 seconds at the mid-thigh region. The skin was stitched with sutures. Pyodine was applied to the wound to avert the infection.

Behavioral analyses

Hotplate test

The recovery of sensory functions was measured by using the hot plate test. In this test, the mouse was adjusted in a position that its ipsilateral hind paw was in direct contact with the hot surface of the hot plate (SCILOGEX MS7-H550-S LED digital 7x7 Hotplate stirrers) until it gave any response. The temperature of the hot plate was adjusted at 56±2℃. The paw withdrawal latency was recorded for every mouse by following the protocol as mentioned in previous studies (Aziz *et al*., 2019; Imran *et al*., 2019; Rasul *et al*., 2019).

Pinprick Test

It is another parameter for measuring the sensory function revival. It was performed by using the protocol as given by Chen *et al*., 2017. Mice were kept on a wire mesh cage and the lateral part of the plantar surface of their ipsilateral hind paw was divided into 5 areas hypothetically. Each area was pinpricked gently by Austerlitz insect pin having size "000" and swiftly removal of the paw was observed as a positive response. The test was applied from the heel (A) to the most lateral toe (E) of the experimental paw. Each area was graded "1" for a positive response and "0" for no response (Chen *et al*., 2017). So that mouse showing score 5 was considered full functional in terms of sensory activity and the mouse with a score '0' was considered having a lack of sensation and scores 1, 2, 3, 4 would show increasing levels of sensory functions.

Grip strength test

The grip strength test is a technique for measuring the muscle function regain following an injury to a peripheral nerve. The grip strength force was measured by a grip-strength meter (Bioseb, Chaville, France) by following the method as previously discussed (Aziz *et al*., 2019; Imran *et al*., 2019). The grip strength force was measured for both limbs of every mouse as contralateral and ipsilateral to the lesion (Hussain *et al*., 2013).

Sciatic Functional Index (SFI)

The motor function revival was also assessed by measuring the SFI that depends on the walking pattern of the animal. In this parameter, the hind limbs of each mouse were colored with the non-toxic ink, and then they were allowed to run on the white surface (paper) of the wooden track to reach their goal. The clearer prints were recorded for further observation and the following formula was used to measure the SFI:

$$SFI = \left(-38.3 \times \frac{EPL - NPL}{NPL}\right) + \left(109.5 \times \frac{ETS - NTS}{NTS}\right) + \left(13.3 \times \frac{EIT - NIT}{NIT}\right) - 8.8$$

Here; the PL (Print length) is the measurement of the distance between the heel and 3rd toe tip, IT is intermediate toe spread i.e. distance b/w 4th and 2nd toe and Toe spread (TS) is the distance b/w 1st and 5th toe. E represents the experimental paw (ipsilateral to the injury site) while N represents the normal (contralateral) paw.

Biochemical analyses

At the end of the experiment, mice were sacrificed after giving deep anesthesia (a mixture of ketamine and

xylazine administered through intraperitoneal injection). The blood was collected and serum was separated and used for the following biochemical analyses.

Total Oxidant Status (TOS)

Abnormally high level of oxidants at the injury site is a major hallmark to worse the pathological changes there. By using this test, the overall status of oxidative stress is evaluated. TOS was measured in the serum samples of animals. It is expressed in terms of µmoles of H_2O_2 $_{Equivalent}$/L. This test was done by following the method previously described (Aziz *et al*., 2019).

Total Antioxidant Capacity (TAC)

The antioxidant capacity is known as the capability of a biological system to fight against the free radicals and associated mal-functioning. The Total Antioxidant Capacity (TAC) was measured in serum samples of animals to assess the antioxidative capability derived by the applied treatment and was expressed in mmol of vitamin $C_{Equivalent}$/L (Aziz *et al*., 2019)

Arylesterase activity (ARE; KU/L)

Arylesterase (ARE) is a thiol enzyme and plays a role in decreasing oxidative stress (Erdem *et al*., 2010). To evaluate arylesterase (ARE) activity in serum samples, phenylacetate was used as a substrate in the reaction solution. During this reaction, the phenylacetate was catalyzed and converted into phenol in the presence of ARE. The conversion rate was taken as a direct measure of the activity of the enzyme. This test was done by mixing the phenylacetate (2.0mmol/L) with methanol (40%) to make the stock solution. The other constituents of this solution include 0.1 M/L Tris-HCL Buffer (8.0 pH) and calcium chloride (2.0mmol/L). The absorbance was measured at 270nm wavelength at room temperature by spectrophotometer and ARE activity was calculated by following already established protocol (Elkiran *et al*., 2007).

Paraoxonase activity (PON-1; U/L)

PON-1, a hydrolytic enzyme, possesses an intrinsic ability to shield the body against lipid peroxidation (Serdar *et al*., 2006). It works in combination with ARE to minimize oxidative stress (Precourt *et al*., 2009). Its activity was measured following the already established protocol (Elkiran *et al*., 2007; Razzaq *et al*., 2020). Its activity depends upon the extent of enzymatic breakdown of paraoxon into the p-nitrophenol in the presence of PON-1. The standard reagent was prepared by mixing paraoxon (2mM/L), Calcium chloride (2mM/L), and tris HCL (0.1M/L). Principally, the formation of p-nitrophenol, on incubation with serum samples, results in the color production which acts as an indicator of the enzymatic activity of PON-1. The absorbance was measured at 405nm wavelength at room temperature by spectrophotometer and enzymatic activity was calculated.

STATISTICAL ANALYSIS

All results were expressed as mean ± SEM. Data were analyzed statistically by using GraphPad Prism (version 8.4.2). A comparison of means of groups was done by applying ANOVA. A value of $p<0.05$ was considered statistically significant.

RESULTS

Effects of C. procera roots on body weight and food consumption

The body weight and food intake of both groups were measured throughout the study. We observed that the diet consumption of mice was not affected after the sciatic nerve lesion and the addition of *C. procera* root in the mice chow (fig. 1 B). Similarly, the body mass percentage of both groups also remained unaltered during the whole period of the experiment (fig. 1 A). The difference in body weight and food intake was statistically non-significant in both groups. This showed that the presence of *C. procera* in the diet did not alter the diet consumption behavior and body weight modification.

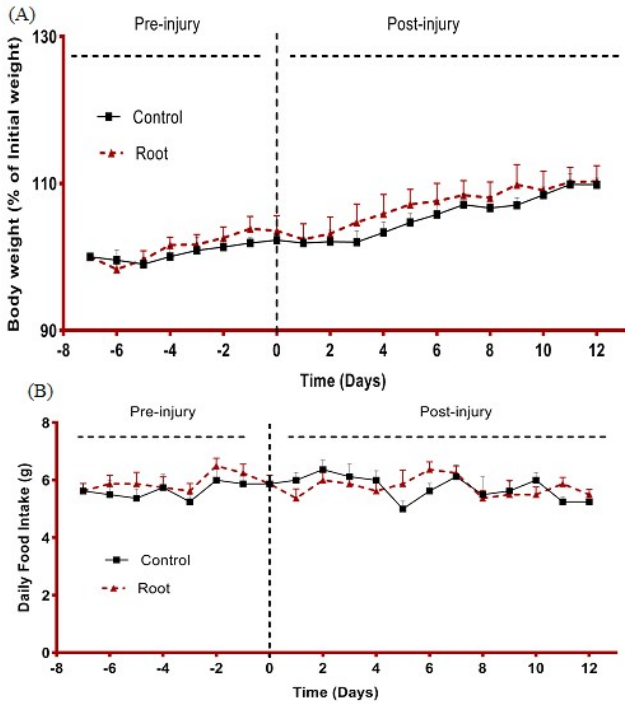

Fig. 1: *C. procera* roots alter neither body mass nor food intake. (A) Time course of body weight of animals fed on normal chow (control group, n=8) or *C. procera* roots containing chow (root group, n=8). The root group was fed on *C. procera* roots containing chow after the onset of sciatic nerve lesion (day '0' post-injury) and throughout the whole study period. Two-way repeated-measure ANOVA showed significant effect of time ($F(19,266)=31.10$, $p<0.001$), non-significant effect of diet ($F(1,14)=0.376$, $p=0.54$) and and a non-significant

interaction between factors (F(19,266)=0.624, p=0.887). (B) Time course of food intake in animals as in A, Two-way repeated-measure ANOVA showed a non-significant effect of time (F(19,266)=1.38, p=0.138), diet (F(1,14)=0.885, p=0.362) and a non-significant interaction between factors (F(19,266)=0.84, p=0.657). Sidak multiple comparisons test (in both A & B) did not show any significant difference between both groups at any time point.

Effects of C. procera roots on sensory function retrieval

The retrieval of sensory functions was measured by performing hot-plate and pinprick tests (fig. 2A and B). We found that *C. procera* root caused an early paw withdrawal response (highly significant differences (p<0.001) on day 7 post-injury) upon thermal stimulation. Similarly, an improved sensory score as assessed by pinprick analysis, in the root group validates the potential of *C. procera* in quickly restoring the sensory functions following the sciatic nerve injury.

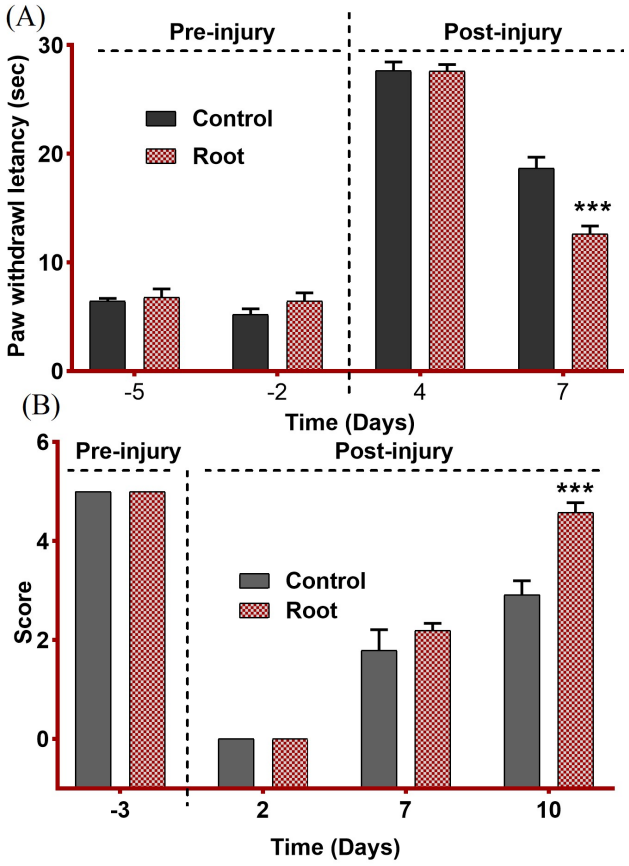

Fig. 2: *C. procera* roots accelerate the sensory function retrieval. (A) Assessment of paw withdrawal latency from the hot surface in animals fed on normal chow (control group, n=8) or *C. procera* roots containing chow (root group, n=8). The root group was fed on *C. procera* roots containing chow since the time of injury induction (day '0' post-injury) and during the whole period of the experiment. Measurements were observed on day -5 and day -3 pre-injury and day 4 and day 7 post-injury. Two-way repeated measure ANOVA showed a significant effect of time (F(3,39)=414, p<0.001), the non-significant effect of diet (F(1,13)=4.24, p=0.06) and a highly significant interaction of factors (F(3,39)=11.1, p<0.001). Sidak multiple comparisons test revealed highly significant differences between the control group and root group at day 7 post-injury (***p<0.001). (B) Paw withdrawal score in reaction to pinprick stimulus in animals as in (A). Measurements were observed at day -3 pre-injury and day 2, 4 and 7 post-injury. Two-way repeated measure ANOVA showed a highly significant effect of time (F(3,39)=9.7, p=0.02), a significant effect of diet (F(1,13)=7.02, p=0.02) and a highly significant interaction of factors (F(3,39)=298, p<0.001). Sidak multiple comparisons test showed a highly significant difference between control and root group at day 10 post-injury (***p<0.001).

Effects of C. procera roots on motor function retrieval

As described in the previous studies, retrieval of motor function can be explored by using grip strength test and SFI. We found significant restoration in the motor functions by using SFI and grip strength force (% of initial force). The *C. procera* root showed significantly earlier motor function retrieval (p<0.001) as compared to the normal chow group (fig. 3A and B).

Effects of C. procera roots on systemic indexes

Following the onset of sciatic nerve injury, the elevated oxidative stress causes further neural damage at the injury site and hereby delays functional retrieval. We measured the level of total oxidant status (TOS), total antioxidant capacity (TAC), enzymatic activities of ARE and PON-1. It was noted that the *C. procera* chow group attenuated TOS levels and enhanced the TAC levels along with the activities of oxidative stress-related enzymes such as ARE and PON-1 with a significant statistical difference (fig. 4A, B, C & D).

DISCUSSION

PNI stands among the most serious health issues being faced by modern society in the present era. Despite the marvelous efforts in this field, functional recovery is still compromised. In the present age, plant-based medicines are getting more attention due to their vast spectrum of health-promoting effects. Our laboratory has previously reported the effects of some medicinal plants on promoting functional rehabilitation. The current study investigates the possible role of a well-known plant, named *Calotropis procera,* of our region.

Calotropis procera (*C. procera*) is a laticiferous plant and belongs to the Apocynaceae family. It has gained fame due to its widely reported antihelmintic, antifungal, antibacterial, and antioxidant properties (Mali *et al.,*

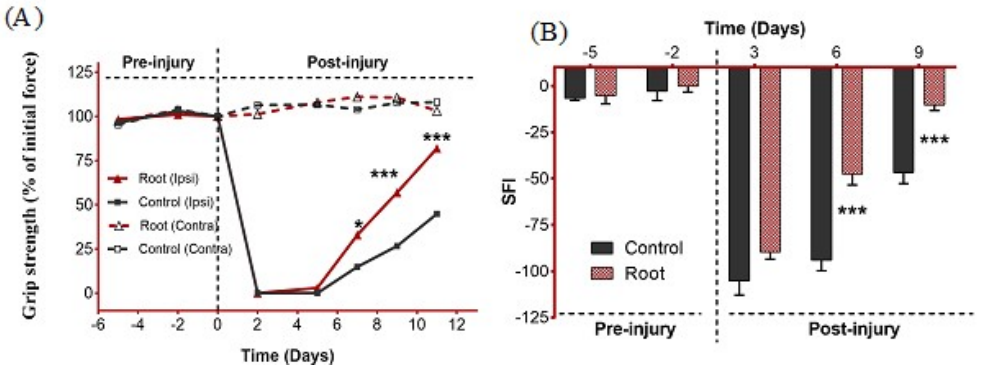

Fig. 3: *C. procera* roots accelerate motor function retrieval following a sciatic nerve lesion. (A) Assessment of improvement in grip force of ipsilateral hind paw in animals fed on normal chow (control group, n=8) or *C. procera* root containing chow (root group, n=8). Observations were recorded for both hind limbs (Ipsilateral (solid lines) and contralateral (dotted lines) to the lesion). Grip-strength is presented as a percentage of the average initial force observed at day -5 and day -2 per animal. Two-way repeated measure ANOVA indicated a highly significant effect of time ($F(7,175)=96.2$, $p<0.001$), diet ($F(3,25)=140$, $p<0.001$), and interaction of factors ($F(21,175)=44.7$, $p<0.001$). Sidak multiple comparisons test revealed significant differences between both groups at day 7 (*p=0.04), day 9 (***p<0.001), and day 11 (***p<0.001) post-injury. **(B)** Assessment of SFI in animals as in (A). Two-way repeated-measure ANOVA indicated a highly significant effect of time ($F(4,48)=192$, $p<0.001$) and diet ($F(1,12)=28.87$, $p<0.001$) and highly significant interaction between factors ($F(4,48)=11.23$, $p<0.001$). Sidak multiple comparisons test at every time point revealed highly significant differences between control and root at day 6 (***p<0.001) and day 9(***p<0.001) post-injury.

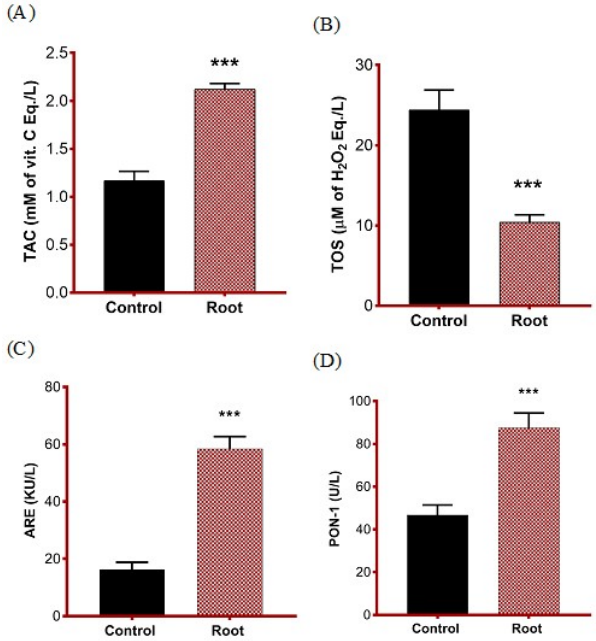

Fig. 4: *C. procera* roots attenuate TOS and enhance TAC levels by modulating oxidative stress-related enzymes' activities (ARE and PON-1). (A) Unpaired t-test showed that the difference in TOS levels was significant statistically between both groups (***p=0.0001 and t=5.29). (B) Unpaired t-test showed that the difference in TAC levels was significant statistically between both groups (***p<0.001 and t=8.3). (C) Unpaired t-test showed that the difference in Arylesterase (ARE) activity was significant statistically between both groups (***p<0.001 and t=8.43). (D) Unpaired t-test showed that the difference in Paraoxonase-1 (PON-1) activity was significant statistically between both groups (***p<0.001 and t=4.84).

2019). There are some pieces of evidence that indicate its effects on neuromodulation, neuroendocrine modulatory pathways, and neuroprotection (Ouedraogo *et al.*, 2014). However, no investigation has been made to explore its effect on the regeneration of a nerve after an injury. The marvelous properties documented by many studies make it an interesting and valuable candidate in promoting functional regain after PNI. Our previous studies on the

plant-based approach (Aziz *et al*., 2019; Imran *et al*., 2019; Rasul *et al*., 2019; Razzaq *et al.,* 2020) lay down a strong ground for explicating the role of *C. procera* in nerve regeneration following a traumatic injury to a peripheral nerve. Thereby, this study was aimed to explore the effect of crude powder of *C. procera* roots on accelerating the regeneration of the peripheral nerve.

Plant material or chemical compounds having strange odor can affect the eating behavior of animals and bodyweight as well. Therefore, we rooted out the possibility that the addition of *C. procera* in the rodent diet could cause a modification in food intake and body weight. It was noted that the average food intake and hereby the bodyweight also remained statistically unaltered suggesting that the observed effects on speedy regain of function was purely due to treatment. As the sciatic nerve is of mixed nature, so the measurement of both sensory and motor functions is equally important to evaluate the pattern of functional recovery. An early retrieval of sensory functions in the *C. procera* root group was noticeable even on day 7 and appeared more prominent on day 10 following the injury. These findings are concomitant to the already reported data (Razzaq *et al*., 2020, Aziz *et al*., 2019). Similarly, the recovery of motor functions could also be observed on day 7 and a trend of accelerated regain was persistent after this point. A regain of 85% motor functional recovery in the treated group as compared to 55% in the untreated animals on day 11 showed the aptitude of *C. procera* in reducing the time for functional recovery following nerve injury.

Oxidative stress is one of the major pathological issues that contribute to the pathogenesis of a variety of diseases. It is one of the most prime underlying factors that contribute significantly to damaging nerve upon injury (Komirishetty *et al*., 2016). We speculated that the antioxidant property of *C. procera* could play a significant role in the accelerated function regain. For this purpose, we measured the TAC and TOS. In this study, reduced TOS level and augmented TAC level provides a promising situation for regenerating nerve. We also found an up-regulated activity of antioxidant enzymes ARE and PON-1 (fig. 4C & D) in the root group. These findings support the antioxidant potential of *C. procera* as a major factor which is also reported previously (Yadav *et al.,* 2014). On these grounds, it is quite tempting to hypothesize that attenuating the oxidative stress by administering *C. procera* can hasten the healing process after sciatic nerve injury. Therefore, the reduction in oxidative stress has been the furthermost target to figure out effective remedies against PNIs.

CONCLUSION

Our findings signify the possible role of *C. procera* roots to accelerate the neuronal regeneration and restoration of functionality following a mechanically induced nerve injury. These outcomes demonstrate that *C. procera* can be a good option to be investigated thoroughly for healing nerve injuries. Though the results of our study are quite preliminary yet they provide a clue about the potency of this plant for having neuroregenerative capacity. Thus, to identify and to explore this plant to find the most effective constituent/constituents, as well as the possible mechanisms involved in this effectiveness in escalating nerve regeneration, would be a future perspective.

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