Neurada procumbens promotes functions regain in a mouse model of mechanically induced sciatic nerve injury

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Abstract: Peripheral nerve injury is a complex condition which results in restricted physical activity. Despite the tremendous efforts to figure out effective remedies, the complete functional retrieval is still a goal to be achieved. So, the need of hour is the exploration of potential natural compounds to recover this functional loss. Here, we have investigated the role of a local plant "*Neurada procumbens"* in ameliorating the functional recovery after an induced nerve compression injury in a mouse model. A dose of *N. procumbens* (50mg/kg of body weight) was administered orally from the day of injury to onwards. The motor functional recovery was assessed by evaluating muscle grip strength and sciatic functional index; while the sensory functions were gauged by the hotplate test. The serological parameters were carried out to analyze the effect of *N. procumbens* on oxidative stress level. The recovery of sensory and motor functions was significantly improved and perceived earlier in the treatment group. Moreover, the elevated antioxidant level was statistically significant in the treatment group. These results indicate that the supplementation of *N. procumbens* accelerates functional recovery after sciatic nerve crush injury.

Keywords: Peripheral nerve injury, sensory functions recovery, motor functions recovery, *Neurada procumbens*. oxidative stress.

INTRODUCTION

Peripheral nerve injuries (PNIs) are considered as life threatening phenomena due to their effects on behavior, mobility, perception, consciousness, sensations and most often a life-long disability for the affected individual (Aziz *et al*., 2019). Different strategies and medicinal interventions have been tested and practiced but the outcomes are still disappointing. Deadly slow recovery of injured nerve is the most common hurdle in 100% functional regain which ultimately aggrevates the muscular atrophy (Tuffaha *et al*., 2016). So, it is still being waited for the exploration of efficient therapeutic interventions which have potential to accelerate the recovery process before the muscle atrophy occurs.

By taking an overview of the unfortunate scenario of any medical intervention against PNI, we took an initial step to explore an effective approach based on natural compounds against injured nerve. While on the similar note, Phytochemicals; the plant-derived compounds are fascinating the modern epoch of the scientific studies by their prodigious demand and least side effects. These compounds act as anti-oxidant, anti-inflammatory, anti-proliferative, anti-nociceptive agents, and have many others health promoting effects. They also have the potential to overcome the health ailments like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and many others *(Hussain et al*., 2018a, 2018b; Hussain *et al.,* 2019).

Neurada procumbens is a desert plant which belongs to the Neuradaceae family and is commonly known as "Alsdan". It has been traditionally used for the treatment of various health ailments such as dysentery and diarrhea. It is also used as a nerve tonic (Marzouk *et al*., 2013; Qureshi *et al*., 2010). Based on available data, nerve regenerative properties of this plant can be speculated. We took advantage of our established model of sciatic nerve injury to explore this potential of our local plant. Currently, there is lack of data regarding *N. procumbens'* pharmacological potential associated with the nervous system. Hence, we decided to take initiative and chose this plant for our study to investigate its therapeutic effects against peripheral nerve injury in the mouse.

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MATERIALS AND METHODS

Animals

The albino mice with an average age of 6-7 weeks and weight 30±4 grams were procured from the animal facility of Department of Physiology, Government College University, Faisalabad. The mice were divided into two groups (Normal chow group n=7, Treatment group n=7). The mice were facilitated with *ad* libitum food and water supply and were kept in standard management conditions (one mouse /cage, 12-h dark/12-h light cycle, 24 ± 2 °C).

Plant preparation and supplementation

The seeds of *N. procumbens* were purchased from the local market and their identifications was confirmed by the department of botany, Government College University Faisalabad. The seeds of *N. procumbens* were shade dried and ground into fine powder. A calculated quantity *N. procumbens* seeds powder was mixed in normal chow diet at a dose of 50mg/kg of body weight. It was made sure that average daily consumed diet of 5gram must contained required dose of plant material. The treatment group was given the plant material containing diet from the day of nerve crush till the termination of the experiment. The average food consumption and body weight were measured daily.

Induction of Sciatic Nerve Crush injury

The sciatic nerve crush was induced in all mice after the acclimatization period of one week. The mice were anesthetized by an intraperitoneal injection of xylazine (5mg/kg body weight) and ketamine (70mg/Kg body weight) mixture. A fine cut of 2cm length was made after smooth cleaning of the skin. Sciatic nerve was exposed and applied with a constant pressure for a period of 15 seconds by using a fine pair of forceps. The nerve compression was ensured that whether the nerve was perfectly crushed or not, and the epineurium had endured intact. The skin was then sutured with 4-0 stitches and pyodine was applied on sutured site for a couple of days to prevent any infection (Hussain *et al*., 2013). From the day of nerve crush, all mice were divided into two groups as Normal chow group (n=7) and *N. procumbens* chow group (n=7).

Behavioral tests

Sciatic functional index (SFI)

The SFI was measured to assess the motor functional recovery. The mouse' hind paws were painted with the blue ink and mice were allowed to walk on the wooden track to reach the darkened box. The SFI was measured by using the given formula as follow for both groups as Normal (N) and Experimental E group.

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

Here; Print length (PL) = Measurement of distance from tip of 3rd toe to heel, Intermediate toe spread (IT) = Measurement of intermediate toe spread i.e. distance b/w the 2nd to 4th toe, and Toe spread (TS) = Toe spread is distance b/w 1st and 5th toe.

Grip strength of muscle

This technique is used to evaluate the mouse muscle strength *in vivo* by using the ability of mice to clamp a horizontal grid or metal bar. It is performed by using grip strength meter (Bioseb, Chaville, France). The animal is placed over metallic grid and it spontaneously stops the unintentional backward movement made by the experimenter. The mice do so until the drawing force weakens their grip strength. The strength meter marks the peak of animal pulling force and a total 3 readings were taken for both hind limbs; Ipsilateral and contralateral to the lesion (Hussain *et al*., 2013).

Hot plate test

The sensory functions recovery of the mice' hind limbs are measured by using the hot plate test. The mice were allowed to stand with the contact of operated hind paw to the surface of the hot plate at a set temperature of 56 ± 2 °C until they showed any response. This time was taken as the hot plate latency (HPL) time period. Following any response, the mouse was removed from the hot plate (Yu *et al*., 2008). To record the latency period, stopwatch was used and three readings were taken with the lap of 2 minutes for each mouse.

Analysis of Systemic Indexes

Total Oxidant Status (TOS)

This term defines the presence of total oxidants level in a living system. This test was performed by using the spectrophotometer (Biolab-310) to assess the level of oxidant status in the serum sample.

a) Principle

In this assay, *o*-dianisidine ferrous ion oxidized and converted into ferric ion due to the oxidants' presence in the sample. A colored complex is formed from xylenol orange by reacting with the ferric ions in an acidic environment. The degree of oxidant molecule (present in the sample) is evaluated by spectrophotometric assessment of the intensity of the previously developed color complex. The hydrogen peroxide (H_2O_2) is used for the calibration of this test, and results are expressed as μmol H_2O_2 Equivalent /L (Motor *et al*., 2014).

b) Reagents

Reagent I

Xylenol orange (114 gm) was mixed with NaCl (8.18 g) in H_2SO_4 (900 ml) (Merck, 25 mM). Then, 100 ml of glycerol was added into the solution to make an ultimate

volume of 1000 ml. The resulting solution now comprised of Xylenol orange (150 µM), glycerol (1.35M), and NaCl (9140 mM. This reagent is valid for > 6 months at 4ºC.

Reagent II

This reagent is prepared by mixing *O*-dianisidinedihydrochloride (3.17g) and Ferrous ammonium sulfate (1.96gm) (Sigma chemicals) in 1000 ml of 25 mM H_2SO_4. Final solution is composed of *O*-dianisidinedihydrochloride (10mM) and ferrous ammonium sulfate (5 mM). The reagent is stable for (> 6 months) at 4ºC.

c) Procedure

This assay is carried out by mixing the sample (35µl) with (225µL) reagent I. Then, the initial absorption was taken. Then, 11µL of reagent 2 was added into the solution and final absorption was taken after the incubation of 4 minutes at room temperature (Anwar *et al*., 2012). Absorptions were taken by using a main wavelength of 560 nm and secondary/differential wavelength of 800 nm with spectrophotometer (Biosystem, BTS-330). The delta change in both absorptions was taken as actual concentration in terms of micro molar H_2O_2 equivalent/L.

Total Antioxidant Capacity (TAC)

TAC was measured by using the method established by Erel (2004) and the assay procedure is described as follows. Trolox equivalent antioxidant capacity or TEAC is the most common direct assay.

a) Principle

The work principle of the TAC assay is described as the formation of a free radical of 2, 2´-azinobis 3 ethylbenzothiazoline-6-sulfonate (ABTS) (ABTS•+) after the incubation of ABTS with H_2O_2. Here, the product ABTS•+ has maximum absorption at 650nm, 734nm, and 820nm and blue-green in color. The TAC of the serum sample is measured by the evaluation of color suppression of ABTS•+ from antioxidants present in the biological sample. The reaction rate is calibrated with the Trolox; a water-soluble derivative of vitamin-E, and the results are measured as mmol Trolox equivalent/L (Rubio *et al*., 2016).

b) Reagents

Reagent 1: Acetate buffer

The solution of sodium acetate (0.4 M/L) was prepared in the deionized H_2O, to have a pH of 5.8. Afterwards, glacial acetic acid (0.4 M/L) solution was prepared in the deionized water. Both of these solutions were mixed, and the final pH was maintained at 5.8. This solution is valid for 6 months and should be stored at 4ºC.

Reagent 2: ABTS

In the deionized water, solution of glacial acetic acid and a solution of acetate buffer (30 mM/L) with a pH of 3.6 were prepared. Then, this mixture was diluted with the solution of commercial H_2O_2 and the final concentration was 2 mM/L. In 100ml of the prepared buffer solution, the ABTS (0.549 g) was dissolved and the resulting concentration of this ABTS in buffer was 10 mM/L. The solution was incubated for 1 hour at room temperature and stored at the temperature of 4ºC. The solution is stable for 6 months at room temperature.

c) Procedure

The spectrophotometric analysis of this test was done by using semi-auto chemistry analyzer (Biosystem, BTS-330). The monochromatic wavelength (650nm) was attuned and a time period of 5 minutes was provided to warm the filter paper. Then, 200ul of reagent 1 was pumped and taken as blank. Afterwards, sample (5 µl) was mixed with reagent 1 (200 µl) and 20 µl of reagent 2 was added into the solution. The absorbance was taken after 5 minutes' incubation at room temperature. The linear type of calibration; derived from standards of vitamin C was used for the results' calculation.

Ethical approval of study

The study design and use of animal for this project was approved by the Institutional Review Board Government College University, Faisalabad, Pakistan. All experiments on rodent model were carried out in accordance with the provided rules, regulations and guidelines.

RESULTS

Effects of N. procumbens on food intake and body weight

The food intake of both groups was measured throughout the experiment. We noticed that the food preference was not affected even after the nerve crush and the presence of *N. procumbens* in diet did not alter the diet consumption (fig.1A). Similarly, the average percentage of body weight of both groups was measured during the whole period of the experiment. It was noticed that presence of *N. procumbens* in the diet did not affect the body weight also (fig.1B). The difference of food intake and body weight was statistically non-significant in both groups.

Effects of N. procumbens on motor functions

Grip strength test is a valuable and reliable approach for evaluating the motor functions retrieval in case of peripheral nerve injury. It allows assessing the level of nerve regeneration indirectly as the functional retrieval is directly connected to the rate of nerve regeneration. Similarly, another behavioral analysis, SFI also helps to evaluate the same nature of function. For both of these parameters, our results indicate that *N. procumbens* group acquires motor functions earlier as compared to Normal chow group (fig. 3A and B). The rate of functional recovery was statistically significant in treatment group.

Effects of N. procumbens on systemic indices

Total Oxidant Status (TOS) and Total Antioxidant Capacity (TAC)

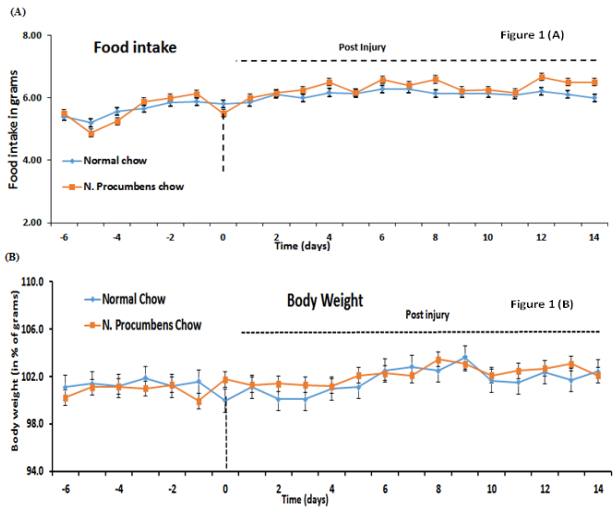

Fig. 1: *N. procumbens* does not alter food intake neither body mass after nerve injury. **(A):** By using independent Student t-test, we found that the results of average diet ingestion were non-significant statistically (p=0.15, t(12) =1.52). This means that the results of both groups i.e. Normal chow (M=6.08, S.D= 0.32, n=7) and *N. procumbens* chow group (M= 5.82, S.D= 0.33, n=7) were non-significantly different. For the differences b/w means, the confidence interval of 95% was -0.11 to 0.64. **(B):** By using independent student t-test, we found that the results of average weight (%) were non-significant statistically (p=0.10, t(12) = -1.76). This means that the results of both groups i.e. Normal chow (M=100.30, S.D=2.60, n=7) and *N. procumbens* chow group (M=102.37, S.D=1.71, n=7) were non-significantly different. For the differences b/w means, the confidence interval of 95% was -4.63 to 0.49.

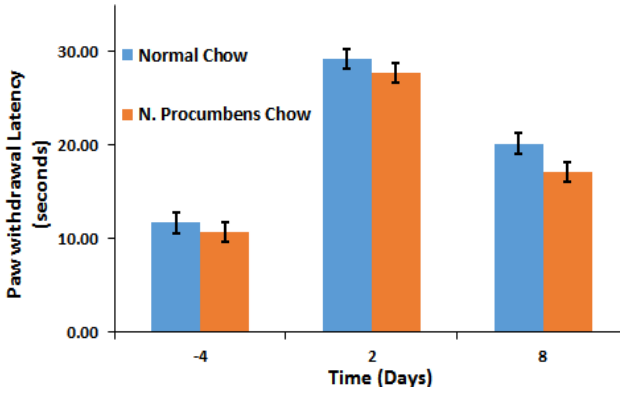

Fig. 2: *N. procumbens* accelerates sensory functions: The results of Hot plate test were statistically analyzed by using independent Student t-test and found to be significant statistically (p=0.02, t(7.13)=2.85). This means that the results of both groups i.e. Normal chow group (M=20.70, S.D=0.82, n=7) and *N. procumbens* chow group (M=17.68, S.D=2.68, n=7) were significantly different. For the differences b/w means, the confidence interval of 95% was 0.52 to 5.51.

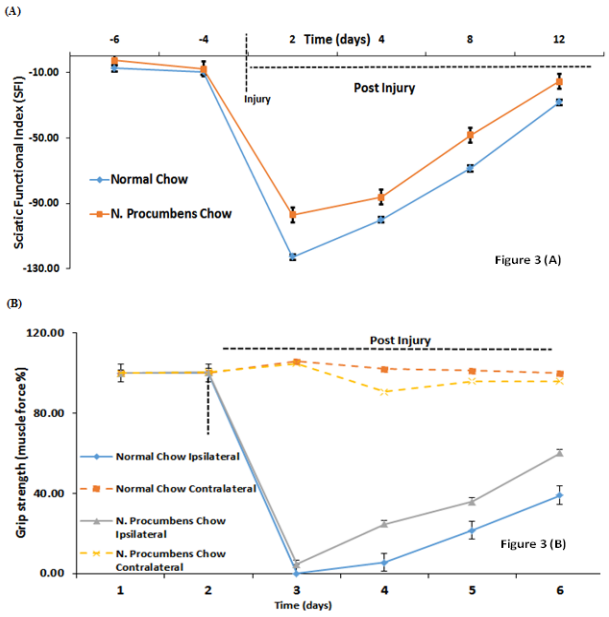

Fig. 3: *N. procumbens* accelerates motor functions recovery. **(A):** By using independent Student t-test, we found that the results of the Sciatic Functional Index were significant statistically (p=0.04, t(7.78)=-2.38). This means that the results of both Normal chow group (M= -53.35, S.D=11.99, n=7) and *N. procumbens* chow group (M=-41.72, S.D=4.67, n=7) were significantly different. For the differences b/w means , the confidence interval of 95% was -22.90 to -0.35. **(B):** By using independent Student t-test, we found that the results of muscle grip strength (Ipsi-lateral hind paw) were significant statistically (p=0.03, t(12)=-2.39). This means that the results of Normal chow group (M=32.07, S.D=5.52, n=7) and *N. procumbens* chow group (M=46.35, S.D=14.78, n=7) were significantly different. For the differences b/w means, the confidence interval of 95% was -27.27 to -1.28.

Oxidative stress is one of the reasons of the neural damage following any type of injury. TOS is usually used to assess the overall status of oxidative stress in the body. Our findings indicate that *N. procumbens* treated group has relatively better TAC as compared to that of Normal group (fig. 4A). Similarly, *N. procumbens* group showed low TOS (fig. 4B). The results of treated groups were clearly improved and statistically significant.

DISCUSSION

To the date, there is no literature available regarding the role of crude *N. procumbens* against peripheral nerve lesion. Therefore, the present study was designed to evaluate the effects of crude *N. procumbens* in accelerating the peripheral nerve regeneration. Unfortunately, we could not find supporting data from the published material for endorsing our findings. The only information regarding the use of this plant as nerve tonic

comes from a Qureshi *et al*., 2010 (Qureshi *et al.,* 2010). Therefore, our study is pioneer effort for opening the new horizons of medicinal use of this local flora. We observed a remarkable effect of *N. procumbens* addition on functional regain. Our findings indicate significant differences in the results of both groups regarding sensory and motor functions' recovery. Moreover, effects on systemic indices such as TAC and TOS were also highly significant. In regard to the motor functions retrieval, the results of both SFI and grip strength were statistically significant. For SFI, the difference in both groups' function was significant even at day 2 after the sciatic nerve injury. The treatment group showed earlier and better motor functional retrieval with statistically significant results. Similarly, the results of grip strength were also significantly different indicating the possible role of *N. procumbens* in accelerating the motor functions and ameliorative effects on PNI associated systemic damages in the treatment group. As the sciatic nerve is of mixed nature, therefore, we also evaluated the restoration of sensory function to evaluate the functional retrieval. Our results indicated that there was an evident decline in withdrawal latency of ipsilateral hind paw in mice of the treated group, and the data were also statistically significant. This fact highlights the protective efficacy of *N. procumbens* and also emboldens further assessment of this plant in relation to other myopathies.

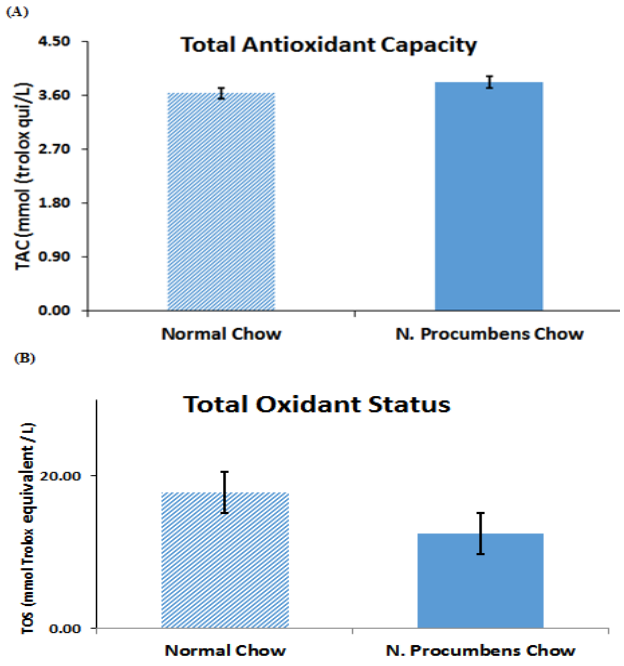

Fig. 4: *N. procumbens* attenuates Total oxidant status. **(A):** By using independent Student t-test, we found that the results of Total oxidant status were significant statistically (p=0.009, t(12)=3.09). This means that the results of both Normal chow group (M=16.94, S.D=3.65, n=7) and *N. Procumbens* chow group (M=11.60, S.D= 2.73, n=7) were significantly different. For the differences b/w means, the confidence interval of 95% was 1.58 to 9.09. **(B):** *N. procumbens* increases Total antioxidant capacity: By using independent Student t-test, we found that the results of Total antioxidant capacity were significant statistically (p=0.02, t(12)=-2.65). This means that the results of both Normal chow group (M=3.64, S.D=0.16, n=7) and *N. procumbens* chow group (M= 3.87, S.D=0.17, n=7) were significantly different. For the differences b/w means, the confidence interval of 95% was -0.42 to -0.04.

Following the sciatic nerve injury, several other pathological features also arise and cause further neuronal damage by evolving the mitochondrial dysfunction. Amongst others oxidative stress is considered as the most pivotal factor. On the similar note, up-regulated anti-oxidative capacity and reduced oxidative stress is taken as a favorable condition resulting in the protective effects following a nerve injury (Komirishetty *et al*., 2016). Anti-oxidative properties of *N. procumbens* have been previously reported (Marzouk *et al*., 2014) and our results also showed higher levels of TAC lower level of TOS in the treatment group. On these grounds, we speculate the strong anti-oxidant effect of *N. procumbens* in case of nerve injury. It is also important to discuss that reducing oxidative stress has been the most interesting target of figuring out the effective remedies. These preliminary findings lay a strong foundation of further studies to elucidate the actual role of *N. procumbens* in promoting nerve regeneration after a traumatic injury. At further step, identification and characterization of actual constituent of this plant may provide a valuable target for drug formulation.

CONCLUSION

In conclusion, this study suggests that the crude powder of *N. procumbens* seeds accelerates the sensory and motor function recovery and indirectly regeneration of the peripheral nerve. Furthermore, the investigation of chief bioactive compounds of the plant, responsible for these effects, may be very important as future prospective. Lastly, it will be extremely valuable to discover the role of altered gene expressions, and associated molecular mechanisms as well as muscle fibers morphology in response to treatment.

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